Università degli studi di Napoli "Federico II"

Dipartimento di Agraria

Tesi di Dottorato ciclo XXXV

"Sustainable Agricultural and Forestry Systems and Food Security"

The use of protein-hydrolysates as biostimulants of vegetable crops: elucidating their mode of action and optimizing their effectiveness through a multidisciplinary approach

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Chapter 1

General Introduction

Pressing matters, the case for food waste reduction

As of November 15th, 2022, the world population has reached a staggering 8 billion people, and whilst population growth is decreasing, it is possible that the target of almost 10 billion is reachable in 2050 [1]. As things stand, it has been estimated that a 25 to 70% increase in food output will be necessary to feed the world's population by that date [2]. Indeed, before even mentioning the agricultural sector wherein biostimulants - the core of this work- lie, it is paramount to assess one of the problems that is putting a grave strain on food supply chains, and that is food waste. In 2014, the EU set out to estimate the amount of food that is wasted by the consumers in the Union and found that 81% of wastage happens between production (39%), and consumption by households (42%) [3].

Total food waste has been put at almost a third of all production, and the amount and type of waste depends on the region [4,5].

As highlighted by Girotto and collaborators [6] in their 2015 work, the causes of food waste in low-income, or developing countries are found in the technical limitations in harvesting techniques, and the often impossibility to maintain proper storage conditions for food products which may include cooling, the use of technological packaging to extend the life of product or simple, fast, and efficient transportation.

This is to say, by working on infrastructure and logistics it could be possible to *greatly* reduce food losses.

In the case of developed countries, the problem is different. The EU consumer requires a constant stream of fresh and widely available products which leads to overproduction, but also the wastage of perfectly edible foodstuff which do not fulfill the high standards set out by the consumer [3,6]. Furthermore, the overly fast and complex lifestyle of the consumers turns reducing waste into a daunting task [4], which in turn increases the use of resources for food production and thus, greenhouse gas emissions [5].

Again, public policy set on working on public sensibility on the subject could manage to reduce food losses.

Concerns for food security

However, this is not to say that there isn't a need for the agricultural sector to address the issues that threaten worldwide food security.

For one, synthetic fertilizer mismanagement has long been under scrutiny for the deleterious effects on the ecosystems and crop yields; in 2019, the FAO published *The international Code of Conduct for the sustainable use and management of fertilizers* [7] where it is stated that "*fertilizer nutrients that are not taken up by plants or retained in soils may be transported to groundwater by leaching causing potential human health impacts, or to waterways by soil erosion or fertilizer misuse, especially nitrogen and phosphorus, causing eutrophication and deterioration of water quality. Excess nutrients may also be released from soils to the atmosphere through ammonia volatilization or as greenhouse gas emissions of nitrous oxide. In addition, excess fertilizer application and losses of nutrients due to the misuse of fertilizers can lower profits of farmers and in some cases can lead to crop failure*". Furthermore, due to economical constraints and extreme increases in fertilizers prices due to geopolitical matters, farmers from vulnerable countries in Africa, Latin America and Asia lack the possibility to properly fertilize their soils, resulting in "*[…] opportunity costs for yield potential, nutritional content, return of carbon to the soil, and enhancement of soil health as well as net nutrient removal from the soil system*" [7,8].

Worrisome for modern agriculture are also abiotic stresses, which are multi-faceted and, in many cases, derived from human-derived land and resource mismanagement at both the microscopic and global level. In their review, Jagadish and collaborators [9] found that the increase in the amount of greenhouse gas emissions effectively increases the energy that is retained in the atmosphere. As a result of what has been called "radiative forcing", it is estimated that global mean annual temperatures will increase by 0.3 – 4-8 °C by 2100, but it is also expected that climate change will lead to more frequent and severe heatwaves, with the ultimate result being yield depression due to decreased plant growth.

Of course, heat stresses do not often come alone, as the loss of water from soils through evaporation, which is driven by high temperature events, high light intensity and dry wind, can exacerbate existing water stresses due to deficient and or/insufficient water supply in drought conditions [10]. The Mediterranean region has been found to be one with high socioeconomic exposure to drought [11] as it contains only 1% of the world's freshwater resources [12]. As the area ran out of renewable water sources *decades ago* [12], there is a strong pressure on surface water resources, which is exacerbated in drought conditions. This ultimately results in aquifer overexploitation, a serious problem in the region [13]. Mediterranean farmers also have to face to increase in soil salinity, as the presence of toxic ions in the soil may affect both irrigated spring/summer crops and non-irrigated winter crops as deficient rainfalls cannot refill aquifers, and/or provide for leaching water volumes to push the damaging salts under the rootzone [14].

Furthermore, the association of low rainfalls and irrigation with marginal and/or salinized water can lead to both reduced water uptake by plants -thus reducing growth and potentially, yield- due to the increased osmotic pressure, but also reduced soil permeability from the modification of soil structure due to the presence of excess salts [14,15].

The new paradigm of product quality

Fruits and vegetables are important sources of vitamins, minerals, dietary fiber, and phytochemicals. Clinical and *in vitro* trials have time and time again shown that a diet with a varied supply of fruits and vegetables can improve quality of life, by reducing the risk of chronic disease and premature mortality [16].

The contribution to human health of fruits and vegetables depends upon their nutritive value and amount consumed, which is greatly influenced by consumer preferences and satisfaction in the product. However, the same preferences are greatly skewed towards flavor, outside appearance and, understandably, price [17]; yet, while better-tasting and visually appealing products are indeed more sought after by the consumers, those parameters are not the end-all, be-all of agricultural produce quality, at least in the context of health improvement.

An increase in *functional quality*, as an example, has been described in 2018 by Kyriacou and Rouphael [18] as the inclusion of bioactive compounds of antioxidant and/or health improving activity and the modulation of mineral contents in the edible parts of the products. The achievement of better functional produce can be reached using a variety of preharvest and postharvest practices, which start from the selection of the appropriate genetic material of higher phytochemical expression, the use of the so-called *eustress* or "good" stress to nudge plants into producing defense compounds such as vitamin C, the management of crop nutrition in order to reduce nitrate, which has been under scrutiny because of high intakes and correlation to disease [19], and the enhancement of the accumulation of calcium and potassium, which are under-consumed in the diets of developing countries [18,20]. Functional quality has always been at the heart of this work, as we strived to provide with our research with a comprehensive panel of phytochemicals of known human-improving qualities, such as vitamin C or ascorbic acid, auxiliary pigments of known bioactivity like carotenoids and anthocyanins, and a plethora of polyphenolic compounds of known antioxidant activity.

Biostimulants in sustainable agriculture

Starting July 16, 2022, the EU regulation 2019/1009, which defines what *is* and what is *not* a biostimulant, is in full force. In brief,

*"[biostimulant products] aim solely at improving the plants' **nutrient use efficiency**, **tolerance to abiotic stress**, **quality traits or increasing the availability of confined nutrients in the soil or rhizosphere**, they are by nature more like fertilizing products than to most categories of plant protection products. They act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates."*

Biostimulants represent "omnibus" products which are meant to address all the points here described and, when integrated in regular agricultural practice, can **help** farmers reduce fertilizer inputs or obtain better product yield with current input use, reduce the yield loss due to stresses like drought and/or soil salinity, all-the-while providing a product which has superior phytochemical contents. The introduction of such products in agricultural practice may prove to be one of the factors to reach the objectives set out by the European Union with the "European Green Deal" in 2020 of developing the member states' economies with a sustainable use of resources [21]. In the context of the Green Deal, EU members developed the Farm to Fork strategy (F2F), which sets out targets for their agricultural systems to adhere to in order to increase their resilience to climate change. One of the outstanding resolutions is the decrease in fertilizer usage by 20% by the year 2030 [22].

But what are biostimulants, exactly?

To understand the multitude of choices that the market offered, in the early 2010s the European Commission requested a literature review study which would aim to provide a categorization of the available formulations. Prof. Patrick du Jardin was assigned to this duty and in 2012, and later in 2015, introduced the concept of "[biostimulant] substances and/or microorganisms", which included humic and fulvic acids, protein hydrolysates, seaweed extracts, chitosan (and other biopolymers), inorganic compounds such as silicon, arbuscular mycorrhizal fungi and plant growth promoting bacteria [23]. In the context of this work, we focused on biostimulant *substances* i.e., ones not deriving from micro-biological sources due to their fascinating, yet highly varied nature. As such, Chapter 2 presents a literature review which was conducted based on answering the most pressing questions surrounding such substances. To do this, we have chosen the most widely available formulates described in scientific literature, which included silicon, seaweed extracts, protein hydrolysates and humic substances, and looked at the literature surrounding their use in the most widely cultivated horticultural crops of cucurbits, leafy vegetables, and nightshades or solanaceous plants. We described *what* the commercial formulations are made of, *how* they work to make the products conform to the EU regulation 2009/1009 in terms of growth, resistance to stress and product quality to the best of the available knowledge, and the *results* of most of the available literature in a span of almost 10 years. We further highlighted the needs and direction

further research must move to in order to shed light on some of the unanswered question regarding these products.

Use of Protein-HydrOlysates as BiOstimulants of vegetable cropS -PHOBOS

Much ado exists on the use of protein hydrolysate biostimulants on food crops, yet caution is still advised when providing a catch-all explanation of their working principles. Whilst Chapter 2 provides a more thorough explanation on what is the state-of-the-art of current scientific literature on these products, on a fundamental level they have been time and time again described as a 'mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources, using partial hydrolysis' [24]. This is to say, aside from any regulatory discourse, every protein matrix can indeed be made into a protein hydrolysate, however no two products are alike [25]. From source material to mode of extraction, from application rate and modality, getting to the bottom of the problem can prove to be difficult as the choices are multiple.

To even out the playing field in the PHOBOS PRIN project, and thus, this work, we have decided to use vegetal-derived protein hydrolysate biostimulants, both commercially available (such as the 'Trainer' and 'Vegamin' formulations), and newly developed for the project ('Cotton-based' and 'Wheat-based' biostimulants). This choice is by no means coincidental for many reasons: first, in their 2017 work, Colantoni and collaborators [26] found through a life cycle assessment that the production of vegetal-derived protein hydrolysate is an environmentally sensible choice when compared to the chemical hydrolysis through which many animal-derived formulations are usually made. Second, it is explicitly stated in the F2F manifesto that the recycling of organic waste into renewable fertilizers is one of the objectives to be reached for sustainable agriculture [22]; this is very much in line with the *ethos* of the vegetal-derived protein hydrolysate. Lastly, we have chosen both commercial formulations as, especially in the case of 'Trainer', they have long been the gold-standard for research on this product category. Just a brief look-up for 'Trainer biostimulant' on the Google Scholar search engine yields around 4260 results! That means, further analysis will prove useful to the achievement of a unified theory on the mode of action of the whole category.

Our research focused on principles found in accordance with the EU Regulation on biostimulants, to:

1) Determine the optimal dosage rate and application modality of protein hydrolysate biostimulants in plants grown in floating system. The research has been performed due to a lack of compelling evidence in the analyzed publications in the literature review. The results of the trail can be found in Chapter 3.

2) Increase nutrient use efficiency i.e., providing increased growth at the optimal *and* suboptimal nitrogen nutrient input. The results of the trials can be found in Chapter 4 and 5.

3) Increase tolerance to abiotic stresses, which in our case consisted of the application of a salt stress. The results of the trials are in Chapter 6 and 7.

4) In all cases, increase phytochemical and nutraceutical contents.

For all trials, we have chosen Lettuce (*Lactuca sativa* L.) as our model crop due to the possibility of cultivation in both substrate and hydroponic growing systems, and in particular as it constitutes Italy's most cultivated leafy green [27]. Furthermore, for Chapters 3 through 7 we have chosen the red cultivar 'Canasta' (Pagano Costantino & F.lli S.R.L, Scafati (SA)) as it is rich in phytochemicals such as vitamins and antioxidants, including anthocyanins, red pigments of health-improving values [28–30].

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Chapter 2

Biostimulant Substances for Sustainable Agriculture: Origin, Operating Mechanisms and Effects on Cucurbits, Leafy Greens, and Nightshade Vegetables Species

Abstract: Climate change is a pressing matter of anthropogenic nature to which agriculture contributes by abusing production inputs such as inorganic fertilizers and fertigation water, thus degrading land and water sources. Moreover, as the increase in the demand of food in 2050 is estimated to be 25 to 70% more than what is currently produced today, a sustainable intensification of agriculture is needed. Biostimulant substances are products that the EU states work by promoting growth, resistance to plant abiotic stress, and increasing produce quality, and may be a valid strategy to enhance sustainable agricultural practice. Presented in this review is a comprehensive look at the scientific literature regarding the widely used and EU-sanctioned biostimulant substances categories of silicon, seaweed extracts, protein hydrolysates, and humic substances. Starting from their origin, the modulation of plants' hormonal networks, physiology, and stress defense systems, their in vivo effects are discussed on some of the most prominent vegetable species of the popular plant groupings of cucurbits, leafy greens, and nightshades. The review concludes by identifying several research areas relevant to biostimulant substances to exploit and enhance the biostimulant action of these substances and signaling molecules in horticulture.

2.1 Introduction

Modern agriculture faces critical challenges that need to be addressed in order to feed the world's population. It is estimated that there are currently over 7 billion people and, based on current trends, in 2050 the global population will reach 9.7 billion [1]. This increase will result in a rise in the demand for foodstuffs that is estimated to be 25 to 70% greater than what is currently produced [2]. Extreme weather events such as extreme heat and cold may be related to global warming-derived climate change [3], a phenomenon to which agriculture contributes by the way of emissions of greenhouse gases [4] and needs to be addressed in order to continue growing foodstuffs and also increasing production. To further add complexity to the matter, there is a necessity for agriculture to cut down on the use of resources, particularly on fertilizers, since the misuse of these chemicals has brought degradation of the land and eutrophication of the water; the misuse of low-quality irrigation water coupled with intensive cropping practices has also brought widespread salinization of the soil [5]. It is estimated that 40% of the total arable land suffers from reduced fertility, and further expansion due to increased needs jeopardizes both plant and animal biodiversity [6]. The scenario

described here poses the problem of increasing yields whilst reducing or minimizing the environmental impact.

The use of plant biostimulants (PBs) seems a valid strategy for the enhancement of sustainable practices. The latest regulatory framework in Europe defines PBs as products that should not be evaluated against their nutrient content; PB effects include increased plant nutrient absorption and use efficiency, tolerance to abiotic stress, and lastly, better produce quality [7]. Regulatory bodies in the US have yet to provide a formal definition; nevertheless, there is one pending approval that is sufficiently similar to what is effective in Europe [8].

Biostimulant substances (BSs), the subject of this work, are a diverse family of products that include silicon, seaweed extracts (SWEs), protein hydrolysates (PHs), and humic substances (HSs). The mechanisms of the biostimulant effects stem from a variety of factors, starting from the source materials and the production methods with which it becomes the final commercial product [9-11]. Product variety notwithstanding, at an abstract level, the stimulation of plant growth and productivity stem from the presence of active molecules such as peptides, algal polymers, and molecular structures that mimic and/or induce the production of phytohormones [11,12], stress-averting antioxidants [13-15], and plant growth regulators [12,16,17]. By modulating the plants' primary and secondary metabolism, PBs elicit a cascade of messages that result in the in vivo results seen in the available literature, which reflect the claims imposed by the European Union for the category [18].

The outlook on the products is generally favorable based on the available literature; however, results are subject to combinatory effects due to the interaction with biostimulant management and environment. The former is due to the application mode criteria that the biostimulant user employs, based upon the mode of application (foliar application, substrate drench, seed coating), dosage regimen, frequency, application timing, and lastly, growing conditions. In their recent review on biostimulant application on fruit trees, Basile and collaborators [19] speculated that the biostimulant effect may not always be consistently efficacious compared to greenhouse-grown plants. The same authors attributed the elevated efficacy in the latter group to a higher application frequency and to the controlled growing environment, which may improve biostimulant uptake, especially when applied via foliar sprays; in particular, this mode of application greatly benefits from high humidity (which can be managed in controlled environments) and leaf porosity, which is a species-dependent characteristic [20].

With these factors in mind, we aimed to collect and sift through the currently available literature on the effects that BSs have on the most widely cultivated species of the horticultural groups of cucurbits, leafy vegetables, and nightshades or solanaceous plants. Other than the familiarity most people have with these groupings, and the clear

economic importance of belonging crops such as tomato (Solanum lycopersicum L.), lettuce (Lactuca sativa L.) and cucumber (Cucumis sativus L.), the large availability of literature on biostimulant applications make them prime candidates for evaluation. In particular, the consulted studies vary in cultivation environment from open field, to greenhouse, to growth chamber, cultivation systems such as soil-based, soilless, and hydroponics, coupled with the plethora of BSs used. To provide an all-encompassing understanding of the mode of actions and/or of the effects these substances have on these important crops, we evaluated the origin, mode of action and the operating mechanisms through which these products modulate plant physiology, and their effects on the wide variety of case studies found in the literature. We tackled the ameliorative effects on abiotic stresses, the increase in plant growth, yield, and product quality attributes. We also envisioned the direction biostimulant science may embody in the future, and where research efforts need to be put forth.

2.2 Origin, Composition, and Mode of Action of Biostimulant Substances

To garner a better understanding of the biostimulant substances found in this review, we have proceeded throughout the body of the work to divide them in silicon, SWEs, PHs, and HSs. The rationale for this order stems from the starting consideration that out of all the considered substances, silicon is the only inorganic biostimulant currently present, and, therefore, should be described first. Furthermore, we have proceeded to divide the organic-derived biostimulants on a market prominence basis, as research shows that SWEs, the most commonly used product category, represent 37% of all the PBs market share, with PHs and HSs following suit, with a combined 50% [21].

2.2.1. Silicon

Silicon is the second most abundant element in the Earth's crust, and while it is universally not considered as an essential nutrient for plant growth, it is proven to have biostimulant action [22]. Sources of this material for use in agriculture include wollastonite (CaSiO3), residues of blast furnaces, and usually, rice-derived straw [23]. Again, though not essential for most plants per se, silicon in the growing medium still provides clear advantages to the grower, such as the mechanical strengthening of tissues that prevents lodging, increases in fruit firmness [24], and also favors the formation of physical barriers to help fend off fungal [25] and insect [26] attacks.

Plant species can be grouped based on their absorption of silicon from the growing medium as high, intermediate accumulators, and excluders. The rationale for this grouping is that depending on the species affinity for the element, absorption may be more, equal or less than what enters through water uptake only; this can be seen by comparing the percent ratio of silicon in dry weights, which is the highest in accumulators [27]. Root absorption of silicon from the nutrient solution is usually made possible by aquaporin-type channels, in particular nodulin 26-like intrinsic proteins

(NIPs) [28]. Interestingly, due to similar structures, arsenite is also transported via the same Si channels [29]. In theory, administering silicon to arsenite-containing mediums could actually alleviate the negative physiological effects of arsenite by way of dilution only.

The literature on the use of silicon is well-centered on its use as a stress alleviator, and a recent review by Zhu and collaborators [30] highlighted its role in regulating ion homeostasis, modulating water balance, and the activity of antioxidant molecules.

Ion homeostasis is fundamental to guarantee adequate growth. A high concentration of salt causes protein and membrane destabilization and also ion imbalances, because Na+ at high concentrations competes for the same high affinity K+ transporter, hence reducing potassium availability [31,32]. Heavy metals such as Al, Mn, and Cu, which were encountered in the available literature, compete with essential elements such as Ca and Mg and, by substituting themselves to the latter ones, disrupt essential reactions [33].

The internal mechanism that plants use to counteract the effects of salt stress is by the exclusion of the dangerous Na+ by expelling it in the apoplast or by moving it to vacuoles. This is conducted by Na+/H+ antiporters of the SOS and NHX type in the plasma membrane and vacuoles, which, when located on the root, can directly expel the toxic ions from the plant [32]. In order for these proteins to function, there is an elevated need for H+ ions to be expelled from the cytosol to form the electro-chemical gradient, which in turn creates the electromotive force that moves sodium away; this is performed by the way of H+-ATPases. Whilst evidence is still uncertain, or at least it seems species-dependent on the role of silicon as a SOS modulator [34,35], its influence has been proven on the upregulation of H+-ATPases [36], even on a plant of horticultural interest such as tomato [37], where LHA1 and LHA2 proteins were upregulated after silicon amendment, and cucumber, where it promoted the expression of vacuolar Na+/H+ exchanger gene NHX1 [38]. In the tomato case, since the plants were grown in a high pH environment (9), it could be inferred that Si could be used to ameliorate pH stress by lowering the rhizosphere pH via the excretion of protons, thus augmenting abiotic stress resistance [37].

Silicon is also used by plants to augment their defenses against the entrance of toxic ions via the root apoplast. Sodium ions manage to cross the symplast through the apoplastic pathway by way of what is called a bypass flow [32]. A bypass flow is formed where the apoplastic barriers, i.e., Casparian bands and suberin lamellae, are not completely developed. Silicon supplementation has been shown to promote the growth of those barriers in some species, including Onion (*Allium cepa* L.), a Si-excluding species of horticultural value [35], thus reducing the bypass flow.

Additionally, related to the apoplast is the mechanisms with which silicon alleviates ion toxicity. The formation of hydroxy-aluminum silicates in the apoplast of the root apex may be the reason for the reduction in apoplastic Al mobility [39], and the binding of excess Mn and Cu to cell walls in cucumber plants [40,41] may explain the increased resistance to an excess of these elements.

Improving water balance is also a way with which silicon alleviates salinity and drought stresses, since the two are alike, as excess soil-borne salt contents increases the osmotic potential of the circulating solution, thus generating a water deficit [42]. As stated before, silicon transport is mediated via AQP-like proteins, which also facilitates cell water intake. The upregulation of silicon-transporting aquaporins as seen in cucumber plants [43], which also translated in higher conductance, may also explain the effect on salinized tomato plants that showed higher water contents compared to the controls [44] and on pepper plants showing an enhanced leaf water potential [45]. The benefits of silicon on plant water balance may also come from the increased amounts of osmoprotectants such as proline and glycine betaine as seen on pepper, cucumber, and tomato plants [46-48].

At last, further explanation on the inner workings of silicon in regard to abiotic stresses is found in ROS response and antioxidant modulation. ROS levels are usually kept in balance by antioxidant molecules, but stressful events can induce plants to produce toxic levels of these molecules that prove costly for growth and yield [49]. Since ROS homeostasis drives organ growth, in particular root growth, and favors germination [50,51], it is then of crucial importance to maintain it. Silicon acts by modulating antioxidant activity and, in particular, improves on the production of ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione peroxidase (GPX) [13,37,52].

As the works included in this review seem to indicate, there is no strong body of evidence suggesting an increase in plant growth, performance, and/or product quality in non-stress situations. Silicon as a biostimulant research seems to be well focused on the amelioration of stresses because, as shown, there is sufficient corroborating evidence in that direction. Regardless, from all the information we have thus far gathered on silicon and its effect on stress control, we can conclude that the use in horticulture seems to be beneficial without any particular side-effects, though there could be a non-defined dose ceiling above which treatments could prove to be detrimental. Contrary to other products, silicon usage is quite easy to recommend since—aside from specific formulations such as silicon nanopowder—it can be easily and conveniently added to nutrient solutions and fertilization regimens by the way of orthosilicic acid and/or silicate salts. Still, as silicon absorption varies on a species-to-species basis, providing a single one size fits all dosage could prove difficult and this could be a new research area,

maybe integrating mixtures of silicon and other BSs with an agonistic molecular approach.

2.2.2. Seaweed Extracts

SWEs are products usually deriving from the water/solvent extraction and hydrolysis from algae biomass of the genera *Ascophyllum*, *Ecklonia*, *Macrocystis*, and *Durvillea* [53], though more are currently under investigation. Production methods are not standardized and often proprietary: in a 2019 review on Ascophyllum nodosum extracts, it was found that eight modes of extraction are currently being employed today [11].

As such, SWEs pose some risks in discussing them as a single entity due to their wildly varied selection of species, the inherent variation of their constituents based on climate and season, and the plethora of extraction modes [11]. Further increasing variation is the inclusion of either multi-species products, such as in the case of 'TAM', a mixture of *Ulva lactuca*, *Jania rubens*, and *Pterocladia capillacea* extracts [54], or non-algal derived matter such as 'Amalgerol', a mixture of oils and Ascophyllum nodosum extracts [55]. In the aptly named paper 'Comparative Transcriptome Analysis of Two Ascophyllum nodosum extract biostimulants: Same Seaweed but Different', Goñi and collaborators [56] showed that two *A. nodosum* extraction methods yield wildly different commercial products, which, in turn, provide for significantly different results in both formulate composition and plant response. To further prove this point, in studies on either lettuce and tomato plants where two *Ascophyllum nodosum* that varied on extraction temperature were tested, Guinan, Dell'Aversana, and their collaborators [57,58] proved that the extracts performed differently in ameliorating salt stress.

Nevertheless, there are some fundamental qualities related to seaweed extracts that can be indicated as responsible for the biostimulant activity and may be shared by the majority of the products. In 2010, when defining the active molecules in SWEs, Cragie [59] divided them into plant hormones such as abscisic acid (ABA), gibberellic acids (GAs), auxins, brassinosteroids [60], and cytokinins (CKs) [11,60,61], growth regulators such as betaines [62] and algal polymers, especially polysaccharides such as alginates, fucoidans, mannitol, and laminarin [11]. Eckol, a bioactive molecule extracted from the seaweed *Ecklonia maxima*, which incidentally has been widely used in a variety of research covered by this work, has shown both an auxin-like and a general growth promoting effect in spinach plants [63]. Furthermore, when evaluating the biostimulant activity of five commercial products derived from the genera Laminaria and Ascophyllum, Ertani and collaborators [61] found that they variably increased root system growth and plant nutrition. In particular, they found that one of the tested Ascophyllum nodosum had higher contents of auxin and cytokinin, which they found to be responsible for the increased lateral root hair production. Other corroborating

evidence of hormone-like effects is found in the enhanced expression of flowering genes in tomato plants [64].

As thus, the hypothesized mechanism of increased plant growth and yield seen in the works detailed here, seem to result from the cascade of signals stemming from the application of the products. By regulating the plants' phytohormone signaling, SWEs can improve nitrogen, carbon metabolism, and the acquisition of important nutrients that result in better physiological states and, thus, better growth [16]. Moreover, as ABA and CKs are of crucial importance in the case of abiotic stresses, they are also related to the quality improvements denoted here. ABA-mediated signaling is linked with the induction of enzymatic and non-enzymatic antioxidant systems [65], which include phenolics, flavonoids, and ascorbic acid that benefit human health [66] and has been seen in the variety of the species covered by this work.

Furthermore, the stress-related biosynthesis of ABA is one of the fastest ways plants respond to unfavorable conditions: an accumulation of ABA reduces water loss by stomata closure, which is crucial in the case of drought and salinity stresses. CKs, having ABA antagonistic effects, may, in some cases, further enhance stress resistance by partially inhibiting ABA accumulation [67]. SWEs rich in ABA-like molecules have proven to inhibit germination and root growth in Arabidopsis, which were reverted when tested on an ABA insensitive mutant [68]. These results are in agreement with a previous 2013 study [69] on A. nodosum extracts, though the concentration of hormones in the extracts were deemed so low that it was speculated that the effects came from hormone production inducing molecules.

Brassinosteroids are a class of phytohormones—or PGRs, depending on the source—that are found in many plant tissues and are needed for plant development and response to stresses. The two known active brassinosteroids, brassinolide (BL) and castasterone (CS), were both found in the commonly used seaweed formulation 'Kelpak' [60]. The mechanisms of the brassinosteroid-mediated amelioration of stresses are still to be completely elucidated, due to the interactions with other factors such as GAs and salicylic acid. However, there is a sufficient amount of evidence pointing to a brassinosteroid-induced modulation of antioxidant activity and a subsequent reduction in oxidative stress induced by drought, salinity, extreme temperatures, and flooding, which was described in a 2015 review by Vardhini and Anjum [70].

Alginate-derived oligosaccharides induced a drought tolerance in tomato plants that determined a higher biomass, lower MDA contents, and a higher proline content and antioxidant activity (SOD and Peroxydase or POD) [14,71].

With the regard of abiotic stresses, we found ourselves agreeing with the hypothesis put forth by Van Oosten and collaborators [72], whereby SWEs with their application

modulates ROS scavenging mechanisms—thus reducing oxidative stresses—reduce ion toxicity by modulating the ABA and CK pathways, therefore, improving membrane stability, and lastly, promoting osmoprotection by increasing the contents of compatible solutes such as proline and providing plants with betaines. By extension, through these mechanisms, SWEs ameliorate stresses in the critical phases of plant biology, which results in an increased plant growth, yield, and antioxidant activity.

A new frontier for SWEs could be as molecular priming agents not unlike what is described by Kerchev and collaborators, which hypothesized that activating phytohormone signaling and antioxidant systems results in better protection when a later stress is applied [73]. Proof of this theory can be found in the results obtained in a recent tomato study [74], where primed tomato seed produced plants showing strong growth and yield when grown in a saline environment. Nonetheless, care should still be taken because, as we emphasized before, the available commercial products are so different in their composition, where providing a catch-all explanation of their workings becomes very hard. A completely new, 'just what works' molecular approach may be needed in order to create standardized products that satisfy the needs of a market that will become more demanding as foodstuff needs become greater and abiotic stresses more common.

2.2.3. Protein Hydrolysates

PHs are commonly defined in the literature as 'mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources, using partial hydrolysis' [75]. Hydrolysis systems include chemical, either acid or basic, and enzymatic by way of proteolysis [9].

The source materials include various protein matrices, which include animal-derived epithelium and connective tissue, plant-derived biomass such as alfalfa and soybean, and algal protein [9]. The interaction between the extraction method and the source material induces a variation among products, which differ by various parameters, such as the free amino acid to protein/peptide rate, amino acid chirality, molecular weight of the constituents and electrical conductivity. When Cavani and collaborators [76] tested 22 different products, they found total amino acidic contents to vary from 5.3 to 52.5%, free amino acidic contents from 0.76 to 19.6%, and electrical conductivity from 3.9 to 20.0 dS m−1. In particular, products stemming from the chemical hydrolysis of the source matrix by way of strong bases or acids under a high temperature and pressure, can lead to products with high free-amino acid contents, racemization, and high electrical conductivity. All of these factors may add up to products, which may prove to cause phytotoxicity symptoms, especially in the case of a high dosage and number of administrations [76,77].

The uptake of PHs biostimulants happens through either foliar or root absorption, and the absorbed peptides and amino acids can be readily transformed in whichever compounds plants need. However, product uptake depends on application, modality, and environmental factors. Substrate application may result in plants taking up only around 6–25% amino acids due to microbial competition [77], whilst foliar uptake is mediated by wind speed, humidity levels, stomata opening and number, and leaf cuticle thickness [20].

As PHs are nitrogen-rich products, the effects on plant growth could very well stem from nitrogen fertilization alone. Whilst large-scale biomass hydrolyzation could be employed in the future to reduce waste, current biostimulant application rates consist of 1–3 L of commercial formulation per hectare of soil, with products themselves having nitrogen contents of 4 to 8% [76]; such figures show that the biostimulation seen in the literature does not depend on nitrogen fertilization alone. As Colla and collaborators [12] summed up in 2017, the mechanisms behind the plant physiological response due to PHs' biostimulant application can be summarized as the increase in root growth due to the hormone-like activities, increased nutrient uptake, the stimulation of carbon and nitrogen metabolism, and the modulation of the antioxidant systems.

The most plausible explanation to the increased root growth is to be found in the presence of signaling peptides, which act as plant growth regulators (PGRs). One such peptide is the root hair promoting peptide, which has been found in a commercially available product [78]. It is probably due to the presence of those molecules that auxin-like activities were multiple studies on either vegetal and animal-derived PHs in both stress and non-stress conditions [15,79], and to further corroborate this hypothesis, new research found out that by molecular fractionation of commercially available products, it possible to obtain specific formulations that could be comparable in efficacy to synthetic auxin in root growth induction [80]. Furthermore, in stressful conditions such as low nutrient availability, protein hydrolysate-dependent root growth may also come from the modulation of salicylic acid production, which may, in turn, induce lateral root growth [81]. However, and understandably, due to dissimilarities in source materials both the increases in root number and growth have proven to show a degree of variance, even when extraction methodology is consistent [82].

Carbon and nitrogen metabolism stimulation by PHs biostimulants is attributed to an increase in the activity of enzymes in the tricarboxylic acid cycle (TCA), and the increase in enzymatic activity in the nitrogen metabolism and uptake, due to the up-regulation of transcript levels related to nitrogen transporters [83].

The modulation of plants' antioxidative systems is probably due to an enhanced cell-to-cell message transduction after the application of the products. In a 2019 metabolomic study on tomato plants, Paul and collaborators [84] found that the plant response to

biostimulant application revolves around the ROS plant signaling network. Among their findings, treated plants showed increased contents of antioxidant compounds such as phenolics and carotenoids. An increased antioxidant molecule content is particularly favorable from a product quality standpoint, and it has been found throughout the consulted literature.

The evidence surrounding the pathways with which PHs work to ameliorate abiotic stress, relates to both product composition and the induction of plants' osmotic regulation and antioxidative systems. PHs contain osmo-regulating molecules to ameliorate drought and salt stresses; some products contain significant quantities of plant compatible osmolytes such as proline, the concentration of which depends on the extraction methodology and protein source—animal or vegetal [85,86]. PHs-mediated plant osmoregulation may also work by the way of eliciting the production of osmolytes such as trehalose that was found upregulated in tomato plants [87]. The second pathway to a better stress resistance is probably due to an enhanced message transduction. Phytohormone and ROS signaling-mediated messages may favor the production of stress-averting antioxidant molecules such as ascorbic acid, tocopherols, and antioxidant enzyme activities, an increase that has been found in several studies [15,86,88], and may further boost the nutraceutical quality of the products.

In conclusion, signaling peptides research may be the future of a particular product category, and since the key to PH biostimulants may very probably lie in these molecules, it would be interesting to see what further research may yield in terms of better performance of the products, and if a different formulation could be made according to satisfy individual needs.

2.2.4. Humic Substances

HSs are described as dark colored, heterogenous aggregates of organic matter, are the result of micro-biotic metabolism, extremely resistant to degradation, and one of the most abundant organic materials on the planet [89]. A traditional—though nowadays criticized—subdivision of this material splits it into the following three groups: humin, the non-water-soluble portion; humic acids (or HAs), soluble in pH > 2 media; fulvic acids (also FAs), which are water soluble [90]. This division, as obsolete as it is, is the most used in all the consulted literature, as it provides a way to produce a meaningful distinction between products, i.e., a humic acid-based product is different than a fulvic acid-based one.

Nevertheless, as Muscolo and collaborators [10] explained in their 2014 article, HSs are now recognized as the structural association of mixtures of small and distinct organic molecules, which are linked together via hydrogen bonds and hydrophobic interactions, and that their diversity is due to different external perturbations and resource usage strategies employed by the ecosystems. This definition suggests that, rather than the

molecular constituents, it is molecular structure and size that seem to be critical in plant–HS interaction. Due to the presence of a high number of oxygenated functional groups (CO2H2, OH phenols, C=O) [91], HSs in the growing media improve plant nutrition by forming complex, stable bonds with micro and macronutrients [17]. While this effect may vary based on the source material, genesis, application dose, and characteristics of the growing medium, it generally results in elevated macro and micronutrient absorption by plants, which may at least partly explain the growth results clearly seen in tomato, pepper, and cucumber plants [92-95].

Indol-3-acetic acid (IAA) and CK content in HS may also explain the improved growth and yield parameters seen in this review. As expected, CK content seems to depend on source material [96] and, likewise, IAA-like activity, but can still be substantial enough to rival the results obtained with synthetic IAA [97], and, thus, elicit increases in root growth [98]. What also may have come into play is the stimulation of root plasma membrane H+ ATPase by auxin-like compounds or nitric oxide-dependent pathways [99], which may create an electrochemical gradient that could facilitate ion uptake [100]. Enhanced nutrient uptake clearly shows an interesting use case for the amelioration of stresses due to alkaline soils, where micronutrients such as Fe are unavailable to plants; water extractable humic fractions have proven to be successful in enhancing the Fe nutrition in tomato plants, even at an elevated soil pH [101], and assisting Fe-deficient cucumber plants in acquiring Fe more efficiently than other organic ligands [102].

What most likely gives the ability of HSs to alleviate abiotic stresses is the interaction with plant roots. While in optimal conditions HSs induce the production of ROS in plants to the point of which excessive doses may actually be detrimental to plant growth [49], it seems that under high stress conditions, they balance excessive ROS response by modulating antioxidant enzymes such as SOD, APX, and POD and determine increases in osmolites such as proline [103-105]. As Garcia and collaborators [99] summarized, the effect of HSs on plant development due to their structure may depend on the induction of signaling networks composed of phytohormones and messengers such as ROS and Ca2+. As with SWEs and PHs, ROS-mediated messages may, in turn, favor the production of human-benefitting phytochemicals, and the evidence points to this being the case in most of the studied species in this work.

In their review, Shah and collaborators [17] called HSs plant tonic for the multitude of effects they have on plant growth and development and advocated for research of the mechanisms that govern HSs-induced effects. We also express the need to further explore HSs' use in stressed horticultural plants, as they represent widely cultivated and often lucrative cash crops. Widespread research in this area may also mean the future widespread adoption of HSs, as knowledge regarding the molecular and biochemical

pathways through which they work may standardize results, thus favoring the adoption of underutilized organic waste for humification, which would prove an environmentally friendly use of resources.

2.3. Cucurbits (*Cucurbitaceae*)

2.3.1. Biostimulants Substances to Increase Cucurbit Resilience to Stress

Cucumber (*Cucumis sativus* L.), the model cucurbit and a Si accumulating species [106], has shown to be responsive to silicon treatments. An addition to the nutrient solution of either sodium silicate (Na2SiO3), sodium silicate-derived metasilicic acid (H2SiO3), or engineered nanosilica at the rate of 0.3 [43,107], 0.8 mM [104], and 200 ppm [108], respectively, have proven to significantly increase the germination rates, fresh and dry weights, decrease the sodium content in roots or leaves, and increase the root hydraulic conductivity of salinized cucumber plants. Moreover, a better physiological status, as in higher photosynthetic rate and Fv/Fm, was recorded than the untreated salt-stressed controls, and comparable results were also obtained when combined heat and salinity stresses were applied [109-111].

This is also true for zucchini squash (*Cucurbita pepo* L.) and watermelon (*Citrullus lanatus* Thunb.), where the application of potassium silicate (K_2O_3Si) and silicic acid at the rate of 1 mM and 4 mM silicon, respectively, via the nutrient solution to greenhouse grown-plants has proven to improve the condition of the stressed controls such as the lower net photosynthesis and fruit yield [52,112], and also reduced the fruit weight loss during storage, though not to an extent that increased market life [113].

In water-stressed cucumber plants, silicon treatments exhibited higher leaf area, fresh and dry biomass, antioxidant activity, and yield [114-116].

Iron deficient [117-119] and micronutrient-deprived [119] cucumber plants also benefitted from silicon supplementation, which prevented Fe deficiency symptoms [118,120], an effect that was more evident at a higher pH (6.0 vs. 5.0) [117]. Moreover, silicon partly ameliorated zinc deficiency symptoms [119]. Furthermore, silicon has also shown a protective effect against the excess concentration of ions, particularly aluminum [120], manganese [41,121-123], and copper [40,124].

Lastly, silicon was found to be effective against the symptoms of cucumber autotoxicity, an intraspecific allelopathy that limits germination rates, seed vigor, and root growth [123,125].

We were able to find evidence on the effects of SWEs based on one article by Rouphael et al. [126] on greenhouse-grown zucchini squash plants subjected to three salinity levels (20, 40, and 60 mM). When averaged over salt treatments, the five, bi-weekly foliar applications of the commercial *Ecklonia maxima* extract 'Kelpak' at the rate of 3 mL L−1 improved marketable yield and shoot dry biomass by 12 and 17.4%,

respectively, compared to the untreated controls. Moreover, salinized plants produced better quality fruits, as expressed by the TSS content and darker color.

Fe-deprived (10% of the full-strength nutrient solution, or 4 μmol) cucumber plants grown in a growth chamber and subjected to two weekly treatments of the 'Trainer' PHs at the rate of 3 mL L−1 showed double the shoot iron contents when compared to the untreated controls. Moreover, whilst the Fe-derived controls showed a 30% reduction in the relative chlorophyll content compared to the full-strength solution, biostimulant-treated plants only showed a 12% reduction [127].

Evidence of the effect of HSs on stressed cucurbits is scarce. In a 1999 study by Demir and collaborators [128], HA was applied to cucumbers grown in soil supplemented with 28 and 56 mM of sodium chloride Kg−1 soil. The plants treated with HA showed a higher yield compared to the non-treated ones, though the exact figures were not published.

Table 1 shows an overview of the effects biostimulant substances have on stressed cucurbit crops.

Table 1. An overview of the abiotic stress amelioration, growth improvement, and fruit quality enhancement by biostimulant substances on cucurbits.

Abiotic Stress Amelioration							
Cucurbit	**Growing Conditions**	**Biostimulant Substance**	**Application Method**	**Dosage**	**Intervention Time**	**Effect of Biostimulant Substance**	**References**
Cucumber	Laboratory and Greenhouse	Silicon as engineered nanosilica	Via irrigation	100, 200, and 300 mg L^{-1}	50% before planting and 50% 7 days after planting	100 and 200 mg L^{-1} treatments were most effective at increasing germination parameters and seedling growth under Saline stress.	Alsaeedi et al., 2018
	Growth chamber	Silicon as silicic acid	Nutrient solution	1.4 mM of Silicon		Silicon ameliorated iron and partially ameliorated Zinc and Manganese deficiency symptoms.	Bityutskii et al., 2014
	Growth chamber	Silicon as silicic acid	Nutrient solution	1.5 mM of Silicon		Silicon ameliorated salinity symptoms by increasing photosynthesis and decreasing leaf fluorescence.	Harizanova and Koleva-Valkova, 2019
	Growth Chamber	Protein Hydrolysate 'Trainer'	Foliar	3 mL L^{-1}	Two spray treatments at weekly intervals.	Treated plants showed higher iron contents and chlorophyll content.	Celletti et al., 2020
Zucchini squash	Greenhouse	Silicon as potassium silicate	Nutrient solution	0.1 and 1 mM Silicon		1 mM Silicon increased fruit number per plant and physiological parameters of salt stressed zucchini.	Savvas et al., 2009
	Greenhouse	Seaweed extract 'Kelpak' (Ecklonia maxima)	Foliar	3 mL L^{-1}	Spray treatments at biweekly intervals, starting from 10 days after transplanting	Treated plants showed higher marketable yields of 12%, when averaged across salinity levels.	Rouphael et al., 2017
Watermelon	Greenhouse	Silicon as silicic acid	Irrigation	4 mM Silicon		Silicon treatments increased plant growth and fruit yield, and decreased salt-related oxidative stress	Bijalwan et al., 2021

Plant Growth and Fruit Yield Enhancement							
Cucurbit	**Growing Conditions**	**Biostimulant Substance**	**Application Method**	**Dosage**	**Intervention Time**	**Effect of Biostimulant Substance**	**References**
Cucumber	Greenhouse	Silicon as wollastonite or K_2SiO_3	Irrigation and soil incorporation	125 mg SiO_2 per plant. 2–4–8 g wollastonite L^{-1} soil.	Irrigation treatments 6 days a week, from planting.	No increase in growth and fruit yield was recorded.	Dorais and Thériault, 2018
	Greenhouse	Seaweed extract 'TAM' (*Ulva lactuca, Jania rubens Pterocladia capillacea*)	Foliar	2.5, 3.5, and 5 mL L^{-1}	Bi-weekly treatments during the growing season.	When used in substitution of 25, 50, and 75% of NPK fertilizer TAM elicited a 51.9% average increase in cucumber fruit yield.	Hassan et al., 2021
	Greenhouse	Humic Acid	Foliar and soil application	Foliar at 10–20–30–40 mL L^{-1}, soil applications at the same rate.	Foliar and soil applications at 15 day intervals four weeks after planting.	20 mL L^{-1} foliar and 30 mL L^{-1} soil application elicited 14.9 and 14.5% yield increases.	Unlu et al., 2011
	Greenhouse	Humic Acid	Soil incorporation	0.5, 1, 3, 5 g kg^{-1} calcium salts of 'Actosol', and 'Actosol'.	Incorporation before planting.	Calcium plus 'Actosol' increased yields by 28.7%, versus 14.4 of 'Actosol' alone.	Ekinci et al., 2015
Fruit Quality Modulation							
Cucurbit	**Growing Conditions**	**Biostimulant Substance**	**Application Method**	**Dosage**	**Intervention Time**	**Effect of Biostimulant Substance**	**References**
Cucumber	Greenhouse	Silicon as wollastonite or K_2SiO_3	Irrigation and soil incorporation	125 mg SiO_2 per plant. 2–4–8 g wollastonite L^{-1} soil.	Irrigation treatments 6 days a week, from planting.	No increase in Total soluble solids, Ascorbic Acid. No difference in peel and pulp color.	Dorais and Thériault, 2018
	Greenhouse	Humic Acid	Foliar and soil application	Foliar at 10–20–30–40 mL L^{-1}, soil applications at the same rate.	Foliar and soil applications at 15 day intervals four weeks after planting.	10 mL L^{-1} treatments increased total soluble sugars. 20 mL L^{-1} treatments increased antioxidant activity, carotenoid, lycopene, and beta carotene contents.	Unlu et al., 2011, Karakurt et al., 2015
Watermelon	Greenhouse	Silicon as silicon Hydroxide $(SiOH)_4$	Irrigation	260 mL of formulate ha^{-1}	Bi-weekly treatments, starting 23 days after planting.	No increase in Total soluble solids, Ascorbic Acid. No difference in peel and pulp color.	Toresano-Sánchez et al., 2010

2.3.2. Implication of Biostimulant Substance Treatments on Cucurbit Growth and Yield

Literature in favor of a role of silicon as a growth-improving substance for cucurbits seems to be lacking. In a 2018 greenhouse cucumber study [129] on silicon treatments, either via the soil incorporation of affordable wollastonite or via irrigation with soluble potassium silicate, did not result in significant increases in either growth or yield. Silicon treatments also do not seem to improve cucumber nutritional status in leaf tissues as no significant differences were noted between the micronutrient and macronutrient contents of treated and untreated plants [130].

Conversely, an increase in per-plant yield of 17.3% was recorded in silicon-treated watermelon plants in the second growing season of a two-year experiment (2005 and 2006), which may suggest species-dependent effects [131].

Ten-percent foliar sprays of seaweed extract from the species *Macrocystis pyrifera*, *Grammatophora* spp., *Bryothamnion triquetrum*, *Ascophyllum nodosum*, and *Macrocystis integrifolia*, the first two laboratory made and the latter being commercial products 'FulvimaxAT', 'SeaplantAT', and 'GaiaAT', respectively, were tested on greenhouse cucumber grown in sand and vermicompost against a control irrigated with Steiner solution, a standardized nutrient solution employed in agriculture. While the SWEs-treated plants showed a lower fruit size and weight compared to the nutrient-solution irrigated control, the *Bryothamnion triquetrum* treatments only showed a 7% reduction in fruit weight and an 8.3% reduction in yield [132].

Similarly, Hassan and collaborators [133] exchanged 25, 50, and 75% mineral fertilization of greenhouse-grown cucumber with bi-weekly foliar sprays at the rate of 2.5, 3.5, and 5 mL L^{-1} of 'TAM', an extract derived from *Ulva lactuca*, *Jania rubens*, and *Pterocladia capillacea*. Researchers found that 'TAM' successfully managed to produce a 51.9% average increase in yield compared to the normally fertilized control. These results, probably due to elevated nutrient use efficiency, show a possible usage of SWEs in reducing the fertilizer input.

HSs testing on cucumber plants goes a long way, as we found a 1981 paper [93] in which varying concentrations of FAs from 20 to 2000 ppm were administered to growth chamber-grown cucumber plants in addition to a Hoagland solution. The study showed significantly improved physiological parameters such as shoot height and length, leaf, and flower number, and also enhanced nutrient (phosphorous, potassium, calcium, magnesium, copper, iron, zinc) concentrations in shoots.

Humic acid trials on cucumber plants in both growth chamber and greenhouse conditions also yielded similar results, with plants showing significantly higher dry weights compared to the untreated controls, suggesting higher nutrient absorption [92,134].

Moreover, greenhouse-grown cucumber treated with HAs via either foliar spray or soil applications at the respective rate of 20 and 30 mL L^{-1} recorded higher fruit yields of 14.9 and 14.5% [135]. Ekinci and collaborators, who tested varying dosage rates of HA soil supplementation (0.5, 1, 3, and 5 g kg^{-1} substrate) found that the addition of 3 g kg^{-1} of a substrate of calcium salts of the commercial HA formulate 'Actosol' provided 28.7% yield increases compared to the untreated control, and 14.4% compared to 'Actosol' alone [134].

Nevertheless, the literature shows that high dosages of HSs may actually be detrimental to the growth of cucurbits. Rauthan and Schnitzer [93] found that an over 300 ppm concentration of nutrient solution-dissolved FAs had proven detrimental to the effectiveness of the treatment, and similar effects were later denoted when cucumber seedlings were grown in greenhouse conditions in potting mix amended with varying concentrations of humates deriving from food waste and pig manure vermi-composts [136]. Whilst food-waste-derived HS was effective at increasing the shoot and root dry weights (28.6 and 18.5%, respectively) compared to the untreated controls at the lower dosage of 50 ppm, the latter pig-derived compost, other than being ineffective at lower dosages, i.e., less than 500 ppm, induced a reduction in the same parameters when applications were higher than 500 ppm, thus showing a variation between humate sources.

Further evidence is found in a watermelon study, where seedlings grown in a shade house and sprayed with various concentrations (0.4, 0.8, 1.19, and 1.59 mL of formulate per seedling) of commercial product 'Humitec' showed higher-than-control growth at the first three dosage regimens that regressed when the dosage was increased [137].

Table 1 shows an overview of the growth and yield-promoting effects biostimulant substances have recorded on cucurbit plants.

2.3.3. Cucurbit Fruit Quality Modulation after Biostimulant Applications

From the limited available literature, silicon does not seem to increase quality parameters such as total soluble solid and ascorbic acid content in greenhouse-grown zucchini squash, cucumber, and watermelon plants [113,129,131]. The visual aspect of the cucumber and watermelon fruits such as peel and pulp color also did not show significant differences following silicon treatments in either study [129,131].

Increased quality parameters in cucumber plants treated with HAs were found in two studies, which tested dosage rates and modes i.e., foliar and soil applications. In a 2011 greenhouse experiment [135], it was found that both applications modes increased fruit firmness, with the highest increment of +17.2% recorded by the 20 mL HA L^{-1} treatment group, when compared to the untreated control.

Nevertheless, the authors found out that foliar treatments might be the best use-case for improving cucumber fruit quality, as a 10 mL L^{-1} foliar regimen yielded the highest increments of total soluble sugars (+14.4%) and reducing sugars (+25.3%). In a later study by the same authors, a further increase to 20 mL L^{-1} foliar applications recorded the highest increases in fruit antioxidant activity, either lipophilic (+31.7%) and hydrophilic (+148%), total carotenoids (+74.2%), lycopene (+120.8%), and β-carotene (+92.8%) contents compared to the untreated controls [138].

Table 1 shows the cucurbit product quality modulation after biostimulant applications.

2.4. Leafy Vegetables

2.4.1. Biostimulants Substances to Increase Leafy Green Resilience to Stress

Lettuce (*Lactuca sativa* L.) seeds treated with 6 mM sodium silicate, in accordance with the results on other species, has proven to either improve or bring seed germination parameters to satisfactory levels when seeds were exposed to saline environments as high as 200 mM NaCl [139]. Moreover, when Milne and collaborators [140] evaluated greenhouse-grown lettuce plants subjected to nutrient solution to which 60 mM NaCl was added, they found that a 2 mM silicon treatment increased shoot and root fresh weights by 71.5 and 75.2%.

In greenhouse spinach (*Spinacia oleracea* L.) plants, the application of 50 mM NaCl kg^{-1} of soil and boron at the rate of 50 mg H_3BO_3 kg^{-1} resulted in higher fresh weights (+16.7 and +19.9%) compared to the unstressed control [47], which could be explained as the species is fairly tolerant to salinity, up to a soil salinity equivalent level of 4.5 dS m^{-1} [141]. Even still, a silicon supply at the rate of 2 mmol silicon kg^{-1} of soil resulted in higher fresh weights, in addition to an improved antioxidant activity compared to both non-saline control, boron, and saline treatments [142].

Further proof of the amelioration of boron toxicity by silicon treatments is found in a study by Gunes and collaborators [143], where silicon improved root dry weights and reduced the severity of leaf symptoms compared to the boron-stressed controls. Silicon-grown plants also showed lower malondialdehyde (MDA) and proline contents, suggesting lower oxidative damage and better osmotic balance [142,143].

Arsenic toxicity in lettuce due to arsenite and arsenate, paired with silicon administration was also evaluated in a 2015 growth chamber study [144]. Nutrient solution applications of arsenite and arsenate on lettuce over 0.1 μmol resulted in a decrease in fresh weight, in particular with the former treatment. Nutrient solution treatments at the rate of 1 mM potassium silicate decreased the effects of the arsenic-containing compounds and, in particular, across arsenate and arsenite treatments, increased the plant dry and fresh weights by 7 and 21% and by 5 and 14% for arsenate and arsenite, respectively.

The *Ascophyllum nodosum*-based, commercial SWE 'Improver' at the 0.3% rate improved the germination rates of heat-stressed (30 °C) spinach seeds by 25%, compared to the control. Moreover, seed priming with the biostimulant resulted in seedlings with a lower hydrogen peroxide and a decreased MDA content, suggesting lower oxidative damage [145]. Salt stress protection by SWEs was tested by using two commercial A. nodosum extracts obtained using two different extraction methods. Greenhouse-grown lettuce plants subjected to 80 mM NaCl stress were treated with an addition to the nutrient solution of varying concentrations of high (>125 °C, 'Super Fifty') and low (<75 °C, 'Ecolicitor') temperature extracts. 'Super Fifty' treatments proved to be the best performing by expressing comparable fresh weight numbers to the non-stressed controls, and a 42.53% increase when compared to the saline-stressed control, when tested at the lowest rate of 0.4 mL biostimulant L^{-1} nutrient solution. Furthermore, by determining the antioxidant activity of the two products, researchers found a 32-fold difference in favor of the high temperature extract, suggesting that the extraction method has a role in determining the extract properties [57].

The effect of a *A. nodosum* extract on potassium deficiency symptoms was also tested on greenhouse-grown lettuce. The foliar application of a solution containing 1% of the extract on potassium-deficient plants resulted in improved growth parameters that were comparable to the non-stressed controls. Moreover, treated plants showed higher photosynthetic activity, even when compared to the non-stressed controls and lower leaf fluorescence (F_v/F_m), thereby indicating a better physiological state [146].

Drought-stressed spinach plants grown in a growth chamber and treated with *A. nodosum* extract 'Stimplex' with various application modes (0.5% solution foliar, 50 mL of 0.5% drench, and combined applications) showed significantly higher leaf fresh and dry weights than the control treatment, with both foliar and drench being equally effective. The physiological parameters such as net photosynthetic rate, stomatal conductance, and transpiration were also increased by all treatments by 25, 71, and 42%, respectively [147]. Evidence of the effect of PHs on stressed leafy vegetables are found in three lettuce studies. Greenhouse-grown and salinized plants treated with either root or combined foliar and root application of PHs 'Trainer' at the rate of 2.5 mL biostimulant L^{-1} showed higher shoot fresh weight compared to the salt stressed and unstressed control.

Furthermore, treated plants showed a higher root growth in length and diameter, which, in the combined treatment, translated into a 76% higher root surface. This, coupled with the 25.8% higher photosystem II quantum efficiency (Fv/Fm) obtained across biostimulant treatments [148], shows the potential of PH in ameliorating stresses.

Similar results were recorded in a 2017 greenhouse study, but whilst the same 'Trainer' biostimulant was employed as a foliar spray at the rate of 2.5 mL of

biostimulant L^{-1} solution, it was augmented with a microbial biostimulant that may have interacted with the product. Nevertheless, a better tolerance to the alkaline (pH 8.1) nutrient solution was recorded with the same metrics (shoot fresh weight, root surface, and PSII quantum efficiency) [21].

Lastly, both lettuce and baby lettuce grown in non-fertilized plots and treated with weekly foliar sprays of the aforementioned biostimulant at a rate of 3 mL of biostimulant L^{-1} showed a comparable yield to lettuce fertilized with 10 kg ha^{-1} of N [149,150]. The treated lettuce plants also showed a better physiological status than their untreated control, as shown by higher soil plant analysis development (SPAD) values and enjoyed better stress protection measure by the higher lipophilic (+23.3%) and hydrophilic (22.4%) antioxidant activities [149].

Table 2 shows an overview of the effects biostimulant substances have on stressed leafy vegetables.

Table 2. An overview of the abiotic stress amelioration, growth improvement, and fruit quality enhancement by biostimulant substances on leafy vegetable crops.

Abiotic Stress Amelioration							
Leafy Vegetable	**Growing Conditions**	**Biostimulant Substance**	**Application Method**	**Dosage**	**Intervention Time**	**Effect of Biostimulant Substance**	**References**
Baby lettuce and Lettuce	Plastic Tunnel	Protein Hydrolysate 'Trainer'	Foliar spray	3 mL L^{-1}	Spray treatments at 7 day intervals, starting from three weeks after sowing.	Unfertilized plants showed comparable yield to lettuce amended with 10 Kg ha^{-1} of N	Di Mola et al., 2019 Di Mola et al, 2020
Lettuce	Laboratory	Silicon as sodium silicate	In solution	6 mM Na_2SiO_3		Improved seed germination parameters compared to salt stressed controls.	Neto et al., 2018
	Greenhouse	Silicon as sodium silicate	Nutrient solution	1,2, and 4 mM silicon		2 mM silicon increased shoot and root fresh weights by 71.5 and 75.2% compared to the 60 mM salt stress control.	Milne et al., 2012
	Growth Chamber	Silicon as potassium silicate	Nutrient Solution	1 mM silicon		Increased plant dry and fresh weights compared to Arsenate and Arsenite stressed controls.	Greger et al., 2015
	Greenhouse	Seaweed extract 'Super Fifty' and 'Ecolicitor' (*Ascophyllum nodosum*)	Nutrient Solution	0.4, 1, 2.5, and 10 mL L^{-1}		'Super Fifty' increased fresh weights of salt-stressed plants up to the non-stressed control.	Guinan et al., 2013
	Greenhouse	Seaweed extract (*Ascophyllum nodosum*)	Foliar	1%	Treatments were administered at 2-week intervals	Treatments ameliorated K-deficiency stress by improving growth to control levels.	Chrysargyris et al., 2018
	Greenhouse	Protein Hydrolysate 'Trainer'	Foliar spray and combined spray and french	2.5 mL L^{-1}	Treatments were administered 5 days after transplanting, and weekly.	Saline-stressed plants recorded fresh weights comparable to unstressed controls. Combined treatments yield 76% higher root surface.	Lucini et al., 2015

Spinach	Laboratory	Seaweed extract 'Improver' (*Ascophyllum nodosum*)	Seed Priming	0.15, 0.3, 0.6, and 1.2%		Improved germination by 25% compared to heat-stressed controls. 0.6% treatment was the best performing	Neto et al., 2020
	Growth chamber	Seaweed extract 'Stimplex' (*Ascophyllum nodosum*)	Foliar spray, substrate drench, combined	0.5% solution for both treatments	Treatments were administered every four days	Both treatment modalities ameliorated drought stress by increasing fresh and dry weights and photosynthetic parameters	Xu e Leskovar, 2015

Plant Growth and Yield Enhancement

Leafy Vegetable	Growing Conditions	Biostimulant Substance	Application Method	Dosage	Intervention Time	Effect of Biostimulant Substance	References
Baby Lettuce	Plastic tunnel	Protein Hydrolysate 'Trainer'	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals. from three weeks after sowing.	Treatments increased yields at below and above optimal nitrogen fertilization levels.	Di Mola et al., 2019
Lettuce	Greenhouse	Protein Hydrolysate 'Trainer'	Nutrient solution, foliar, and combined	0.15 and 0.3 mL 'Trainer' L^{-1} nutrient solution applications, foliar at 3 mL L^{-1}	Foliar treatments after transplanting, and every 6 days. Nutrient solution application when refilled.	Nutrient solution only applications increased green butterhead yield by 82.7%. Combined applications increased red crisphead yield by 55.4%.	Cristofano et al., 2021
	Open Field	Humic acid	Foliar	2 and 4 mL L^{-1}	Spray treatments at 4 and 6 weeks after transplanting.	The 4 mL L^{-1} treatments increased yield by 75.2 and 30.3% in the two growing seasons.	Fouad Fawzy, 2010
	Open Field	Humic Substance	Foliar, soil, and combined applications	3.8 and 3.3 L ha^{-1} soil applications and 1.9 and 1.7 L ha^{-1} foliar for compost and biogas manure-extracted humic substance, respectively.	Treatments were administered at 3, 6, and 9 weeks after transplanting.	Foliar applications of compost-derived humic substance elicited 31.3% in fresh yields in only one cultivar.	Shahein et al., 2014
Spinach	Greenhouse	Seaweed extract (*Ascophyllum nodosum*-based 'Amalgerol' and	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals, starting from 17 days after sowing.	On average, both biostimulants increased yields by 48.3%.	Rouphael et al., 2018

		Ecklonia maxima 'Kelpak')					
	Plastic tunnel	Protein Hydrolysate 'Trainer'	Foliar	4 mL L^{-1}	Spray treatments at six-day intervals, starting at 21 days after sowing.	Across crop cycles and nitrogen fertilizer applications, treatments increased yields by 23.5% and improved nitrogen use and uptake efficiency.	Di Mola et al., 2020
Rocket	Greenhouse	Protein Hydrolysate 'Trainer'	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals, from leaf length above 6 cm.	Across two successive crop cycles treatments increase yield by 11.4%	Caruso et al., 2019

Product Quality Modulation

Leafy vegetable	Growing Conditions	Biostimulant Substance	Application Method	Dosage	Intervention Time	Effect of Biostimulant Substance	References
Baby Lettuce	Plastic tunnel	Seaweed extract 'Kelpak' (*Ecklonia maxima*)	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals, from three weeks after sowing.	33.6% Leaf ascorbic acid increase in non-fertilized plots.	Di Mola et al., 2019
	Plastic Tunnel	Protein Hydrolysate 'Trainer'	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals, from three weeks after sowing.	Higher leaf succulence and leaf carotenoid contents. Antioxidant activity was nitrogen fertilization dependent.	Di Mola et al., 2019
Lettuce	Greenhouse	Protein Hydrolysate 'Trainer'	Nutrient solution, foliar, and combined	0.15 and 0.3 mL 'Trainer' L^{-1} nutrient solution applications, foliar at 3 mL L^{-1}	Foliar treatments after transplanting, and every 6 days. Nutrient solution application when refilled.	Foliar applications only increased total ascorbic acid in green butterhead by 51.2%. Combined applications increased red crisphead hydrophilic antioxidant activity by 21.9%, and total ascorbic acid 5.6-fold.	Cristofano et al., 2021
	Open Field	Humic acid	Foliar	2 and 4 mL L^{-1}	Spray treatments at 4 and 6 weeks after transplanting.	The 4 mL L^{-1} treatments increased leaf phosphorous, potassium zinc, and magnesium contents. Leaf nitrate content was decreased.	Fouad Fawzy, 2010
Spinach	Greenhouse	Seaweed extract (*Ascophyllum*	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals,	Increased leaf potassium and magnesium contents by 25.6 and	Rouphael et al., 2018

		nodosum-based 'Amalgerol' and *Ecklonia maxima* 'Kelpak')			starting from 17 days after sowing.	20.1%, increased phenolic and ascorbic acid contents by 30.7 and 79.1%. Leaf nitrate levels only 4.3% below EU limit.	
	Open Field	Humic acid	Foliar	10% solution at 160 L ha^{-1}	40, 50, and 60 days after germination.	Phenolic and carotenoid increases were deemed significant after the second and third treatment.	Aslam et al., 2016
Rocket	Greenhouse	Protein Hydrolysate 'Trainer'	Foliar	3 mL L^{-1}	Spray treatments at weekly intervals, from leaf length above 6 cm.	Averaged across two crop cycles, higher phosphorous, calcium, polyphenolic contents. Leaf Vitamin C and antioxidant activity were also recorded.	Caruso et al., 2019

2.4.2. Implication of Biostimulant Substance Treatments on Leafy Green Growth and Yield

SWEs seem to significantly enhance the agronomic performance of leafy vegetables. In a 1992 growth chamber spinach study [151], a foliar spray of the *A. nodosum* extract 'Goemar GA 14' showed significantly higher fresh matter production compared to the untreated controls by 12 to 15%. The weight increase was linked to an increase in spinach leaf area and not leaf number, which is the same result that was obtained in 2018 by Rouphael and collaborators [55], who tested 'Amalgerol', a blend of *A. nodosum* and oils, and the 'Kelpak' seaweed extract in greenhouse conditions. A weekly foliar application of both products at the rate of 3 mL of biostimulant L^{-1} of solution resulted in equally increased yields by 48.3% and leaf area by 15.4%. The results were linked to higher SPAD values, thus indicating better photosynthetic performance, and better potassium and magnesium nutrition. The results are shared—though a lower (14.4%) increase in yield was obtained—with La Bella and collaborators [152], who similarly employed the E. maxima extract 'Kelpstar' in a protected environment.

Interestingly, the same results were not obtained in a separate growth chamber study by Xu et al. [147] using a different, but still *A. nodosum*-based, biostimulant, 'Stimplex'. No significant differences were denoted in leaf number and leaf area between the treatments at the manufacturer suggested rate of 5 mL of biostimulant L^{-1} of solution, thus furthering the argument about the different seaweed products being variably effective.

The *E. maxima* extract 'Kelpak' on lettuce showed similar results to what was obtained on spinach, with the SW treatments producing the highest marketable yield at the highest (equivalent to 30 kg N ha^{-1}) fertilization levels, compared to a control and two biostimulants, one being PHs 'Trainer' and the other being a tropical plant extract, and generally significantly higher than the control results at lower fertilization levels [149].

A 2013 assessment [153] showed that foliar sprays at 2.5 mL L^{-1} of the 'Trainer' PH did not seem to have meaningful effects on lettuce grown in a floating system with full strength nutrient solution. The findings seem to be confirmed by a later study [154] though, and contrary to biostimulant ethos, the authors preferred to replace the nutrient solution inorganic nitrogen with the Amino16 PHs. Nevertheless, what emerged from this study is that crop uniformity was substantially increased by the application of the product, with lettuce in the 200–249 g weight class being significantly higher in number compared to the inorganic N supplementation (55% vs. 24–28% of control, which provided nitrogen in inorganic form).

Xou and Mou [155] provided proof of an increase in fresh biomass due to fish-derived PHs by recording significantly higher leaf numbers (+27%), and shoot and root weights;

whilst leaf numbers could be a reasonable indicator of higher marketable yields, they are not an absolute measure. However, Di Mola and collaborators later recorded increases in the yields of tunnel grown baby lettuce after treatments with 'Trainer', which were significant at below-and-above-optimal levels of fertilization (0, 10, and 30 kg N ha^{-1}). Significant differences were also denoted in the growth-related parameters such as leaf area index (LAI) and SPAD values, though no exact figures were published [149].

However, more recent evidence has shown that results may differ from the genotype, dosage, and application mode. By greenhouse-testing two varieties differing by shape and pigmentation in a floating raft system, Cristofano and collaborators [156] found that the green butterhead 'Ballerina' cultivar favored nutrient solution applications of the 'Trainer' biostimulant, with the 0.15 mL L^{-1} nutrient solution treatment showing an 82.7% higher yield compared to its control. Conversely, the red crisphead 'Canasta' preferred combined foliar and nutrient solution treatments, which at the rate of 3 mL L^{-1} foliar and 0.35 mL L^{-1} nutrient solution, promoted a 55.4% increase in fresh yield.

Two 2019 greenhouse-rocket studies by both Caruso and Di Mola [157,158] showed a different trend; in the former study and when averaged over two successive crop cycles (winter and winter–spring), plants treated with 'Trainer' PHs at the 3 mL L^{-1} rate showed a significant (11.4%) increase in marketable yields, with the higher recorded values of SPAD in line with the consulted literature [157]. In the latter, also employing the same formulate, a 33% increase in baby rocket's marketable yield was found, even when averaged across nitrogen treatments and successive harvests [158]. The results were also confirmed by a subsequent study by Giordano and collaborators [159], who tested a 4 mL L^{-1} dosage of the same biostimulant and obtained a 50.7% increase in rocket yield when averaged across three consecutive harvests.

Two different spinach studies using the 'Trainer' biostimulant showed similar yet distinct results [55,160]; while the latter found significantly higher yields (57%), SPAD values, and nitrate contents compared to the untreated controls, the former found yield increases only at suboptimal levels of nitrogen fertilization (0–15 kg N ha^{-1}), but, nevertheless, the spinach yield at the 15 kg N ha^{-1} level was not significantly different than the 30 kg ha^{-1} control treatment. A more recent study by Di Mola and collaborators [161] shed light over the previous results obtained by the previous authors by also trialing spinach growth and nitrogen applications and finding that foliar applications of 'Trainer' at the rate of 4 mL L^{-1} increased yields by 23.5%, but, interestingly, improved N-use efficiency and N-uptake efficiency compared to the untreated plants, by 18.8 and 73.3%, respectively.

Evidence found in the literature regarding the use of HSs on lettuce, point to it being generally effective in increasing growth performance, though some specifications are to

be made. Dosage rates were evaluated in a 2010 study [162] on open field-grown lettuce sprayed with HAs at the rate of 2 and 4 mL HA L^{-1}. Across two growing seasons, the best performing 4 mL L^{-1} group recorded a 75.2 and 30.3% yield increase, respectively, compared to the untreated control. The treatments also showed, on average, 30.3% higher leaf numbers, a result shared with what was found by Hernandez and collaborators [163]. Moreover, the results were coupled with higher dry weights and lower nitrate contents than the control, and the higher dosage also yielded higher potassium (+16 and +12.2%) and phosphorous (+24 and 12.9%) contents in both growing seasons [162].

Similar results were obtained in two other lettuce studies by Mirdad and Kiran [164,165], who both tested combinations of fertilization and HSs applications. The former author tested soil applications of Has at varying rates (30 through 90 L ha^{-1}), which were evaluated at two different nitrogen fertilization levels representing non fertilized and optimally fertilized. In the non-fertilized plots, 90 L ha^{-1} HA determined an increase in growth parameters such as stem length (+28.8%), root length (+38%), shoot fresh weight (+150.8%), and dry weight (+159%) and also an increase in leaf nitrogen (24.15%), potassium (67.1%), magnesium (29.6%), and manganese (+75%) contents.

Interestingly, in both studies, a growth regression was denoted when combined HA and fertilizer were applied. Shahein and collaborators [166] also found differences in the cultivar response to biostimulant applications, as the two tested lettuce cultivars 'Dark Green' and 'Big Bell' reacted differently to mixtures of HSs derived from different matrices and supplied as a substitution for 50% of the mineral fertilizer. In fact, the foliar treatments of compost-derived humic substances elicited significantly higher fresh weights only in 'Big Bell', with a 31.3% increase in fresh yields across two growing seasons. Similar results to lettuce plants were obtained in 2015 [167] on spinach plants treated with foliar sprays of HA at the rate of 4.76 and 9.52 L ha^{-1}. Averaged across N fertilization, HA treatments were the highest yielding across two different growing seasons, with the highest concentration being most effective at increasing the plant fresh weight (+24.6 and 63% in the 2013 and 2014 seasons). Still, and contrary to lettuce studies, no macronutrient differences were denoted between treatments for phosphorous and potassium, except for nitrogen (+9.7 and +9.6% for each season).

Table 2 shows an overview of the growth and yield-promoting effects biostimulant substances have recorded on leafy vegetable crops.

2.4.3. Leaf Quality Modulation after Biostimulant Applications

Rouphael and collaborators [55] found, in a 2018 greenhouse study, that weekly foliar treatments of the 'Kelpak' extract and the combined seaweed and oil extract 'Amalgerol' at the rate of 3 mL L^{-1} increased spinach leaves' potassium and magnesium contents by 25.6 and 20.1%, respectively. The same study also found a 30.7% increase in leaf

phenolics and a 79.1% increase in total ascorbic acids. However, both biostimulants also recorded an average 41.1% increase in leaf nitrate contents, which, while still remaining under the EU limit of 3500 mg kg^{-1}, was just 4.3% below.

Later research on greenhouse-grown lettuce plants found significantly increased leaf succulence by 7.8% and carotenoids content by 16.8% after plants were foliarly treated with a 3 mL L^{-1} solution of the 'Kelpak' extract. The total ascorbic acid content increase was found to be N-fertilization-dependent, as it was significantly (+33.6%) higher than the untreated control only in non-fertilized plots [149].

The PH 'Trainer' was the treatment of choice for the consulted literature on this particular biostimulant grouping.

When averaged across four fertilization levels (0–10–20 and 30 kg ha^{-1}), tunnel-grown baby leaf lettuce treated with a 3 mL biostimulant L^{-1} foliar spray showed 16.4% higher leaf succulence compared to the untreated controls and increased leaf pigment contents, with carotenoids and chlorophyll being 11.6 and 12.8% higher, respectively [149].

The same authors also noted that the antioxidant activity increases were nitrogen-fertilization-dependent as lipophilic and hydrophilic activity were 23.3 and 22.4%, respectively, higher in the plants grown in unfertilized plots, whereas the hydrophilic activity was 40.6% higher at the highest fertilization level.

Furthermore, Cristofano and collaborators [156], who tested the same formulation on lettuce grown in a floating raft system in greenhouse conditions, found the increases in lettuce quality parameters to be genotype-, treatment dosage-, and modality-dependent. In fact, of the two tested cultivars, the red butterhead 'Canasta' recorded the highest hydrophilic antioxidant activity (+21.9%) and total ascorbic acid contents (a 5.6-fold increase) when both the foliar and nutrient solution treatments were applied, whilst the green butterhead 'Ballerina' recorded its highest contents of ascorbic acid (+51.2%) when only foliarly treated.

When weekly foliar sprays of the same formulate at the 3 mL L^{-1} rate on greenhouse-grown spinach were investigated, a 36.4% increase in potassium contents was found, coupled with a 30.7% increase in leaf phenolic contents and a 79.1% increase in the total ascorbic acid content [55]. A later experiment, also on spinach, was undertaken by Carillo and collaborators [160], who also investigated nitrogen fertilization (0–15–30–45 kg ha^{-1}) and 'Trainer' applications at the 4 mL L^{-1} rate. Researchers found that across fertilizer levels, the treated plants showed 12.9% higher leaf phosphorous, 12.8% higher calcium, 10.3% higher magnesium, but 10.8% lower polyphenolic contents. Moreover, a 43.5% increase in amino acid content was denoted, of which the essential amino acids glutamic acid and alanine received a boost of 17.2 and 39.7%, respectively.

Consistently with previous studies and when averaged across consecutive winter and winter–spring crop cycles, foliar sprays of 'Trainer' on rocket yielded plants with higher phosphorous, calcium, and polyphenolic contents (+11.9, 9.5, and 10.8%, respectively), but also of higher ascorbic acid contents (+11.9%) and hydrophilic (+18%) and lipophilic activities (34.4%) [157]. However, the same results were not obtained by a subsequent study [159], using the same biostimulant (albeit at the higher 4 mL L^{-1} dosage regime) and greenhouse conditions, also in the winter–spring cycle. After three consecutive harvests, researchers found that the leaf potassium increase was only significant at the third harvest, with a 44.8% increase and, likewise, magnesium, which increased by 43.4%. The leaf tissue calcium increase was more consistent, with a 30.6% increase when averaged across successive harvests.

Lettuce plants treated with HSs showed no significant increases in total soluble solids content in two separate studies [162,166]. However, Fawzy found that across two growing seasons, two bi-weekly foliar applications of HAs at the rate of 4 mL L^{-1} on open field-grown lettuce increased leaf phosphorous, potassium, zinc, and magnesium contents by 18.5, 14.2, 9.9, and 30%, respectively, and consistently decreased the leaf nitrate content by 19.6% [162]. Aslam and collaborators [168] also tested the foliar application of 10% HA on spinach. The results from the open-field trial suggested that treatment repetition may be an important factor when product quality is a consideration: the recorded increases in phenolics (28.9% on average) and carotenoids (76.5%) were only deemed significant after the second and third treatments, respectively.

Table 2 shows an overview of the leaf quality modulation after biostimulant applications.

2.5. Nightshades (*Solanaceae* Juss.)

2.5.1. Biostimulants Substances to Increase Nightshade Resilience to Stress

Most research is focused on pepper (*Capsicum annum* L.) and tomato (*Solanum lycopersicum* L.) plants, and it is shown that excess salinity causes decreases in many physiological and growth parameters from germination to fruit yield. From the consulted literature, nutrient solution application of soluble silicon is the most studied and varied by rate (0.5 through 3 mM) and type i.e., engineered nanosilica vs. silicate salts such as sodium and potassium silicate.

Salt stress studies show that low (0.5 mM) silicon supplementation can roll back germination percentages up to control levels, when up to a 150 mM NaCl stress is applied [169]. The increased plant growth parameters of salt stressed tomato plants such as plant fresh and dry weights [44,170] are further outlined by the increased mineral contents [171]. The ameliorative effect of silicon supplementation has been found to be cultivar-dependent, as a growth chamber study by Wasti and collaborators [172] on two tomato cultivars, 'Rio Grande' and 'Moneymaker', illustrated that calcium silicate

treatments were more effective, and at lower dosages (2 mM vs. 4 mM), in the former cultivar at reducing the effects of 100 mM NaCl stress. Silicon supplementation at the rate of 1 mM significantly increased tomato yield compared to the non-stressed and salt-stressed controls (12.4% when averaged across salt treatments) and significantly decreased the blossom-end rot symptoms by 46.5% [171].

Studies on pepper plants show similar results: in a 2018 growth chamber study [45], it was found that salt stressed plants supplemented with 2 mM soluble silicate showed a higher dry weight (results also found by Manivannan and collaborators [173]), leaf area, and photosynthetic rate compared to the stressed control. Again, the efficacy of the treatment was found to be cultivar-dependent, as it was more pronounced in the salt-tolerant 'Karaisoli' versus the sensitive 'Demre'.

Silicon treatments were also proven to be beneficial in the case of drought stress. The treatments improved the growth of drought-stressed (simulated through polyethylene glycol 6000) tomato plants in a genotype dependent manner, by differently modulating the stress response mechanisms. In particular, researchers found that silicon supplementation was more advantageous to the drought sensitive 'FERUM' line, compared to the resistant 'LA0147' line [174]. The effects of silicon supplementation also include increased tomato plant shoot and root growth, chlorophyll contents, and quantum efficiency compared to drought-stressed controls; increased transpiration rates versus the control; and improved leaf relative water content (RWC) in stressed pepper plants, whilst maintaining nitrogen metabolism, which manifests as higher nitrate reductase activity [46,175,176,177].

A. nodosum commercial extracts, 'Rygex' and 'Super Fifty', were tested in a greenhouse experiment on nutrient deprived (70% of the basic nutrient solution) and salinized 'Microtom' tomato plants in a 2018 study [178]. Both treatments mitigated the effects of salinity by increasing the potassium, calcium, and nitrate contents and lowering the sodium and chloride contents compared to the stressed controls; however, when averaged across nutrient and salt stresses, the 'Rygex' treatments caused a reduction in the fruit fresh weight of 17.1%. GC-MS analysis conducted in the previous research showed that the two biostimulants, whilst produced from the same biomass, yielded different products. The discovered differences in the amounts of bioactive compounds and minerals, with 'Super Fifty' delivering four times more potassium and magnesium, whilst 'Rygex' delivered seven times more calcium, are indicative of some variation between the two treatments.

In a later study, the same two commercial products were found to be successful in open field conditions at priming tomato plants before a saline stress was applied: biostimulant drip-irrigated plants were found to have improved water efficiency, improved shoot-to-to root ratio, and were ultimately better yielding (48.7 and 70% for

Rygex and Super Fifty, respectively) than the untreated controls [74]. An A. nodosum extract was also tested in salt-stressed pepper plants in a greenhouse study [179]. Compared to the salt stressed (100 mM NaCl) controls, the plants treated by drip irrigation with the biostimulant showed a significantly higher dry matter and fruit yield (though the exact figures were not published) and, interestingly, a reduction in the plant proline content and ROS-scavenging mechanisms such as SOD and CAT, which may suggest a better plant oxidative state.

Drought-stressed tomato plants also showed similar results, with foliar treatments of the *Ascophyllum nodosum* extract 'Bio-algeen S92' eliciting the production of antioxidants and phenolics and with treated plants showing a 21.6% higher leaf area and a 20.3% leaf chlorophyll content. Moreover, the plants treated with the product had a significant boost in fruit yield of 65.4% compared to the stressed controls, and also manifested the highest fruit lycopene and flavonoid contents [180].

Tomato plants grown in a growth chamber with an iron-derived nutrient solution (4 μmol, or one tenth of the full-strength solution) and subject to two weekly treatments of PH 'Trainer' at the rate of 3 mL L^{-1} showed a 50% increase in biomass when compared to the untreated plants [127]. Iron reductase activity was reverted to the condition of control plants supplied with a complete nutrient solution when the biostimulant was applied, whereas the untreated, iron deficient controls registered a 70% increase. Interestingly, the iron stored in biostimulant-treated shoot tissues in the low iron group was almost two-fold higher than the untreated control.

Francesca and collaborators [88] employed the PH 'CycloFlow' on open field-grown tomato plants, to which a 50% water deficit was applied. Root drench treatments yielded plants with 51% more pollen viability and 70% higher fruits per plant, which were, on average, weighing 95% more. All in all, the researchers obtained a six-fold increase in the final yield compared to the untreated controls.

A 2004 study [181] on salt-stressed tomato plants showed that HA supplementation to the growing medium at a rate of 500 through 2000 mg HA kg^{-1} significantly improved seed germination, shoot length, and leaf numbers. Moreover, HAs increased both the shoot and root micro (copper, iron, manganese, and zinc) and macronutrient (nitrogen, phosphorus, potassium, sulfur) contents in a dose dependent manner, with the 1000 mg kg^{-1} dose being the most effective.

A later greenhouse study [182] confirmed the findings, but also demonstrated that repeated root applications of HAs at the rate of 750 mg L^{-1} significantly increases the number of fruits per plant and fruit mass, thus increasing, on average, the fruit yield by 27.5%. Conversely, increasing HA treatments significantly lowered quality traits such as

TSS and fruit juice EC (an average 11.1 and 12.2% reduction, respectively) compared to the saline-stressed controls.

The results obtained on tomato plants were also found on hot pepper (Capsicum annum L.) plants: the application of HAs yielded better growth and yield parameters compared to the stressed and non-stressed controls, while also improving on the plants' nutrient status (higher nitrogen, phosphorous, and potassium) [183,184]. The best results were obtained by adding calcium nitrate to the HA treatments. However, it has to be noted that two consecutive studies (2016 and 2017) from Bacilio and collaborators [184,185] under greenhouse and field conditions on pepper cultivars 'Jupiter' and 'Ancho San Luis' provide evidence of HA treatments being particularly beneficial only to salt-susceptible cultivars at high dosages.

Table 3 shows an overview of the effects biostimulant substances have on stressed solanaceous crops.

Table 3. An overview of the abiotic stress amelioration, growth improvement, and fruit quality enhancement by biostimulant substances on Solanaceous vegetable crops.

Abiotic Stress Amelioration							
Solanaceous Crop	**Growing Conditions**	**Biostimulant Substance**	**Application Method**	**Dosage**	**Intervention Time**	**Effect of Biostimulant Substance**	**References**
Tomato	Laboratory	Silicon as silicon Nanopowder	Priming	0.5, 1, 2, and 3 mM		0.5 mM silicon rolled back germination rates of 150 mM NaCl stressed seeds up to control (0 NaCl) levels.	Almutairi, 2016
	Growth Chamber	Silicon as calcium silicate	Nutrient solution	$CaSiO_3$ at the rate of 2 and 4 mM		Silicon amelioration of salt (100 mM) stress was found to be cultivar and dosage-dependent.	Wasti et al., 2017
	Greenhouse	Seaweed Extracts 'Rygex' and 'Super Fifty' (*Ascophyllum nodosum*)	Substrate Drench	0.25 and 0.20% solution for 'Rygex' and 'Super Fifty'	Treatments were applied every two weeks	'Super Fifty' increased plant fresh weight by 6%, no yield increase. 'Rygex' decreased fruit fresh weight by 17.1%.	Di Stasio et al., 2018
	Greenhouse	Seaweed Extract 'Bio-algeen S92' (*Ascophyllum nodosum*)	Foliar Spray	0.20%	Two treatments were applied immediately after transplanting, and fifteen days later.	Compared to drought stressed plants, treated plants had higher plant growth and fruit yield	Murtic et al., 2018
	Growth Chamber	Protein Hydrolysate 'Trainer'	Foliar Spray	3 mL L^{-1}	Two treatments were applied at 8 and 15 days after planting	Compared to iron deficiency-stressed plants, treatments reduced iron reductase activity and increased shoot iron contents.	Celletti et al., 2020
	Open Field	Protein Hydrolysate 'CycloFlow'	Soil applications	3 g L^{-1}	Treatments were applied at transplanting, and every 15 days	Compared to drought stressed plants, treatments increased pollen viability. Plant yield increased six-fold.	Francesca et al., 2021
	Greenhouse	Humic Acid	Soil applications	750 and 1500 mg L^{-1}	Treatments were applied at 10, 25, and 40 days after transplanting	750 mg L^{-1} treatments increased fruit yield by 27.5% compared to salt-stressed plants.	Feleafel and Mirdad, 2014

Pepper	Growth Chamber	Silicon as potassium silicate	Nutrient Solution	2 mM K_2SiO_3		Silicon increased dry weights, leaf area, and photosynthesis, the effect was cultivar dependent.	Altuntas et al., 2018
	Greenhouse	Seaweed Extract (*Ascophyllum nodosum*)	Soil Drench	1, 2, and 3 g L^{-1}	Treatments were applied every week with irrigation	When compared to 100 mM NaCl salt-stressed controls, treated plant showed higher yield and lower stress related parameters.	Yildiztekin et al., 2018
Hot Pepper	Greenhouse	Humic Acid	Substrate incorporation	750 and 1500 mg L^{-1} and combined HA and calcium nitrate		750 mg L^{-1} treatments, alone and combined with calcium increased growth and yield parameters compared to 100 mM salt stressed controls.	Akladious and Mohamed, 2018

Plant Growth and Yield Enhancement

Solanaceous Crop	Growing Conditions	Biostimulant Substance	Application Method	Dosage	Intervention Time	Effect of Biostimulant Substance	References
Tomato	Greenhouse	Seaweed extract (*Chaetomorpha antennina*)	Seed Priming	Seaweed extract at concentration from 20 to 100%		100% Extract increased plant growth. Tomato yield was increased by 135.9%	Muthu-Pandian Chanthini et al., 2019
	Field and Greenhouse	Seaweed extract (*Ascophyllum nodosum*)	Foliar spray and soil drench	0.2 and 0.5%	Treatments were administered 15 days after transplanting, and every 15 days thereafter.	0.5% spray treatment was the most effective, increasing yield by 63% in field, and 54% in greenhouse.	Ali et al., 2016
	Open Field	Protein Hydrolysate 'Trainer'	Foliar Spray	3 mL L^{-1}	Weekly spray intervals, starting from the early growth of the first fruit truss.	Treated plants recorded 18.6% higher yields due to 19.3% higher fruit numbers.	Caruso et al., 2019
	Greenhouse	Protein Hydrolysate 'Trainer'	Foliar spray	2.5 and 5 mL L^{-1}	Spray treatments at 10-day intervals, starting from 15 days after transplanting.	5 mL L^{-1} differentially increased yields in both tested tomato cultivars. 'Akyra' recorded 13.9% higher fruit numbers, 'Sir Elyan' 28.7 heavier than their respective controls.	Rouphael et al., 2017

Tomato	Greenhouse	Humic substance 'Humicop'	Substrate incorporation	100 L ha^{-1}		Treated plants recorded increased yields by 18.1%.	Abou Chehade et al., 2018
	Open Field	Humic Acid	Soil incorporation	40–80–120–160–200 L ha^{-1}		160 and 200 L ha^{-1} treatments increased yields by 35.2% and leaf nutrition.	Asri et al., 2015
Pepper	Greenhouse	Seaweed extract 'Wokozim' (*Ascophyllum nodosum)*	Foliar spray	2 and 4 mL L^{-1}	Spray treatments at 15 day intervals.	4 mL L^{-1} sprays increased yields by 83 and 46.4% in cultivars 'Sven Rz' and 'Red Knight'.	Khan et al., 2018
	Greenhouse	Humic substance 'Solum H80'	Foliar Spray	0.5, 1, and 1.5 g L^{-1}	Spray treatments at 20-day intervals, starting from 20 days after transplanting.	1.5 g L^{-1} elicited 15.7, 7.2, and 14.1% higher yields from the three tested cultivars due to a modulation of yield parameters.	Ibrahim et al., 2019
Eggplant	Open Field	Seaweed extract 'Göemar BM-86' (*Ascophyllum nodosum)*	Foliar spray	1.5 L biostimulant ha^{-1}	Spray treatments every two weeks, staring from 2 weeks after transplanting.	Of the 6 tested cultivars, 'Epic', 'Flavine', and 'Wa 6020 F10' registered yield increases across two growing seasons.	Pohl et al., 2019

Fruit quality modulation

Solanaceous Crop	Growing Conditions	Biostimulant Substance	Application Method	Dosage	Intervention Time	Effect of Biostimulant Substance	References
Tomato	Greenhouse	Seaweed extract (*Chaetomorpha antennina*)	Seed Priming	Seaweed extract at concentration from 20 to 100%		Increased total soluble solids (+8.5%), phenolics (+74.6%), and ascorbic acid contents (+38.9%).	Muthu-Pandian Chanthini et al., 2019
	Greenhouse	Seaweed Extract 'Bio-algeen S92' (*Ascophyllum nodosum*)	Foliar Spray	0.20%	Two treatments were applied immediately after transplanting, and fifteen days later.	Increased total soluble solids (+3.1%), phenolics (+10.8%), and FRAP antioxidant activity (10.2%).	Murtic et al., 2018
	Greenhouse	Seaweed extract 'Kelpak' (*Ecklonia maxima*)	Foliar Spray	3 mL L^{-1}	10-day spray intervals, starting from the early growth of the first fruit truss.	No increase in total soluble solids, juice pH, antioxidant activity, total phenol contents, ascorbic acid, lycopene.	Colla et al., 2017

	Greenhouse	Protein Hydrolysate 'Trainer'	Foliar spray	2.5 and 5 mL L^{-1}	Spray treatments at 10-day intervals, starting from 15 days after transplanting.	5 mL treatment performed best, by increasing fruit total soluble solid, +10.7%; lipophilic, +260%; and hydrophilic, +61.9% antioxidant activity. Lycopene increased by 34.9%.	Rouphael et al., 2017
	Open Field	Protein Hydrolysate 'Trainer'	Foliar Spray	3 mL L^{-1}	Weekly spray intervals, starting from the early growth of the first fruit truss.	Treatments increased fruit total soluble solids, 10.1%; lipophilic antioxidants, 56.9%; lycopene, 30.7%; and ascorbic acid, 106.2%	Caruso et al., 2019
	Open Field	Humic Acid	Soil incorporation	40–80–120–160–200 L ha^{-1}		No increase in TSS across two growing seasons. Titratable acidity increase across two growing seasons was 10.3%	Asri et al., 2015
Pepper	Greenhouse	Humic substance 'Solum H80'	Foliar Spray	0.5, 1, and 1.5 g L^{-1}	Spray treatments at 20-day intervals, starting from 20 days after transplanting.	The 1.5 g L^{-1} treatment was the most performing, by increasing ascorbic acid content, titratable acidity, total soluble solids, and total sugar. Increase was cultivar-dependent.	Ibrahim et al., 2019

2.5.2. Implication of Biostimulant Substance Treatments on Nightshade Green Growth and Fruit Yield

SWEs determine a variety of growth-promoting effects on solanaceous plants. Tomato and pepper seeds treated with such products showed a higher germination rate, lower germination time, and amplified germination energy [186,187,188]; Muthu-Pandian Chanthini and collaborators [186] also found, in 2019, that the tomato seeds treated with pure *Chaetomorfa antennina* water extract gave rise to pot-grown plants that exhibited higher growth parameters such as 16% higher plant height, 110.5% more branches, 40.1% higher leaf numbers, and were, ultimately, 135.9% higher yielding than the untreated counterparts. Renaut and collaborators [189] found bi-weekly ANE 'Stella Maris' treatments to increase the fruit number in tomato plants (this one amended with hen manure) and pepper plants by 46 and 195% respectively; the tomato plants did not result in an increased average fresh weight, whilst the pepper plants recorded a 35% increase, and also increased root and shoot fresh weights.

The mode of application and cultivar selection seem to be important when deciding to employ seaweed extracts. When Ali and collaborators [190] grew greenhouse tomato plants with a 0.5% foliar spray of an ANE, they found that it was more effective at increasing yields than the substrate drench treatment that brought more fruit bearing clusters (+81% compared to control), higher (+54%) per plant yield, and heavier fruits (55% in the >70 g category vs. 18% of the control). A later study [191], also in greenhouse conditions, confirmed the efficacy of ANE 'Stimplex' foliar treatments at the 0.5% rate, which averaged a +71.5% and +80.9% yield increase in tomatoes and peppers, respectively. Dobromilska and Gubarewicz [192] grew tomato plants in greenhouse and open field-conditions using 'Bio-algeen S90' over three growing seasons at the rate of 0.3% at four different growing stages. When the plants were sprayed three times, at the two-three true leaves stage, before planting and the beginning of flowering, a 49.5% yield increase was recorded, coupled with increased fruit nitrogen, phosphorous, and potassium contents and increased photosynthetic parameters.

Similar results were obtained by Li and Mattson [193], who found that 20 mL L^{-1} foliar treatments of ANE 'Stimplex' elicited a 15.9% increase in tomato transplant weight compared to a −43% decrease in the drench group, both compared to the untreated control.

Nevertheless, it is plausible that some dosage issues may have been at play, especially in the latter study, as either A. nodosum treatments via fertigation on open field and greenhouse-grown tomato plants and combined pre-transplant soak and foliar spray on both tomato and pepper plants recorded significantly higher fruit yields [54,86].

Genotypical variation may also be a factor at play. Khan and collaborators [194] found that greenhouse grown pepper cultivars sprayed with ANE 'Wokozim' at 15-day intervals at the rate of 2 and 4 mL L^{-1} behaved differently, as the 'Sven Rz' cultivar showed an 83% increase in yields, whereas 'Red knight' showed a 46.4% increase, both compared to the respective untreated controls. More recent research conducted by Melo and collaborators [195] recorded in-between results by 'Elisa' peppers sprayed with a 0.5% solution of 'Reabilit Algas'. The mixture of Kappaphycus alvarezii and Sargasum vulgare increased the 1000 plant yield by an estimated 68.8%, compared to its untreated control.

Conversely, Arthur and collaborators [196], while also testing three pepper cultivars (yellow 'Orobelle', red 'Indra', and 'King Arthur') using 'Kelpak', found increases in fruit numbers and average fruit weight to be significant only in 'Indra' and with combined pre-transplant soak and foliar spray.

Genotype-dependent efficacy is not only limited to pepper plants, as out of six eggplants (Solanum melongena L.) cultivars grown in open field conditions and sprayed with ANE Göemar BM-86 at the rate of 1.5 L of biostimulant ha^{-1} only 'Epic'. 'Flavine' and 'WA 6020 F1' registered significant increases in yield across two growing seasons. 'Epic' and 'Flavine' had a significantly higher fruit number, whereas 'WA 6020 F1' registered higher fruit weight [197].

Treating tomato plants with PH products increased tomato growth and yield in four separate instances [86,198,199,200]. Foliar treatments at a rate of 3 and 5 mL L^{-1} with the commercial PH 'Trainer' significantly increased growth in tomato plants grown in either open field and greenhouse studies. In open-field, Caruso and collaborators recorded a 14.6% total aerial biomass, and 18.6% higher yields stemming from 19.3% higher fruit numbers in 'Vesuvian Piennolo Tomato'. Similar results were obtained by Colla and collaborators in greenhouse conditions [198,199]. Open field-grown plants treated with the animal-biomass-derived 'Pepton' recorded dose-dependent increased growth parameters such as height, stem diameter, and 31.1% higher leaf number at the highest supplied dosage of 300 g 'Pepton' L^{-1}. In a similar dose-dependent way, 'Pepton' treatments also significantly increased tomato yield, which, at its highest dosage, reached an estimated 27.5% increase compared to the untreated controls [86].

Genotype and dosage-dependent efficacy was also proven by Rouphael and collaborators [200] in greenhouse-grown tomato plants, by testing two treatment rates of 2.5 and 5 mL L^{-1} on tomato cultivars 'Akyra' and 'Sir Elyan'; the researchers found the highest concentration to be the most effective in improving both plant growth and average tomato yield (+21.3%, compared to the control), but also to variably increase yield parameters. In fact, at the best performing treatment rate, 'Akyra' recorded a 13.9%

higher fruit number, whereas 'Sir Elyan' bore fruits that were 28.7% heavier than the control treatments.

Dose-dependent results were also obtained in a growth tunnel study on alfalfa-based treatments on hot pepper plants; plants sprayed with two dosages (2.5 and 5%) of a solution of alfalfa hydrolysate showed an increased fruit number, which was highest at the elevated dosage [201].

There is a substantial body of evidence confirming the validity of HS treatments on nightshade plants. The effects include an increased rate of seed emergence in tomato and eggplant plants and seedling growth in tomato and pepper plants when HA was added to the growing medium at low concentrations (0.5 g per L^{-1} and 0.2%, respectively [95,202]); effects also include increased plant vegetative growth parameters such as the fresh and dry biomass of shoots and fruits, LAI, and plant height [203,204,205,206]. HA application was also found to increase the leaf nutrient concentration of nitrogen, phosphorous, and potassium of tomato plants [207] and nutrient transfer from the growing medium in tomato and eggplant plants [95].

The most glaring effect of the treatments on nightshades is the increased fruit yield [203-210], which is usually dose and application mode-dependent.

From the consulted tomato studies in either open field or greenhouse conditions it is found that optimal soil applications soil application may lie at around 100 to 200 L of HS per hectare. Abou Chehade and collaborators [208] who tried the former dosage regimen (100 L ha^{-1}), recorded increasing fruit yields in a greenhouse by 18.1%, whereas Asri et al. [207] found the ranges between 160 to 200 L ha^{-1} giving rise to increased yields (+35.2%), leaf macro, and micronutrients levels (nitrogen, phosphorous, potassium, iron, zinc, and manganese). The evidence also seems to suggest that when foliar spray and substrate drench treatments are pitted against each other, it is usually the former being the most effective. Tomato and pepper plants grown in greenhouse and open field conditions, respectively, and sprayed with 20 mL HA L^{-1} recorded higher yields (+27.5 and 29.5%), due to an increased mean fruit weight (+30.4 and 22.5%) and, in the case of tomato plants, fruit number (+30.4%) [209,210].

Yield and growth increases, consistently with other studies, showed a tendency to decrease at higher dosage levels [205,209] and there is also still evidence of the treatments not being effective in increasing yields in some cases.

A three-year investigation by Suman and collaborators [209] found that adding 0.5 L humic acid ha^{-1} via fertigation to open-field-grown tomato plants did not enhance growth and yield when fertilization was 100% of the recommended dosage, which is also consistent with what Monda and collaborators [211] recently found. When fertilization was 80% of the recommended dosage, it performed significantly better than

its untreated control (12.6% higher yielding), and statistically equal to the 100% fertilization group; the same results were also recorded when 25 and 50 mg of HAs were added to a full-strength nutrient solution [212], which may point to differences in either the plant, the experimental setup, and/or the HA source material and dosage.

When testing commercial HA product 'Solum H80', Ibrahim and collaborators [204] found that there is a significant degree of variance from cultivar to cultivar regarding the effectiveness of the treatments. Open-field-tested pepper cultivars 'Barbero', 'Ferrari', and 'Imperio' treated with commercial HS 'Solum H80' at the rate of 1.5 g L^{-1} recorded 15.7, 7.2, and 14.1% yield increases, respectively, when compared to the untreated plants, but more interestingly, also recorded differences in the yield parameters. 'Barbero' and 'Imperio' had more and bigger average fruits, whereas 'Ferrari' produced the same amount of fruit that was higher in weight compared to the untreated counterpart.

Lastly, and worth noting, research carried out by Hartz and Bottoms [213] in the 2008 and 2009 growing season on tomato plants grown in open field conditions found no significant differences in either growth and nutrient application between five commercial HAs formulations at the rate of 1.1 and 3.4 kg HA ha^{-1} and an untreated control. The authors attributed the cause of this behavior to the doses being insufficient for the biostimulant effect.

Table 3 shows an overview of the growth and yield-promoting effects biostimulant substances have recorded on solanaceous crops.

2.5.3. Nightshade Fruit Quality Modulation after Biostimulant Applications

Treating greenhouse-grown tomato plants with 'Siliforce', a commercial formulation of orthosilicic acid at the rate of 300 mL of formulate ha^{-1} brought an increase in fruit firmness, but only when the product was applied one day before harvest [24]. Such increases may nevertheless come with disadvantages: in an open field study, the treated fruits showed significantly less total soluble solids content and total acidity [214]. This is coupled with the sometimes excessive dosage regimens. In the consulted literature, an instance was found where the authors suggested silicon amendments of 400 kg ha^{-1} of silicon salts (calcium, potassium and sodium silicate) [215].

Such a proposition may render silicon treatments unpalatable to those who want to increase tomato fruit quality.

Tomato and pepper plants treated with SWEs yielded fruits that were higher in vitamin C and total soluble solids [180,186,216,217]. Murtic and collaborators [180] found foliar treatments of A. nodosum-based 'Bio-algeen S92' on greenhouse-grown tomato plants at the 0.2% concentration to increase the fruit total soluble solids, phenolic,

flavonoid contents, and ferric reducing antioxidant activity (FRAP) by 3.1, 10.8, 10.5, and 10.2%, respectively, compared to the untreated controls [180].

Similar results were also obtained in separate studies using different source materials, such as undiluted *Chaetomorpha antennina* water extract, 5% *Kappaphycus alvarezii* extract, and *Sargassum johnstonii* [216,217]. Interestingly, it was noted that the root-zone drench treatment of *Sargassum johnstonii* extracts was more effective at increasing TSS, fruit phenolic contents, and lycopene and at lower concentrations, compared to foliar treatments [216].

Nevertheless, not all the literature seems agree on SWEs providing beneficial effects to fruit quality. No quality parameter improvements were recorded when Colla and collaborators [199] applied *Ecklonia maxima* extract 'Kelpak' at the rate of 3 mL L^{-1} on greenhouse-grown tomato plants; similar results were obtained by Di Stasio, who similarly tested ANEs 'Rygex' and 'Super Fifty' and only found increases in fruit calcium, potassium, and magnesium contents (31 and 22%, 17 and 45%, and 32%, respectively) and essential amino acids content [178]. Still, SWEs may found utilities at the post-harvest level, as increased fruit firmness retention during cold storage, coupled with a lesser oxidative increase in fruit TSS were recorded in pepper fruits [194,218]

Three instances of quality improvements using PH biostimulants on tomato plants were found in the available literature, and in each instance, the 'Trainer' legume-derived PH was employed [198,199,200].

Both greenhouse and field studies found that foliar treatments every 7–10 days of such product in the range of 3 and 5 mL of formulate L^{-1} consistently increased the fruit quality parameters with an average 11.7% increase in the total soluble solids across the three studies being the most repeatable effect across the literature. Tomato fruit antioxidant activity increases may be a factor of application rates, as Rouphael et al. [200], who employed 5 mL L^{-1}, recorded increases in lipophilic activity of 260% and hydrophilic activity of 61.9% across the two tested cultivars 'Akyra' and 'Sir Elyan', vs. the 24.6% hydrophilic activity recorded by Colla and collaborators [199], who administered a rate of 3 mL L^{-1} on the same crop.

Other fruit quality parameters enhanced by treatments include increased potassium, ascorbic acid, and lycopene contents, the latter of which was found increased in a field study by Caruso and collaborators [198] by 106.2% over its control.

Foliar alfalfa–hydrolysate treatments at the rate of 25 mL L^{-1} on hot pepper plants grown in a growth tunnel were the most effective at increasing pepper phenol concentration (+44.8 and +140.2%), FRAP antioxidant activity (+36.8 and 27.1%), and ascorbic acid (16.1 and 153.4%) contents in red and green fruits, respectively;

nonetheless, the highest tested dosage of 50 mL L^{-1} substantially increased the capsaicin concentration of red peppers by 598% [201].

From the consulted tomato plant studies, there is no consensus for HSs to elicit significant increases in quality parameters. Both Abou Chehade, Asri, and their collaborators [207,208] found that delivering HS to the soil in either greenhouse or open field conditions, respectively, at a rate of 40 through 200 L $hectare^{-1}$ did not substantially increase any tested fruit quality indicator (titratable acidity, total soluble solids, ascorbic acid, lycopene, phenolic contents an antioxidant activity), save for a single-year when a 10.3% increase in titratable acidity was recorded by the latter authors.

Conversely, foliar treatments of HA on greenhouse-grown tomato plants at a rate of 20 mL L^{-1} increased ascorbic acid by 50.3% and total soluble solids by 18% when averaged across the experiment's two growing seasons, thus indicating that treatment modality may be a factor when HSs are used for product quality improvement [210]. The available research on pepper plants paints a different picture, with open field studies and greenhouse studies both indicating that foliar treatments of either FA at 6% [219] and HA 'Solum H80' at the rate of 1.5 g L^{-1} [204] were the most effective at increasing the ascorbic acid contents of pepper fruits.

Ibrahim and collaborators [204] also found out that the increase in quality parameters were cultivar-dependent, as the three tested cultivars 'Barbero', 'Ferrari', and 'Imperio' recorded a respective +11, +6, and +8% increase in vitamin C contents, a +14, +8, and +10% increase in titratable acidity, and a +18, +7, and +10% increase in the fruit total soluble content. Dosage-dependent product quality improvements were also denoted in open field-grown hot pepper plants. Out of the four tested soil application regimens (50–200–350–500 kg ha^{-1}), the 350 kg ha^{-1} registered an increase in antioxidant activity of 22.3% and an increase in fruit capsaicin contents by 36.8%, whereas lycopene content was highest at 200 kg ha^{-1} by +43.3% and beta carotene at 350 kg ha^{-1}, with an 89.1% increase [220].

Table 3 shows an overview of the nightshade fruit quality modulation after biostimulant applications.

2.6. Chapter 2 Conclusions

The ever-more pressing issue of climate change and the effects that agriculture has on the environment has posed the dilemma of rapidly finding new answers for the sustainable intensification of crop practices.

Whilst these problems are multifaceted and may require a complete rethinking of how agriculture should be managed worldwide, the introduction of biostimulant substances have brought a valid interim solution toward the future of agriculture. These substances derive from, or are generated by, industrial waste or waste biomass, therefore, limiting the recourse to newly and wastefully generated fertilizers. Furthermore, they prove their worth by increasing plant growth, reducing plant stress, and increasing produce quality at low dosage applications, thus earning their namesake.

However, there is still space for arguing about some of the sore points that have been found in the consulted biostimulant literature. The incredibly wide selection of source materials, from seaweed species through the plethora of waste streams that can be made into humic substances, creates a variety of products that contain a plethora of active ingredients. The same active ingredients have been hard to discover, certainly not helped by the great number of often proprietary production methods, and the list of the ones we currently know is by no means exhaustive and still leaves some doubts.

Agronomic factors such as cultivar selection and biostimulant management i.e., how much biostimulant to use, when to use it, where to use it (greenhouse or open field), and in which modality it is administered (foliar, drench, seed treatment, nutrient solution), sometimes are the make-or-break decisions that may or may not express the crops' and products' full potential, and have to be carefully considered.

The picture depicted here shows the need for interdisciplinary biostimulant research: products need to be scrutinized at the molecular level, which could be performed by the way of fractionation or separation; rapidly and repeatedly screened via metabolomic, genomic, and physiological analysis; and then tested against widely used crop benchmarks in order to assay their performance. Thus, a top-down approach might be needed going forwards, and judging from the consulted literature, it is currently happening, and it is a welcomed change for agriculture worldwide.

2.7 References for Chapter 2

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Chapter 3

Foliar and Root Applications of Vegetal-Derived Protein Hydrolysates Differentially Enhance the Yield and Qualitative Attributes of Two Lettuce Cultivars Grown in Floating System

Abstract: Lettuce (Lactuca sativa L.) is a leafy vegetable cultivated widely for its fast and year-round production and its beneficial phytochemical content, which may be boosted further by plant biostimulants that are considered eco-sustainable means for enhancing horticultural crop production. A greenhouse experiment was carried out to evaluate the yield and qualitative parameters of two differently pigmented lettuce cultivars grown in a floating raft system either untreated or treated (leaf, root or leaf/root application) with vegetal protein hydrolysates (PHs). For foliar ap-plication (F), lettuce plants were sprayed at a dose of 3 mL L-1, whereas for root application, 0.15 (T1) or 0.3 (T2) mL L-1 was applied to the nutrient solution alone or in combination with foliar spray (T1 + F and T2 + F) with the same foliar concentration. Bio-morphometric and production data were collected after harvest. Physiological and plant nutrition assays included leaf gas ex-change, leaf fluorescence, SPAD index, mineral content, carotenoids, total phenols, total ascorbic acid content and antioxidant activities. Cultivar-specific reactions to biostimulant application were noted: whilst the green pigmented cultivar thrived under nutrient solution applications and recorded higher yield by 82.7% (T1) or (T1 + F) and 71.7% (T2), the red cultivar thrived under combined treatments, yielding 55.4% (T2 + F) higher than control and providing the most concentrated phytochemical content. These latter treatments also recored the highest SPAD index, Fv/Fm ratio, CO2 assimilation, stomatal conductance and transpiration. In addition, the T2 + F treatment boosted 'Canasta' hydrophilic antioxidant activity (21.9%) and total ascorbic acid (5.6-fold). Nutrient solution treatments alone proved advantageous when compared to foliar treatments, while mixed treatments proved genotype-specific. New research on genotype specificity of biostimulant effects is warranted for future use, in order to rationalize biostimulant application modes and dosages.

3.1. Introduction

Lettuce (Lactuca sativa L.) is one of the most grown vegetable crops at over 29 million tons harvested in 2019 [1] and in particular it embodies Italy's most cultivated leafy green [2]. Lettuce consumers benefit from a variety of health improvements, starting from the general lowered risk of diseases due to the consumption of vegetables [3] and the elevated intake of phytochemicals such as vitamins, polyunsaturated fatty acids (PUFA) and antioxidants pertained to this leafy vegetable [4,5]. Whilst commonly grown in soil-based systems, concerns over land, fertilizers abuse and specialized soil-borne

pathogens due to intensive cropping [6–8] has favored the introduction of soilless farming, of which the floating raft system represent a notable example. Ad-vantages of growing Lettuce in floating systems include low maintenance, higher yield, nutrient and water efficiency and continuous cropping throughout the year [9,10]. Moreover, by virtue of soil absence, floating systems can be employed in urban agriculture projects that provide better food availability, local social and economic development and reduced environmental impact [11]. Lastly, the controlled growing environment makes the obtainment of quality products easier, as the fine tuning of pre-harvest factors like the nutrient solution composition makes increments of the above-mentioned phytochemicals possible [12,13].

Plant biostimulants provide a good fit with floating systems, since their purpose, as defined by the EU Commission, consist of improving nutrient use efficiency, tolerance to abiotic stress, quality traits and availability of confined nutrients in soil or rhizo-sphere [14]. Protein hydrolysates (PHs) biostimulants are a rather interesting addition to the group: generally employed as foliar spray or substrate drench treatments. PHs products include bioactive molecules like readily absorbed amino acids and a category of small molecules known as signaling peptides [15]. Such molecules provide for a plethora of plant growth and physiological effects, including hormone-simile effects, due to auxin and gibberellin-like activity [16], upregulation of carbon and nitrogen metabolism [16–19] and induction of secondary metabolites production like phenolics and flavonoids having antioxidant capacities of interest for human health [20,21]. Evi-dence on the use of PHs biostimulants on leafy vegetables seem to give credit to their plant-growth enhancing prowess, as elevated yield and yield parameters such as leaf numbers [22,23] were denoted in lettuce plants and higher marketable yield were seen in rocket [20]. Scientific literature also points out at PHs increasing nutrient efficiency and plant growth when grown in a floating system [24], but to this day application modes and dosages in this particular growing system are still not well defined. PHs application to both roots and leaves has proven to be beneficial to lettuce plants grown in sand substrate [25], but no information is available regarding a growing system with higher root nutrient availability such as the floating system. Furthermore, there is a lack of information about a dosage ceiling or application mode on lettuce, whereby the bi-ostimulant could be either ineffective or downright detrimental to plant growth and quality and whether it is cultivar-specific or not. This last question stems from the availability of multiple lettuce types that provide considerable variation in the Lactuca sativa L. species, from head shape and size [26]. This variation is even more accentuated by the presence of different pigmentations, which may provide health-promoting benefit to consumers; such as red pigments indicating the presence of powerful radical oxygen species (ROS) scavenging molecules [27].

To evaluate these research questions, a greenhouse study was conducted with two differently-pigmented lettuce cultivars, grown in a floating raft system and subjected to either foliar spray, nutrient solution application or combined applications of a vege-tal derived PHs biostimulant. Crop response to treatments was evaluated in terms of morpho-physiological traits, mineral contents and antioxidant activity. The results displayed in this study may provide new horizon for PHs utilization and will contribute in meliorating lettuce quali-quantitative features in hydroponic systems.

3.2. Materials and Methods

3.2.1 Growth Conditions, Experimental Design and Plant Material

A greenhouse experiment was carried out at the University of Naples "Federico II" — Department of Agriculture in a passively ventilated greenhouse situated in Portici (Province of Naples, Italy; 40°48′ N, 14°20′ E, 29 m.s.l.) from 20 April until 7 May 2020, for a total of 17 days. Relative humidity and temperature were recorded continuously using WatchDog A150 data loggers (Spectrum Technologies Inc., Aurora, IL, USA; 3%/0.6 °C RH/Temp accuracy) placed at canopy level at different locations of the experimental area (**Figure S1**). A bi-factorial experimental design was employed, consisting of two lettuce (Lactuca sativa L.) cultivars, a green butterhead 'Ballerina' (Rijk Zwaan Italia S.R.L., Bologna (BO), Italy) and a red crisphead 'Canasta' (Pagano Costantino & F.lli S.R.L, Scafati (SA), Italy), an untreated control and five distinct levels/modes of biostimulant application. Each treatment was replicated three times and the 12 combinatorial treatments (2 × 6) were arranged in a randomized complete-block design. The two-cultivars seedlings were transplanted, into 24-hole polystyrene trays (52 × 33 cm) at a density of 70 plants m^{-2}. Each tray maintained 12 plants and corresponded to an experimental unit (**Figure S2**), accounting in total for 36 experimental units. The trays were floating in plastic tanks (35 L maximum capacity) filled with 30 L of nutrient solution (NS) containing the following macro- and micro-nutrients: 9.0 mM Nitrate, 1 mM Phosphorous, 2.0 mM Sulfur, 1.0 mM Ammonium, 4 mM Potassium, 4 mM Calcium, 1 mM Magnesium, 15 μM Iron, 9.0 μM Manganese, 0.3 μM Copper, 1.6 μM Zinc, 20.0 μM Boron and 0.3 μM Molybdenum, accounting for an electrical conductivity of 1.3 mS cm^{-1}. Each experimental unit was supplied with an immersion air pump to prevent plant roots anoxia and NS was checked for pH fluctuations on a daily-basis with a portable pH meter (HI 991301, Hanna Instruments (Italia S.R.L., Ronchi di Villafranca Padovana (PD), Italy) and when needed, it was adjusted at the 5.8 ± 0.2 pH level. The tanks were topped up with freshly prepared NS on a weekly basis.

3.2.2. Biostimulant Application

The vegetal-derived protein hydrolysates Trainer® (Hello Nature Italia S.R.L., Rivoli Veronese (VR), Italy), a commercially available product obtained through enzymatic hydrolysis of legume biomasses was used for this trial. The components of the PHs are

amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val) and soluble peptides which comprise 5% of the total nitrogen content, along with phenolics and soluble sugars. Detailed analysis of the product was reported by Paul et al. [28] and Rouphael et al. [21], who pointed out that no plant hormones were found in the product. Biostimulant treatments consisted of five distinct levels/modes of application: Foliar application (F) at the rate of 3 mL biostimulant L^{-1} of solution. For root application, 0.15 (T1) or 0.3 (T2) mL L^{-1} was applied to the nutrient solution alone or in combination with foliar spray (T1 + F and T2 + F) with the same foliar concentration. Foliar applications were done by the means of a 10 L steel-bottle sprayer. Three treatments during the growing season were adopted starting directly after transplanting and every six days. Equally, for the PHs application in the NS, it was added on transplanting and successively added with the NS when the refill of the tanks was done.

3.2.3. Sampling, Yield and Growth Assessment

At the end of the experiment, nine plants from each experimental unit were chosen for the biometric measurements, consisting of leaf number, leaf area and shoot fresh yield (leaves + stem). Leaf area of each plant was estimated using ImageJ software 1.50 version (U.S. National Institutes of Health, Bethesda, MD, USA) and quantified in cm^2. The aforementioned plants were put in a forced air-drying oven at 60 °C until constant weight was reached for the successive determination of shoot dry biomass (leaves + stem) and dry matter percentage (DM %, calculated as (leaf dry weight/leaf fresh weight) × 100). For qualitative analysis, a pool of two plants per experimental unit were harvested and conserved at −80 °C and later on freeze dried in a lyophilizer (model Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

3.2.4. Soil Plant Analysis Development (SPAD) Index, Chlorophyll Fluorescence and Photosynthetic Parameters

Soil Plant Analysis Development (SPAD) Index was assessed by taking 24 measurements per experimental unit using a Minolta Chlorophyll Meter (model SPAD-502, Minolta Camera Co. Ltd., Osaka, Japan). Leaf chlorophyll fluorescence measurements, expressed as the maximum quantum efficiency of PSII photochemistry (F_v/F_m) were taken using a portable leaf fluorometer (model F_v/F_m meter, Opti-Sciences, Hudson, NH, USA) onto leaves at the same developmental stage. Seven measurements per experimental unit were taken. As for the leaf gas exchange, the measurements per experimental unit were carried out onto fully expanded leaves using a portable gas exchange analyzer (model Li-6400, LI-COR Biosciences, Lincoln, NE, USA) equipped with a 6 cm^2 leaf chamber and a programmable LED light source (model 6400-02b). Photosynthetically active radiation (PAR) was kept steady at 2000 μmol m^{-2} s^{-1}, relative humidity (RH) and CO_2 concentration were kept at ambient values, flow rate of air was maintained at 500 mL s^{-1}. Measured parameters consisted of assimilated CO_2 (A_{CO2}),

stomatal conductance (g_s) and transpiration rate (E). All of the physiological measurements were carried out in the 9:00 to 11:00 am timeslot on harvest day.

3.2.5. Leaf Mineral Content Analysis

Total leaf nitrogen content analyses were conducted on dry, milled samples using the Kjeldahl method [29]. Based on Pannico et al. [30] protocol, a 250 mg aliquot of milled (model MF10.1, IKA-Werke GmbH & Co. KG, Staufen, Germany) dry leaf sample was used for the determination of leaf mineral (NO_3, P, K, Ca, Mg, S and Na) composition. Mineral analysis was then carried out after 0.45 μm filtering using an ion chromatographer (model ICS-3000, Dionex, Sunnyvale, CA, USA), quantified using an electrical conductivity detector equipped with an IonPac CS12A and IonPac AS11-HC analytical columns for the analysis of cationic and anionic contents, respectively (Dionex, Sunnyvale, CA, USA). All the minerals were expressed as mg g^{-1} on dry weight (DW) basis except for nitrate that was expressed as mg kg^{-1} on fresh weight (FW) basis, based on each sample DM%.

3.2.6. Leaf Total Chlorophylls and Carotenoids

Leaf pigments content were determined using one g of fresh leaf samples which were extracted in pure acetone and kept in darkness for 15 min. After centrifuging the extracts at 3000 g for five minutes, pigments content was determined by their light absorbance at 662, 645 and 470 nm for chlorophyll a, b and total carotenoids, using a Hach DR 2000 spectrophotometer (Hach Company, Loveland, CO, USA). Total Chlorophylls was calculated as the sum of chlorophyll a and b. The extinction coefficients used for pigment determination were described in Lichtenthaler and Buschmann work [31].

3.2.7. Total Phenols and Total Ascorbic Acid Analysis

Antioxidant molecules assays were performed on freeze-dried leaf tissue using the Folin–Ciocalteau [32] method for Total Phenols Content and on fresh leaf material using the Kampfenkel [33] method for the determination of Total Ascorbic Acid (TAA). Spectrophotometric measurements of the solutions were carried out at 765 and 525 nm, respectively.

3.2.8. Antioxidant Activity Analysis

A total of 200 mg of freeze-dried material was analyzed by means of two antioxidant essays. The 2,20′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS in short) method was employed as described in Pellegrini et al. [34] and the N,N-dimethyl-p-phenylenediamine (DMPD in short) was implemented as described by Fogliano et al. [35]. In order to measure the reduction in absorbance of the solutions a spectrophotometric assay was carried out at 734 and 505 nm wavelengths, respectively.

3.2.9. Statistical Processing of the Data

A two-way analysis of variance (Two-way ANOVA) was performed using SPSS 20 for Windows (IBM Corp., Armonk, NY, USA), in order to access the interaction between

the two factors (Cultivar-C and Biostimulant-B). The mean effect of the cultivar (C) was compared by Student t-test. Separation of the means was obtained using Duncan's Multiple Range Test (DMRT). Differences between treatments were deemed significant at $p = 0.05$.

3.3. Results

3.3.1. Growth and Yield Assessment

Table 1 shows the data of the growth and yield of both lettuce cultivars ('Ballerina' and 'Canasta') grown in floating raft system and subjected to diverse biostimulant application modes and doses. Significant cultivar-specific difference was denoted in some of the evaluated parameters, such as leaf number that was not influenced by biostimulant treatments, with 'Ballerina' developing more leaves than the other cultivar. A significant interaction cultivar × biostimulant was denoted for all the studied parameters with the sole exception of leaf number. When considering foliar application only, 'Ballerina' recorded 51% higher yield than control, whilst 'Canasta' showed a modest 12.5% increase. Nutrient solution additions also showed a different behavior of the two cultivars, as the T1 and T2 treatments exhibited 82.7% and 71.7% increases over the control treatment in 'Ballerina', compared to the 7.1% and 23.4% increase obtained by 'Canasta'. It is when foliar and nutrient solution applications were combined that an interesting phenomenon arose; while a slight increase was recorded in 'Ballerina' at the T1 + F level, the T2 + F dosage determined a significant decrease in yield figures when compared to T1 + F by 18.3%, while still being 59.2% higher than the control treatment. Conversely, 'Canasta' thrived under the combined treatments, as the T1 + F and T2 + F treatments boosted yield by 42.5 and 55.4%, with the latter treatment being the best performing overall. In addition, plant yield components such as leaf area showed more or less the same range of increase with all the five treatments in 'Ballerina', whereas 'Canasta' exhibited a gradual increase when passing from nutrient application to combination with foliar. As for leaf dry matter %, 'Ballerina' and 'Canasta' × control were amongst the highest treatments, in addition to 'Ballerina' × combined treatments; all ranging 5.7% on average.

Table 1. Leaf number, leaf area, fresh and dry biomass and leaf dry matter of 'Canasta' and 'Ballerina' lettuce as influenced by the biostimulant application.

Source of Variance	Leaf Number (no. plant^{-1})	Leaf Area (cm^2 plant^{-1})	Shoot Fresh Yield (g plant^{-1})	Dry Shoot Biomass (g plant^{-1})	Leaf Dry Matter (%)
Cultivar (C)					
Ballerina	22.39 ± 0.32	1603 ± 50	80.28 ± 3.6	4.42 ± 0.21	5.52 ± 0.08
Canasta	17.33 ± 0.57	1522 ± 35	79.86 ± 3.17	4.34 ± 0.16	5.43 ± 0.05
t-test	***	ns	ns	ns	ns
Biostimulant (B)					
Control	20.50 ± 1.67	1264 ± 44 c	57.70 ± 3.17 d	3.30 ± 0.19 d	5.81 ± 0.04 a
F	19.50 ± 1.57	1515 ± 34 b	74.68 ± 1.44 c	3.81 ± 0.05 c	5.10 ± 0.06 d
T1	20.00 ± 1.81	1523 ± 70 b	81.01 ± 5.32 b	4.39 ± 0.28 b	5.39 ± 0.07 c
T2	20.17 ± 0.95	1675 ± 54 a	83.45 ± 2.01 b	4.59 ± 0.11 b	5.47 ± 0.05 bc
T1 + F	19.83 ± 1.14	1695 ± 13 a	92.95 ± 2.01 a	5.14 ± 0.16 a	5.51 ± 0.13 bc
T2 + F	19.17 ± 1.05	1702 ± 20 a	90.65 ± 4.46 a	5.06 ± 0.23 a	5.58 ± 0.07 b
	ns	***	***	***	***
C × B					
Ballerina × Control	23.67 ± 0.88	1193 ± 55 f	50.79 ± 1.58 h	2.88 ± 0.10 f	5.86 ± 0.06 a
Ballerina × F	22.67 ± 0.33	1557 ± 58 bc	76.69 ± 2.30 de	3.82 ± 0.10 e	5.00 ± 0.06 f
Ballerina × T1	23.33 ± 0.67	1665 ± 33 ab	92.81 ± 1.58 b	5.02 ± 0.02 b	5.36 ± 0.10 de
Ballerina × T2	21.67 ± 0.88	1783 ± 52 a	87.19 ± 2.59 c	4.82 ± 0.08 bc	5.51 ± 0.09 bcd
Ballerina × T1 + F	22.00 ± 0.58	1694 ± 28 a	93.81 ± 4.03 b	5.42 ± 0.23 a	5.76 ± 0.14 ab
Ballerina × T2 + F	21.00 ± 0.58	1729 ± 32 a	80.86 ± 1.15 de	4.58 ± 0.14 cd	5.65 ± 0.12 abc
Canasta × Control	17.33 ± 1.76	1335 ± 41 e	64.60 ± 0.72 g	3.72 ± 0.07 e	5.76 ± 0.02 ab
Canasta × F	16.33 ± 1.45	1473 ± 26 cd	72.66 ± 0.58 ef	3.80 ± 0.02 e	5.19 ± 0.05 ef
Canasta × T1	16.67 ± 2.19	1382 ± 57 de	69.21 ± 1.01 fg	3.77 ± 0.10 e	5.43 ± 0.10 cde
Canasta × T2	18.67 ± 1.20	1568 ± 24 bc	79.71 ± 0.43 de	4.35 ± 0.05 d	5.42 ± 0.06 cde
Canasta × T1 + F	17.67 ± 1.20	1696 ± 8 a	92.09 ± 1.58 bc	4.87 ± 0.03 bc	5.27 ± 0.06 de
Canasta × T2 + F	17.33 ± 1.33	1676 ± 19 ab	100.43 ± 1.29 a	5.54 ± 0.09 a	5.51 ± 0.09 bcd
	ns	***	***	***	**

All data are expressed as mean ± standard error, n = 3. ns, **, *** non-significant or significant at $p \leq 0.01$ and 0.001, respectively. Cultivars means were compared by *t*-Test. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p = 0.05). F: foliar treatment (3 mL L^{-1}), T1: nutrient solution treatment 0.15 mL L^{-1}, T2: nutrient solution treatment 0.3 mL L^{-1}.

3.3.2. SPAD Index, Chlorophyll Fluorescence, Photosyntethic Parameters

SPAD index records (**Table 2**) showed a significant cultivar × treatment interaction. T1 and T1 + F recorded the highest values for 'Ballerina', which were 8.2% higher than the untreated control, whereas T1 + F and T2 + F recorded the highest values for 'Canasta'. Cultivar × biostimulant data showed a distinct behavior of the two tested genotypes in relationship to the tested dosage, as 'Ballerina' seemed to favor the T1 and T1 + F treatments, whilst 'Canasta' in line with previous data (i.e., yield) recorded the highest values at the most concentrated biostimulant applications, with the T2 + F treatments showing the highest SPAD values overall and an 8.6% increase compared to its control.

Table 2. SPAD index, F_v/F_m ratio and leaf gas exchange (assimilated CO_2: A_{CO2}, stomatal conductance: g_s and transpiration rate: E) of 'Canasta' and 'Ballerina' lettuce as influenced by the biostimulant application.

Source of Variance	SPAD Index	Fluorescence F_v/F_m Ratio	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	g_s (mol H_2O m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})
Cultivar (C)					
Ballerina	32.56 ± 0.25	0.837 ± 0.00	19.64 ± 0.48	0.64 ± 0.04	8.86 ± 0.29
Canasta	33.01 ± 0.29	0.830 ± 0.00	20.41 ± 0.39	0.67 ± 0.04	9.29 ± 0.24
t-test	ns	***	ns	ns	ns
Biostimulant (B)					
Control	31.70 ± 0.40 d	0.831 ± 0.00 bc	18.27 ± 0.67 c	0.50 ± 0.04 c	8.41 ± 0.38 c
F	32.13 ± 0.24 d	0.830 ± 0.00 c	18.33 ± 0.67 c	0.64 ± 0.07 b	8.00 ± 0.41 c
T1	33.03 ± 0.29 bc	0.836 ± 0.00 a	19.94 ± 0.50 b	0.62 ± 0.03 b	9.73 ± 0.15 ab
T2	32.35 ± 0.14 cd	0.835 ± 0.00 ab	20.31 ± 0.25 b	0.67 ± 0.04 b	9.10 ± 0.33 b
T1 + F	33.98 ± 0.18 a	0.836 ± 0.00 a	21.54 ± 0.31 a	0.85 ± 0.09 a	10.00 ± 0.35 a
T2 + F	33.52 ± 0.66 ab	0.835 ± 0.00 ab	21.76 ± 0.72 a	0.66 ± 0.07 b	9.22 ± 0.59 b
	***	*	***	***	***
C × B					
Ballerina × Control	31.19 ± 0.52 d	0.834 ± 0.00 bcd	17.12 ± 0.81 f	0.58 ± 0.04 cdef	8.99 ± 0.28 cde
Ballerina × F	32.30 ± 0.31 c	0.834 ± 0.00 bcd	17.21 ± 0.83 f	0.52 ± 0.03 def	7.39 ± 0.52 f
Ballerina × T1	33.62 ± 0.18 b	0.842 ± 0.00 a	20.93 ± 0.53 bcd	0.68 ± 0.04 bcde	9.65 ± 0.33 abc
Ballerina × T2	32.30 ± 0.20 c	0.838 ± 0.00 ab	20.64 ± 0.42 bcd	0.58 ± 0.01 cdef	8.41 ± 0.00 def
Ballerina × T1 + F	33.88 ± 0.30 b	0.842 ± 0.00 a	21.78 ± 0.43 b	1.00 ± 0.10 a	10.65 ± 0.30 a
Ballerina × T2 + F	32.08 ± 0.17 cd	0.833 ± 0.00 bcd	20.16 ± 0.20 cde	0.50 ± 0.02 ef	8.07 ± 0.61 ef
Canasta × Control	32.21 ± 0.51 c	0.828 ± 0.00 de	19.42 ± 0.53 de	0.41 ± 0.03 f	7.82 ± 0.55 ef
Canasta × F	31.96 ± 0.41 cd	0.826 ± 0.00 e	19.45 ± 0.52 de	0.76 ± 0.11 bc	8.61 ± 0.45 cde
Canasta × T1	32.45 ± 0.21 c	0.831 ± 0.00 cde	18.95 ± 0.12 e	0.57 ± 0.01 def	9.81 ± 0.06 abc
Canasta × T2	32.41 ± 0.23 c	0.831 ± 0.00 cde	19.98 ± 0.16 cde	0.75 ± 0.04 bc	9.78 ± 0.27 abc
Canasta × T1 + F	34.08 ± 0.26 ab	0.830 ± 0.00 cde	21.31 ± 0.49 bc	0.71 ± 0.08 bcd	9.35 ± 0.32 bcd
Canasta × T2 + F	34.97 ± 0.26 a	0.836 ± 0.00 abc	23.36 ± 0.02 a	0.81 ± 0.05 b	10.36 ± 0.27 ab
	***	*	***	***	***

All data are expressed as mean ± standard error, n = 3. ns, *, *** non-significant or significant at $p \leq 0.05$ and 0.001, respectively. Cultivars means were compared by *t*-Test. Different letters within each column indicate significant differences according to Duncan's multiple-range test (p = 0.05). F: foliar treatment (3 mL L^{-1}), T1: nutrient solution treatment 0.15 mL L^{-1}, T2: nutrient solution treatment 0.3 mL L^{-1}.

Fluorescence data showed significant interaction between the cultivars and biostimulant applications. Again, the 'Ballerina' cultivar reported its highest figures at the T1 and T1 + F treatment levels, which were overall the highest recorded along 'Canasta' × T2 + F. The latter treatment resulting significantly higher than control and F × 'Canasta'. In addition, leaf gas exchange measurements (Table 2) showed significant interactions for the CO_2 assimilation rate, stomatal conductance and transpiration rate. Similarly to above mentioned data, a familiar pattern arose from the interaction data, as for every studied parameter (A_{CO2}, g_s and E) a performance regression was noted at the T2 + F level compared to the T1 + F for the green 'Ballerina' cultivar and a general upward trend was noted for the 'Canasta' cultivar. A different trend emerged in the latter two indices, as stomatal conductance and transpiration in 'Ballerina' were the

highest at the T1 + F level compared to all studied combinations, with an increase of 72.0% and 18.4% when compared to its control average.

3.3.3 Leaf Total Nitrogen, Nitrate and Mineral Content

Leaf mineral content records in **Table 3** showed significant cultivar differences in the assimilation of nitrate, calcium and sulfur, with 'Ballerina' cultivar being the highest accumulator of nitrate and calcium. Nonetheless, nitrate accumulation was ruled by the interaction of C × B. Where 'Ballerina' accumulated the most at T1 + F and T2 + F (around 1769 mg kg−1 FW) and 'Canasta' accumulated the most at only T2 + F (1621 mg kg−1 FW). Noting that 'Canasta' × control registered 35.3% less nitrate than 'Ballerina' × control. In addition, notable, are the recorded decreases in nitrate content in both cultivars at the T2 level, with 'Ballerina' accumulating 19% less than its control, in addition to the foliar treatment for 'Canasta', though the decrease was not deemed significant for this cultivar. At any rate, none of the tested treatments exceeded the nitrate threshold set by the EU Regulation 1258/2011. Sodium accumulation in both cultivars was the highest at T1 level, with the 'Canasta' cultivar showing the highest overall figures and relative increase of 39.4% compared to its control. Nonetheless, T1 + F and control × 'Ballerina' were equally rich in sodium. When averaged across cultivars, biostimulant application significantly affected mineral contents, as it is clear for P, K and Mg except for T2 + F treatment. These three macro-minerals increased on average 14.4, 7.7 and 12.0%, respectively, when both cultivars were treated with biostimulants. As for sulfur, only T1 + F and T2 + F induced significant higher accumulation in comparison to the control, which was the same case for total nitrogen %, in addition to T1 treatment.

Table 3. Total nitrogen and mineral leaf content (nitrate, P, K, Ca, Mg, S and Na) of 'Canasta' and 'Ballerina' lettuce as influenced by the biostimulant application.

Source of Variance	Total N (%)	Nitrate (mg kg^{-1} FW)	P (mg g^{-1} DW)	K (mg g^{-1} DW)	Ca (mg g^{-1} DW)	Mg (mg g^{-1} DW)	S (mg g^{-1} DW)	Na (mg g^{-1} DW)
Cultivar (C)								
Ballerina	3.88 ± 0.04	1446 ± 67	4.23 ± 0.09	43.83 ± 0.64	16.58 ± 0.33	5.91 ± 0.11	1.04 ± 0.02	4.45 ± 0.22
Canasta	3.99 ± 0.06	1106 ± 83	4.02 ± 0.08	44.13 ± 0.58	14.86 ± 0.20	5.78 ± 0.08	1.20 ± 0.02	4.36 ± 0.18
t-test	ns	**	ns	ns	***	ns	***	ns
Biostimulant (B)								
Control	3.77 ± 0.04 c	1087 ± 113 c	3.78 ± 0.11 b	42.09 ± 0.81 b	15.11 ± 0.56 bc	5.37 ± 0.10 d	1.07 ± 0.05 b	4.45 ± 0.38 b
F	3.83 ± 0.07 bc	1090 ± 157 c	4.29 ± 0.12 a	45.49 ± 0.83 a	16.00 ± 0.54 ab	6.04 ± 0.03 ab	1.13 ± 0.04 ab	4.28 ± 0.29 bc
T1	4.00 ± 0.09 ab	1346 ± 40 b	4.40 ± 0.08 a	45.79 ± 0.72 a	16.21 ± 0.64 ab	6.27 ± 0.23 a	1.04 ± 0.03 b	5.50 ± 0.17 a
T2	3.89 ± 0.09 bc	919 ± 72 d	4.18 ± 0.14 a	44.48 ± 0.81 a	14.58 ± 0.47 c	5.93 ± 0.11 abc	1.12 ± 0.04 ab	3.56 ± 0.20 d
T1 + F	4.09 ± 0.09 a	1480 ± 101 b	4.43 ± 0.10 a	45.46 ± 0.61 a	16.55 ± 0.61 a	5.82 ± 0.09 bc	1.17 ± 0.06 a	4.76 ± 0.28 b
T2 + F	4.03 ± 0.06 ab	1733 ± 76 a	3.67 ± 0.08 b	40.56 ± 0.29 b	15.88 ± 0.49 ab	5.62 ± 0.10 cd	1.20 ± 0.05 a	3.89 ± 0.08 cd
	**	***	***	***	*	***	*	***
C × B								
Ballerina × Control	3.76 ± 0.07	1319 ± 28 c	3.84 ± 0.16	41.85 ± 1.11	16.14 ± 0.55	5.31 ± 0.15	1.00 ± 0.01	5.07 ± 0.57 ab
Ballerina × F	3.83 ± 0.13	1410 ± 91 bc	4.42 ± 0.21	45.87 ± 1.81	17.15 ± 0.33	6.00 ± 0.03	1.05 ± 0.00	3.75 ± 0.07 de
Ballerina × T1	3.93 ± 0.10	1338 ± 66 c	4.52 ± 0.07	45.07 ± 1.15	17.54 ± 0.46	6.63 ± 0.32	0.97 ± 0.02	5.37 ± 0.24 ab
Ballerina × T2	3.76 ± 0.06	1069 ± 48 de	4.41 ± 0.19	44.14 ± 1.61	14.34 ± 0.55	5.90 ± 0.13	1.08 ± 0.04	3.16 ± 0.12 e
Ballerina × T1 + F	4.00 ± 0.05	1692 ± 70 a	4.46 ± 0.06	45.59 ± 0.60	17.78 ± 0.31	5.87 ± 0.07	1.05 ± 0.02	5.30 ± 0.12 ab
Ballerina × T2 + F	4.00 ± 0.07	1846 ± 123 a	3.71 ± 0.10	40.48 ± 0.33	16.54 ± 0.76	5.73 ± 0.11	1.11 ± 0.05	4.03 ± 0.01 d
Canasta × Control	3.77 ± 0.04	854 ± 96 ef	3.73 ± 0.18	42.33 ± 1.41	14.08 ± 0.44	5.44 ± 0.14	1.15 ± 0.08	3.83 ± 0.03 de
Canasta × F	3.83 ± 0.09	770 ± 116 f	4.15 ± 0.07	45.11 ± 0.12	14.85 ± 0.14	6.08 ± 0.06	1.21 ± 0.03	4.81 ± 0.37 bc
Canasta × T1	4.07 ± 0.15	1354 ± 59 c	4.28 ± 0.10	46.51 ± 0.88	14.89 ± 0.31	5.92 ± 0.19	1.12 ± 0.00	5.62 ± 0.28 a
Canasta × T2	4.02 ± 0.14	770 ± 32 f	3.96 ± 0.11	44.83 ± 0.74	14.83 ± 0.85	5.96 ± 0.21	1.16 ± 0.07	3.97 ± 0.12 d
Canasta × T1 + F	4.18 ± 0.17	1269 ± 34 cd	4.40 ± 0.21	45.34 ± 1.21	15.33 ± 0.53	5.77 ± 0.19	1.29 ± 0.03	4.22 ± 0.28 cd
Canasta × T2 + F	4.07 ± 0.12	1621 ± 36 ab	3.63 ± 0.15	40.64 ± 0.55	15.21 ± 0.42	5.51 ± 0.16	1.28 ± 0.03	3.74 ± 0.09 de
	ns	**	ns	ns	ns	ns	ns	***

All data are expressed as mean ± standard error, n= 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Cultivars means were compared by *t*-Test. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p = 0.05$). F: foliar treatment (3 mL L^{-1}), T1: nutrient solution treatment 0.15 mL L^{-1}, T2: nutrient solution treatment 0.3 mL L^{-1}.

3.3.4. Leaf Pigments and Qualitative Parameters

Chlorophyll pigments (**Table 4**) demonstrated only significant genotype differences, where 'Ballerina' cultivar exhibited significantly higher total chlorophyll by 22.1%, respectively compared to 'Canasta'. Identically, total phenols were as well dominated by the cultivar effect, with 'Canasta' being 36.8% denser. Moreover, biostimulant treatments significantly affected total ascorbic acid content, Hydrophilic antioxidant activity, ABTS antioxidant activity and carotenoids content, with significant interactions recorded for every parameter. Stark genotype-derived differences were denoted especially when considering total ascorbic acid content, the combination 'Ballerina' and biostimulant foliar application (F) yielded the highest overall total ascorbic acid content, with an increase of 51.2% compared to its control; however, it is the 'Canasta' cultivar that recorded the most substantial relative increase, as the T1 + F treatment increase over its untreated control was almost 7-fold and T2 + F 5.6-fold. As for the hydrophilic antioxidant activity, T2 + F treatment boosted by 55.9% the content in 'Ballerina' and by 21.9% the content in 'Canasta', this later cultivar had as well a great boost by T2 treatment (around 31.1%), with 'Canasta' being overall richer in HAA. On the other hand, ABTS results depicted a different trend, as 'Ballerina' showed a steady decrease in antioxidant activity beyond the control and F treatment, where biostimulant treatments causing a 15.3% overall decrease. The same cannot be said of 'Canasta', where one significantly higher-than-control treatment was found (T2). As for carotenoids content, it largely seemed unaffected by the applied treatments, save for the T1 × 'Ballerina', which yielded a 33.3% improvement when considering the average of the control treatments.

Table 4. Leaf pigments (chlorophylls and carotenoids), total ascorbic acid (TAA), hydrophilic antioxidant activity (HAA), ABTS antioxidant activity (ABTS) and total phenols of 'Canasta' and 'Ballerina' lettuce as influenced by the biostimulant application.

Source of Variance	Total Chlorophylls (mg g-1 FW)	TAA (mg AA 100 g-1 FW)	HAA (mmol AA eq. 100 g-1 DW)	Carotenoids (mg g-1 FW)	ABTS (mmol Trolox eq. 100 g-1 DW)	Total Phenols (mg gallic acid eq. g-1 DW)
Cultivar (C)						
Ballerina	1.49 ± 0.02	145.9 ± 7.26	3.77 ± 0.28	0.34 ± 0.01	27.30 ± 0.66	3.64 ± 0.14
Canasta	1.22 ± 0.03	84.45 ± 15.3	7.40 ± 0.26	0.34 ± 0.00	24.67 ± 0.78	4.98 ± 0.20
t-test	***	***	***	ns	ns	***
Biostimulant (B)						
Control	1.40 ± 0.05	81.86 ± 24.6 d	5.35 ± 0.73 c	0.33 ± 0.00 b	27.86 ± 1.31 a	4.85 ± 0.68
F	1.33 ± 0.09	123.7 ± 37.1 c	4.75 ± 0.71 c	0.32 ± 0.01 b	24.92 ± 2.47 b	4.18 ± 0.24
T1	1.38 ± 0.10	86.02 ± 16.8 d	4.83 ± 0.98 c	0.39 ± 0.03 a	24.71 ± 1.20 b	4.48 ± 0.27
T2	1.32 ± 0.05	86.42 ± 17.8 d	6.41 ± 1.18 b	0.34 ± 0.00 b	27.36 ± 0.91 a	4.07 ± 0.50
T1 + F	1.34 ± 0.05	164.3 ± 12.2 a	5.00 ± 0.88 c	0.33 ± 0.00 b	24.25 ± 0.34 b	3.73 ± 0.28
T2 + F	1.33 ± 0.06	148.9 ± 6.39 b	7.16 ± 0.55 a	0.34 ± 0.00 b	26.79 ± 0.31 a	4.54 ± 0.27
	ns	***	***	***	***	ns
C × B						
Ballerina × Control	1.48 ± 0.03	136.6 ± 4.69 bcd	3.83 ± 0.57 c	0.33 ± 0.00 b	30.54 ± 1.12 a	3.52 ± 0.48
Ballerina × F	1.52 ± 0.02	206.5 ± 3.11 a	3.23 ± 0.47 cd	0.30 ± 0.00 c	30.29 ± 1.32 a	4.00 ± 0.40
Ballerina × T1	1.59 ± 0.00	121.8 ± 8.29 d	2.68 ± 0.08 d	0.44 ± 0.02 a	27.19 ± 0.64 bc	3.90 ± 0.12
Ballerina × T2	1.44 ± 0.02	125.3 ± 8.10 cd	3.80 ± 0.16 c	0.34 ± 0.01 b	25.54 ± 0.66 cde	3.10 ± 0.23
Ballerina × T1 + F	1.45 ± 0.02	139.3 ± 8.60 bcd	3.12 ± 0.34 cd	0.32 ± 0.00 bc	23.81 ± 0.52 ef	3.25 ± 0.22
Ballerina × T2 + F	1.43 ± 0.03	146.2 ± 7.03 bc	5.97 ± 0.22 b	0.33 ± 0.00 b	26.43 ± 0.52 cd	4.04 ± 0.21
Canasta × Control	1.32 ± 0.08	27.09 ± 2.06 f	6.88 ± 0.22 b	0.34 ± 0.01 b	25.19 ± 0.45 cde	6.18 ± 0.55
Canasta × F	1.13 ± 0.07	41.02 ± 4.83 ef	6.27 ± 0.15 b	0.35 ± 0.01 b	19.56 ± 0.18 g	4.35 ± 0.32
Canasta × T1	1.18 ± 0.04	50.22 ± 7.49 e	6.97 ± 0.40 b	0.34 ± 0.01 b	22.24 ± 0.79 f	5.06 ± 0.12
Canasta × T2	1.20 ± 0.02	47.52 ± 1.32 ef	9.02 ± 0.38 a	0.34 ± 0.01 b	29.18 ± 0.60 ab	5.05 ± 0.53
Canasta × T1 + F	1.24 ± 0.06	189.3 ± 7.01 a	6.88 ± 0.44 b	0.34 ± 0.01 b	24.70 ± 0.33 de	4.20 ± 0.32
Canasta× T2 + F	1.24 ± 0.08	151.6 ± 12.2 b	8.36 ± 0.23 a	0.34 ± 0.00 b	27.14 ± 0.28 bc	5.03 ± 0.27
	ns	***	**	***	***	ns

All data are expressed as mean ± standard error, n = 3. ns, **, *** non-significant or significant at $p \leq 0.01$ and 0.001, respectively. Cultivars means were compared by *t*-Test. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($p = 0.05$). F: foliar treatment (3 mL L^{-1}), T1: nutrient solution treatment 0.15 mL L^{-1}, T2: nutrient solution treatment 0.3 mL L^{-1}.

3.3.5. Principal Component Analysis

A comprehensive view of the biometric, mineral, qualitative and physiological aspects and subdivision of 'Canasta' and 'Ballerina' lettuce in response to PHs was acquired via principal component analysis (PCA), which helped to further explain the differences in the biostimulant treatment dosage and mode of application. Out of all the obtained principal components (PCs), the first three explained 69.8% of the total variance, where PC1 and PC2 (Figure 1), explained 55.6% of the cumulative variance and were associated with eigen values higher than1. PC1 explained 30.1% of the cumulative variance and was positively correlated with leaf area, shoot fresh and dry weight, all the studied minerals except for sulfur. In addition, it was positively correlated to F_v/F_m, g_s, TAA and carotenoids. In contrast, it was negatively correlated with total phenols. On the other hand, PC2 explained 25.5% of the cumulative variance and was found positively correlated with total nitrogen, sulfur, SPAD index, photosynthetic parameters such as CO_2 assimilation (ACO_2) and transpiration rate (E) and HAA. Whilst it was negatively correlated with leaf number, DM%, total chlorophylls content and ABTS antioxidant activity. Based on the loading matrix, the PCA illustrated that the Shoot FW and DW were closely aligned with SPAD index and photosynthetic parameters (ACO_2, E and gs). In addition, the score plot issued from the PCA obviously separated the application mode and dose of the PHs, resulting in 'Canasta' × T1 + F or T2 + F and 'Ballerina' × T1 + F in the upper right quadrant with high shoot FW and DW, SPAD, ACO_2, E and g_s. On the other hand, both cultivars × control were diagonally opposite in the lower left quadrant.

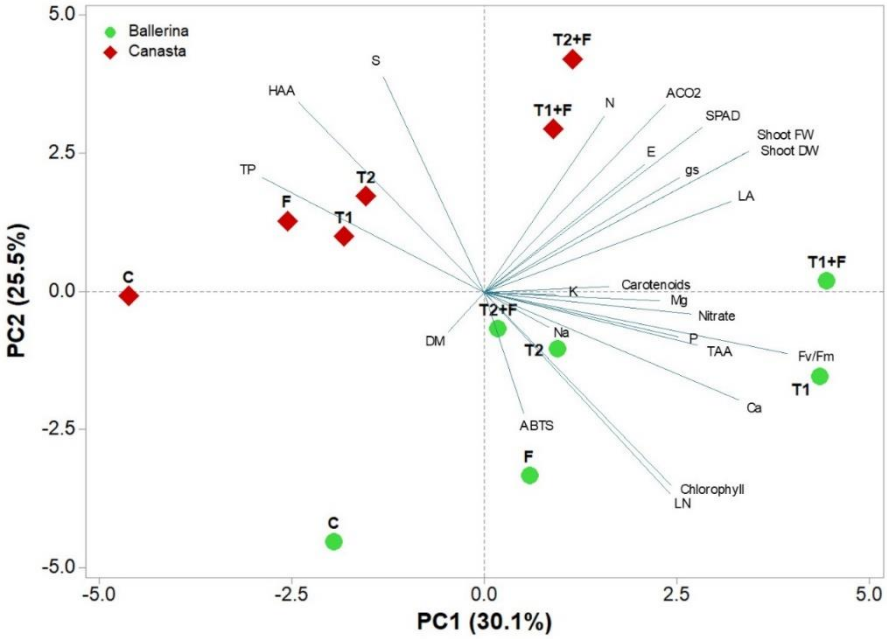

Figure 1. Principal component loading plot and scores of principal component analysis (PCA) on biometric (shoot fresh weight (FW), shoot dry weight (DW), dry matter % (DM) leaf number (LF) and Leaf Area (LA)), mineral (Nitrate, P, K, Ca, Mg, S and Na), qualitative (Total chlorophylls, Hydrophilic antioxidant activity (HAA), ABTS antioxidant activity, carotenoids, total phenols (TP) and total ascorbic acid (TAA)) and physiological aspects (SPAD index, fluorescence (F_v:F_m ratio), assimilated CO_2 (A_{CO2}), stomatal conductance (g_s) and transpiration rate (E)) of 'Canasta' and 'Ballerina' lettuce as influenced by the biostimulant application mode and dose (F: foliar treatment (3 mL L^{-1}), T1: nutrient solution treatment 0.15 mL L^{-1}, T2: nutrient solution treatment 0.3 mL L^{-1}).

3.4. Discussion

The aim of this paper was to depict the effect of the utilization of PHs in boosting the yield of floating system-grown lettuce, in addition to detecting any physiological and qualitative improvement, especially when testing new combinations of biostimulant application. The obtained results showed that the biostimulant application did indeed prove to be beneficial in boosting crop yield, as T1 and T1 + F treatments on the green 'Ballerina' cultivar and the T2 + F on the red 'Canasta' cultivar, recorded the highest marketable fresh yield compared to every other treatment and their untreated controls. Such increases can be explained by the modulation of yield parameters by the biostimulant, as they are consistent with leaf area, leaf fresh weight and stem fresh weights (data not shown) increases, which in turn provided for higher dry weights figures (data not shown). Yield increases after PHs biostimulant treatments are in line with currently available literature, as there is evidence of higher lettuce and spinach yield when treated with PHs, independently from nitrogen fertilization levels [21,23]. Physiological results are also in accordance with previous studies, as the increases of photosynthetic and physiological parameters were also recorded in tomato plants treated with the same commercial formulation and were also found to be dosage-dependent [36]. Interestingly, the only biometric parameter proven to be unaffected by the treatments on both cultivars was the leaf number, which comes in contrast with what is found in rocket, spinach and even lettuce studies [20,22,37]. Such a difference may be at least in part explained by addressing two important factors that directly modulate this plant feature. First, as our results clearly showed, cultivar-specific variation had a direct influence on lettuce leaf number and even then, cultivar-specific sensibility to nutrient contents in the growing medium may also come into play, as some varieties may favor leaf expansion over new leaf growth [38]. This finding is supported by the increased leaf area of both cultivars when treated with PHs. Second, the very different growing systems also have an impact on this parameter: a consensus can be found in the available literature of leafy vegetables and in lettuce in particular grown in hydroponics having—other than the already mentioned advantages—higher leaf numbers compared to traditional soil and substrate-based systems [39–41], which is due to a variety of factors that are inherent to soil cultivation, such as suboptimal oxygen and moisture contents, competition from soil organisms and biotic and/or abiotic stresses [42–45] that are the prime culprits of yield losses.

The postulated mechanism for the biostimulant effect of PHs can be traced back to product composition and in particular to the presence of bioactive molecules such as the so-called signaling peptides. Of those, the root hair growth promoting peptide [46] is one of the most widely known and is contained in the tested product [47]. As its name implies, products containing such peptide provide modifications of root architecture in density, length and increases in the number of lateral roots [15]. Nonetheless, the

explanation of the inner workings of PHs biostimulants prove more complex than that, as the increases in root growth may partially explain the elevated mineral (N, P, K, Ca, Mg, S) contents seen in this trial by the means of higher effective availability, but does not fully elucidate the recorded, whole-plant effects. A more involved explanation of the inner workings comes from their ability to act as plant physiological primers, by inducing transcription changes that favor the biosynthesis of phytohormones like indol-3-acetic acid (IAA) and abscisic acid (ABA) [16,48] and significantly impact gene expression in areas of plant development and metabolism [18,19], thus stimulating plant growth and yield. PH biostimulants, such as the one used in this trial, are also known to up-regulate nitrogen enzyme transcription both at the transporter and assimilation level [17,18], thereby increasing availability of this critical nutrient for plant growth for metabolic processes and can explain the improved photosynthetic activity which contributed to plant growth. Nevertheless, what this research also provides is a clear insight of a genotype-dependent response to PHs application, in both application mode and dosage. First, a comparatively higher growth response was denoted in the nutrient solution treatments, especially for the 'Ballerina' cultivar at its highest performing at T1 level. Differences between foliar and root-zone treatments was previously observed in a tomato study [49], whereby substrate drench application of a PHs biostimulant elicited increases in nitrogen metabolism and nitrogen leaf contents compared to the foliar treatment. The denoted differences in the biostimulant effect of the two application modes may be due to mechanisms at play when considering the means with which the product is taken up by the plants. Roots absorb amino-acids via specialized transporters [50] which, when coupled with the growing system used in this study, renders the availability of the biostimulant easier and daily throughout the growing cycle. Conversely, leaf absorption is a passive process that is mediated by climatic conditions such as wind and humidity levels that influence plant biological responses like stomata opening and cuticle thickness [51] and thus affecting the biostimulant absorption when applied in foliar mode. Therefore, placing T1 and T2 treatments in advantage when compared with F for 'Ballerina' and 'Canasta', respectively. In these regards, economic factors may also come into play when considering foliar treatments and especially combined applications. Colla [52] and Giordano [53] in their respective papers similarly employed weekly foliar treatments of the Trainer biostimulant on tomato and rocket plants and by operating a partial budget analysis, found that biostimulant-treated plants yielded increases in added net returns per hectare, from ~1260€ for tomato, stemming from a 7% increase in marketable yield, to ~9945€ for rocket which benefitted from a 50.7% yield increase. In this study, the T2 + F treatment elicited a 55.4% increase in yield for "Canasta", while for "Ballerina" the T1 treatment elicited an 82.7% increase in yield, which prove to be economically advantageous. When these percentages increase of production are calculated per hectare, the additional yield obtained amid the

biostimulant treatment render the boosting in tons very clear, where it increases from 35.6 to 65 tons ha^{-1} and from 45.2 to 70.3 tons ha^{-1} in "Ballerina" and "Canasta", respectively.

On the other hand, significant yield decreases were also recorded at the T2 and T2 + F level for the 'Ballerina' cultivar compared to the best performing T1 level, which comes to a sharp contrast to what was obtained in 'Canasta'. Insight into the matter comes from the physiological data, as for every studied parameter there was a reduction in the T2 + F × 'Ballerina' data compared to the best yielding T1 + F treatment, whereas 'Canasta' at the T2 + F level thrived with the elevated dosage by recording the highest recorded data. Growth inhibition by excessive exogenous amino-acid application has been postulated in literature as the phenomenon of "general amino acid inhibition" [54], whereby excess amino acid contents may either interfere with plant growth by inhibiting amino acid biosynthetic pathways [55] or, in a similar fashion, cause a strong phloematic load which in turn may cause plants to reduce nitrate absorption or reduction [47]. The latter case may explain why, in the 'Ballerina' × T2 combination the availability of amino acid and peptide contents in the nutrient solution might have reduced nitrate uptake and ultimately growth, phenomenon which was further accentuated by the increase in amino acid content provided by the T2 + F treatment. In the case of the 'Canasta' cultivar, amino-acid related stress symptoms may be averted by a combination of factors, all of which may relate to genotype-dependent stress-combating strategies. First and as seen in hydrophilic antioxidant activity data, 'Canasta' might originally have adapted a higher degree of stress related defenses due to the presence of anthocyanins, antioxidant molecules known to be induced by stress conditions [56–58], that were indirectly revealed by the melioration of the a* red color parameter detected at the same treatment (data not shown). Biostimulant treatments, due to the modulation of the ROS signaling network may have caused a change in antioxidant compounds [59] which manifested as increased HAA which may have provided stress protection. Similarly to anthocyanins, the drastic change at higher biostimulant dosages in the content of total ascorbic acid, a powerful ROS scavenging molecule [60,61] may have contributed to better protection against performance-decreasing dosage issues. Evidence seems to favor the use of PHs biostimulants to increase the functional quality of produce.

Increased antioxidant activity, which is linked to the combined effects of multiple antioxidants, like vitamin C and phenolic compounds [62], was found in rocket [20], lettuce [23] and may be cultivar and dosage dependent, as seen with tomato fruits [36]. In this trial, quality improvements manifested in higher hydrophilic antioxidants known for their health benefits [63] and vitamin C that take part in this category, is an essential phytochemical for human health [64]. Moreover, enhanced root mineral uptake from the roots also increased leaf K and Mg contents, therefore increasing the nutritional value of the leaves. Our results also showed a significant increase of leaf nitrate contents, which

plants use for nitrogen storage in leaves [65] and may have stemmed from the increase in root nitrogen availability and increased nitrogen metabolism after the application of the biostimulant. Nitrate contents in leafy greens is a cause for concern in today's agriculture, as one of the main dietary sources for humans is vegetable consumption [66] and while there's conflicting evidence on the role of nitrate on health risks due to long term consumption, a reduction in vegetable-borne contents may be favorable [67]. Still, no treatment out of all the tested combinations exceeded the nitrate threshold set by the EU Regulation 1258/2011, which for lettuce grown in protected environments is set at 4000 mg NO_3 kg^{-1}. PCA plotting has being used in previous studies [36,52] to better convey information regarding cultivars and biostimulant applications, especially with the regards of product quality. In this current study, the PCA reflected cultivar-specific varied response to biostimulant treatments tangible. In particular, 'Canasta' formed for two distinct groups in the upper quadrants, of which the right one includes higher quality produce with increased antioxidant activity, ascorbic acid and mineral contents. The lower right quadrant includes every other 'Ballerina' treatment, save for the control, which are characterized by elevated mineral and ascorbic acid contents, especially the T1 treatment, and low sulfur, phenolic and hydrophilic antioxidant activity. The different changes in functional quality of the two lettuce cultivars after being subjected to biostimulant application can further the hypothesis of these products acting by fine tuning ROS-mediated signaling [59], therefore being variably effective due to different leaf composition in the regards of pigments, ascorbate and phenolic contents.

3.5. Chapter 3 Conclusions

The results obtained in this trial suggest that the application of a legume-derived protein hydrolysates biostimulant on *L. sativa* has positive effects on crop performance, seen as elevated yield, physiology, and quality parameters. In depth, we recorded that the magnitude of the biostimulant effect is cultivar-specific, as the green 'Ballerina' cultivar recorded its highest growth performance at the lowest nutrient solution biostimulant application rate, with (T1 + F) or without foliar application (T1), resulting in the latter a staggering additional yield of 29.4 tons ha^{-1}, whereas red 'Canasta' exhibited the highest yield and nutraceutical content (in terms of total ascorbic acid and hydrophilic antioxidant activity) at the highest nutrient solution application rate combined with foliar PHs application (T2 + F), resulting as well a staggering additional yield of 25.1 tons ha^{-1}. In the case of 'Ballerina', as biostimulant usage has to be pondered against its costs to benefit ratio, these results could translate into monetary savings, in a commercial environment. In fact, not only the T1 treatment saves raw material compared to T2, but compared to F, it requires no further use of machines and manpower for the weekly foliar treatments and still enhance better shoot fresh yield. To conclude, more studies may be needed to figure out which genotypic features may impact performance when biostimulants are used, as to rationalize biostimulant application modes and dosages and guarantee the best crop growth and quality in a persistent manner.

3.6 Supplementary Material for Chapter 3

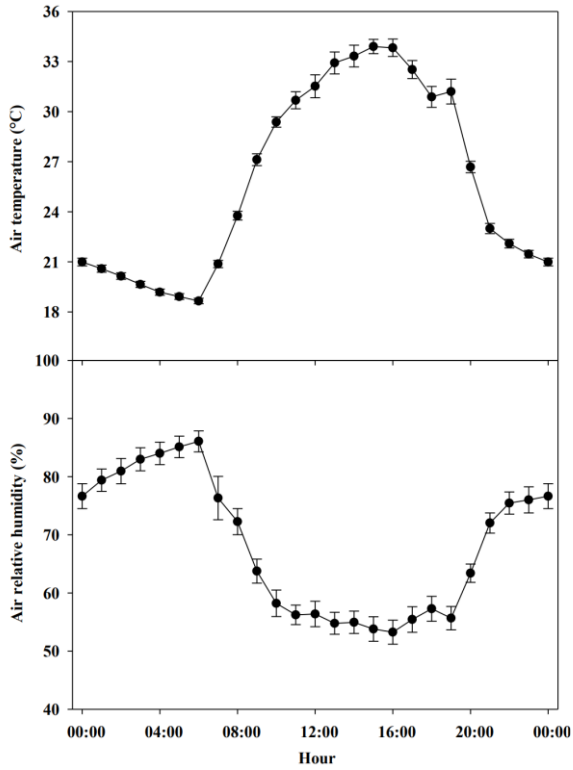

Figure S1. Hourly average air temperature and relative humidity values recorded throughout the lettuce crop cycle

Figure S2. Depiction of the experimental unit adopted for the trial

3.7 References for Chapter 3

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Chapter 4

Vegetal-derived Biostimulants Distinctively Command Physiological and Metabolic Signature of Lettuce Grown in Depleted Nitrogen Condition

Abstract: Biostimulants are sustainable inputs that can be used to reduce chemical fertilizers dependency while reinforcing nutrient uptake, yield and quality of crops, and modulate plant metabolic processes. Protein hydrolysates (PH) are prominent biostimulants that guarantee a reduction in yield loss under sub-optimal N conditions. On these bases, a new Malvaceae-derived PH product was tested along a commercial legume-derived PH on soilless greenhouse-grown lettuce, to comparatively assess their activity under nitrogen depletion conditions (1 mM NO_3^-). Both PHs increased biometric parameters under optimal but to a lesser extent under depleted N conditions. Legume-derived PH promoted greater Fv/Fm, lutein and β-carotene under optimal N conditions and higher catalase and total phenolic acids. In contrast, Malvaceae-derived PH did not affect phenolic acids but increased leaf concentration of Ca, Mg and catalase while reducing H_2O_2. Biochemical changes were then evaluated through untargeted metabolomics. Metabolomics showed a hierarchically prevalent effect of the N level, with the PH showing distinctive reprogramming under optimal and depleted N conditions. Among others, phenylpropanoids were mainly down-accumulated in stressed plants, while PUFA accumulated following the application of PHs. Notwithstanding, the severe depletion of N cannot be compensated by PH treatment since biostimulants are used to complement fertilizers use and not to replace it.

4.1. Introduction

Traditional agriculture relies extensively on synthetic chemicals [1], whereas greenhouse horticulture sets forth the finest corroboration of resource-intensive agriculture [2]. Indeed, synthetic nitrogen inputs represent the key factor that boosts the production of crops [2–4] across the seasons under optimal and sub-optimal conditions [3] by enhancing resource use efficiency [5]. Notwithstanding the utility of nitrogen application, its irrational and unversed use has been causing environmental impacts [2,4,6] and embodies the highest input cost other than reducing its efficiency use [1]. Accordingly, management plans are required to ameliorate nitrogen use efficiency and to deviate towards environmentally friendly and sustainable practices, where the use of biostimulants may represent an adequate solution [1,3,4,6].

Biostimulants from natural origin can support a sustainable production coupled with a diminution of agrochemicals input [5], fostering growth and development of crops in optimal or limited conditions [7]. Nonetheless, they represent an eco-friendly approach to reduce chemical fertilizers dependency and reinforcing nutrient uptake and yields

[8,9]. Such an increase in nutrient uptake can be attributed to several factors, like the modification of root architecture or the amelioration of soil enzymatic and microbial activities [10]. Although plant-based biostimulants are encouraged to be used in organic farming, they are finding an excellent space for application in conventional agriculture [11] to cope with stresses induced by climate change or depleted fertilizer conditions [9]. Noteworthy, some biostimulants can be obtained from different industrial by-products, reducing and recycling waste and thus generating advantages for the food industry, growers and consumers [8,12]. Protein hydrolysates, a prominent group of biostimulants, are attracting attention due to the positive responses in terms of crop performances [11]. Their use is proven to diminish yield reduction under deficient nitrogen levels in horticultural crops like rocket, spinach and lettuce [2,3,13,14]

Biostimulants have been reported to modulate plants' metabolic and enzymatic processes [8], triggering a biochemical reprogramming that supports plant performance. Metabolomics provides a characterization of the pool of metabolites in plant tissues, including those involved in the response with environment. It has been thrivingly adapted for molecular phenotypes in plants as a response to stress, to discern patterns related to stress coping and to distinguish any chemical fingerprint left by cellular processes [15]. Indeed, high-throughput analytics like metabolomics have exemplified huge progress in plant science, allowing us to understand plants' kickback or acclimatization to minerals availability [16], as well as to shed light on the mechanisms underlying the mode of action of different biostimulants [17]. In this sense, previous evidence has highlighted that biostimulants may play a role in regulating the biosynthesis of specialized metabolites implicated in the defense and fortification of plant organisms. These secondary metabolites are the communication tools for plants' interaction with the surroundings and vary significantly across species, thus also playing an ecological outcome [18]. Unravelling the biochemical processes involved in plant response to biostimulants is important to develop tailored biostimulant strategies, thus paving the way towards the efficient implementation of biostimulant-based agronomic strategies.

In this work, a soilless greenhouse experiment was performed on lettuce (*Lactuca sativa* L.) to decipher the biostimulant impact of two vegetal-based protein hydrolysates derived from malvaceae and legumes under either optimal or depleted nitrogen administration. The work aimed to investigate in a soilless greenhouse experiment the modulation of lettuce growth and metabolomics reprogramming in response to low nitrogen levels and to two different vegetal-derived protein hydrolysates. In particular, the aftermath of a PH from malvaceae biomass was compared to a commercial legume-derived product in terms of growth, mineral composition, biochemicals and metabolites under nitrogen deficiency. For this purpose, a combined phenolic profiling and untargeted metabolomics approach is proposed, to gain a deep insight into the effects

of both biostimulants on stressed lettuce plants. Overall, this approach will assist in the discovery of new natural biostimulants to enhance the production of economically attractive crops under relevant environmental stresses, as is the case of nitrogen deficiency.

4.2. Materials and Methods

4.2.1. Experimental setup and design

A greenhouse experiment was set up on October 2nd 2020 for a total of 42 days. The investigation was carried out in an unheated greenhouse at the Department of Agricultural Sciences of the University of Naples Federico II. The seedlings of *Lactuca sativa* L. cv. "Maravilla De Verano Canasta" were transplanted into plastic pots (1.6 L) filled with a 90:10 (*v*/*v*) mixture of 3 mm quartz sand (Vaga, Sabbie e Ghiaie Silicee, Costa de'Nobili (PV) Italy) and perlite, respectively. The planting density consisted of 14 plants per m^2. The experimental design consisted of a split-plot system, where the nitrogen level in the nutrient solution was the main factor (2 levels), and a sub-factor consisting of the biostimulants treatments (2 protein hydrolysates and an untreated Control). Inside the main blocks, the biostimulant treatments were organized in a randomized block design with 3 replicates. Each replicate consisted of five plants.

The nutrient solution of the optimal level of nitrogen consisted of 8 mM nitrate, 1.5 mM phosphorus, 4 mM potassium, 4 mM calcium, 2.5 mM sulfur, 1.25 mM magnesium, 20 μM iron, 9 μM manganese, 0.3 μM copper, 1.6 μM zinc, 20 μM boron, and 0.3 μM molybdenum. As for the nutrient solution of the low level of nitrogen, the following composition was used: 1 mM nitrate, 0.5 mM calcium supplied by calcium nitrate but to ensure equal calcium concentration and guarantee iso-osmosis with the other nutrient solution (optimum nitrogen), calcium chloride was added, whereas the rest of nutrients remained unchanged. Both nutrient solutions had an electrical conductivity of 1.6 ± 0.1 dS m^{-1} and the pH of the solutions was 5.8 ± 0.2.

4.2.2. Protein hydrolysates treatments

The protein hydrolysates biostimulants chosen for this trial included a legume-derived PH (LPH) commercial formulation ('Trainer', Hello Nature Italia S.R.L., Rivoli Veronese, Verona, Italy), consisting of mixtures of amino acids and soluble peptides, described in detail by Paul and collaborators [19], containing 5% N (31.2% aminoacids and peptides) and 17.6% total carbon. As recommended, the foliar application of the biostimulant was made at a rate of 3 mL L^{-1} solution via a steel-bottle sprayer. A total of five treatments were applied during the experiment. The first treatment was done 10 days after the transplant (BBCH-scale 19) and was repeated each week.

The second test item was a malvaceae-derived PH (CPH), the aminogram of which was as follows (expressed in μmol mL^{-1}): Ala (9.39), Arg (3.17), Asn (8.82), Asp (3.76), GABA (0.93), Gln (0.17), Glu (4.89), Gly (4.67), His (1.38), Ile (2.05), Leu (4.7), Lys (3.42),

MEA (0.75), Met (1.99), Orn (4.06), Phe (1.28), Pro (4.97), Ser (11.04), Thr (4.12), Trp (2.61), Tyr (3.09), Val (3.33), total amino acids (84.6), minor amino acids (27.02) and branch-chained amino acids (10.07). As for the minerals and organic acids analysis, CPH contained (expressed in μmol mL^{-1}): Na^+ (35.01), NH_4^+ (46.69), K^+ (41.49), Mg^{2+} (112.58), Ca^{2+} (64.61), Cl^- (13.86), NO_2^- (1.91), NO_3^- (0.08), SO_4^{2-} (191.58), acetate (99.91), and malate (0.36). This CPH contained 4.67% N (29.2% aminoacids and peptides) and 16.9% total carbon. It was sprayed in a concentration to supply the same amount of N as the LPH.

4.2.3. Sampling and biometric measurements

At the end of the experiment, three plants per experimental unit were chosen to determine leaf number, leaf area, growth index, shoot fresh weight, and shoot dry weight. Then, the leaf dry matter percentage was calculated as DM = (shoot dry weight/shoot fresh weight) × 100. Just before harvesting, plant growth index (GI) was calculated as $GI = \pi (W/2)^2 \times H$ and expressed in cm^3 $plant^{-1}$. Height (H) was determined as the distance from the substrate surface to the plant top, and width (diameter: W) as the average of two perpendicular measurements. Leaf area measurements were carried out via leaf photography and further quantified using the ImageJ v1.52a software (U.S. National Institutes of Health, Bethesda, MD, USA) and expressed in cm^2. After the fresh weight measurements, all plant matter was dried in a forced-convection oven at 60 °C until a constant weight was reached.

The obtained dry matter was further processed using a grinding mill (MF10.1 model, IKA-Werke GmbH & Co. KG, Staufen, Germany) for leaf mineral content determination and total nitrogen. A pool of four leaves from two plants per experimental unit was immediately quenched in liquid nitrogen and later stored at -80 °C for the determination of oxidative stress markers (malondialdehyde acetate (MDA) and hydrogen peroxide (H_2O_2)), and antioxidant enzymes activity (catalase (CAT) and ascorbate peroxidase (APX)). Furthermore, an additional pool of four leaves from two plants per experimental unit was also quenched in liquid nitrogen for the metabolomic assays, carotenoids, and polyphenolic determination.

4.2.4. Leaf gas exchange and chlorophyll fluorescence

Leaf gas exchange measurements were carried out before harvesting between 11 am and 1 pm, on five young and fully expanded leaves per each experimental unit. To this aim, an LCi T compact photosynthesis system (ADC Bioscientific Ltd., Herts, UK) equipped with a broad-leaf chamber and a programmable LED light was used. Photosynthetic photon flux density (PPFD) inside the chamber was set as 1000 μmol m^{-2} s^{-1}; relative humidity and CO_2 concentration were kept at ambient levels. The data recorded included CO_2 net assimilation rate (A_{CO2}; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs; mmol H_2O m^{-2} s^{-1}) and transpiration (E; mol H_2O m^{-2} s^{-1}). A fourth derived measurement, instantaneous water use efficiency or WUEi was calculated as

WUEi = A_{CO2}/E (WUEi; μmol CO_2 mol^{-1} H_2O). On the same day, chlorophyll fluorescence was measured by a portable fluorometer Fv/Fm Meter (Opti-Sciences Inc., Hudson, United States). Chlorophyll fluorescence was performed on the leaves of five plants per experimental unit after their dark adaptation by leaf clips for 20 min. According to the maximum quantum efficiency of Photosystem II (PSII), Fv/Fm was calculated as (Fm - F_0)/Fm, where F_0 and Fm were the ground fluorescence signal and the maximal fluorescence intensities in the dark-adapted state, respectively [20].

4.2.5. Biochemical parameters determination

Plant extraction was carried out in 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) per 0.20 g of tissue powder. Homogenates were centrifuged for 15 min at 12,000 × *g* at 4 °C, and supernatants were recovered. Lipid peroxidation was estimated through malondialdehyde (MDA) determinations by the thiobarbituric acid reaction, according to the protocol reported by Dhindsa and collaborators [21]. Briefly, 0.5 mL of the supernatant was mixed with 1 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) and heated for 30 min at 95 °C. The non-specific background absorbance reading at 600 nm was subtracted from the specific absorbance reading at 532 nm. The MDA content was estimated using the extinction coefficient of 155 mM^{-1} cm^{-1}, and the concentration was expressed as nmol g^{-1} fw.

Hydrogen peroxide (H_2O_2) concentration was quantified according to Sergiev and collaborators [22]. The supernatant was treated with 0.5 M potassium phosphate buffer (pH 7.0) and 1 M potassium iodide (KI), and the absorbance of the mixture was measured at 390 nm. The H_2O_2 concentration was determined using a calibration curve obtained with different concentrations of H_2O_2 and expressed as μmol g^{-1} fw.

For antioxidant enzyme determination, frozen leaf samples (0.5 g) were ground in 5 mL of extraction buffer (100 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA). The homogenate was centrifuged at 10,000 × g, and the supernatant was collected and immediately used for subsequent analyses of catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11), as well as for the determination of the total protein content in accordance to what described by the Lowry and collaborators' [23] method with bovine serum albumin as a standard. Catalase activity was determined by following the enzyme-driven consumption of H_2O_2. The reaction mixture of 3 mL was prepared by mixing 1.5 mL phosphate buffer (100 mM, pH 7), 0.5 mL H_2O_2 (60 mM), 50 μL enzyme extract, and 0.95 mL distilled water. The absorbance was measured at 240 nm every 10 s for 2 min. The CAT activity was calculated using the molar extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed in μmol H_2O_2 mg^{-1} protein min^{-1}.

Ascorbate peroxidase activity was assessed by following the consumption of ascorbic acid at 290 nm [24]. The 3 mL of the reaction mixture was prepared by mixing 1.5 mL phosphate buffer (100 mM, pH 7), 0.3 mL ascorbic acid (5 mM), 0.1 mL EDTA (3 mM),

0.1 mL H_2O_2 (60 mM), 0.1 mL enzyme extract, and 0.9 mL distilled water. A decrease in the absorbance was assessed spectrophotometrically at 290 nm every 10 s for 2 min. The APX activity was calculated using the molar extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed in μmol ascorbate mg^{-1} protein min^{-1}.

4.2.5. Leaf total nitrogen and mineral analysis

The total leaf nitrogen assay was conducted on 1 g of dried leaf samples using the Kjeldahl method after mineralization with 96% sulfuric acid, 30% hydrogen peroxide, and selenium + potassium sulfate + copper oxide as catalyzer buffer, as described previously [25].

A further set of minerals, namely P, K, S, Ca, and Mg were determined using the ICS-3000 ion chromatography system (Dionex, Sunnyvale, CA, USA) after water extraction of 0.250 g of dry sample matter in a heated bath at 80 °C for 10 minutes. After separation using the IonPac AS11-HC and IonPac CS12A analytical columns, the minerals were quantified against analytical standards as described in detail in a previous work [26]. All leaf mineral contents were expressed as mg g^{-1} dry weight (dw).

2.6. Lutein and β-carotene and total ascorbic acid analysis

Leaf lutein and β-carotene determinations assays were done using 100 mg of lyophilized leaf matter. As described by Kyriacou and collaborators [27], a first sample extraction was performed in 0.1% BHT in ethanol, followed by a saponification step using KOH. Pigment extraction was performed in *n*-hexane, which was later evaporated under a nitrogen atmosphere. Afterwards, 1 mL of chloroform was added to this residue, and the mixture was separated through a reverse Phase-HPLC-DAD using a Shimadzu Model LC 10 chromatographer (Shimadzu, Osaka, Japan) equipped with a 250 × 4.6 mm, 5 μm Gemini C18 column (Phenomenex, Torrance, CA, USA). The absorbance of the eluent was measured at 450 nm. Authentic lutein and β-carotene were used to evaluate their quantity in the sample based on external calibration curves ranging 5–100 μg mL^{-1} including a minimum of six levels of concentration. Carotenoid content was quantified as μg g^{-1} dw.

The total ascorbic acid, defined as the sum of ascorbic and dehydroascorbic acids, was assessed by spectrophotometric assays based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl [28]. Dehydroascorbate was first reduced to ascorbic acid by pre-incubating the sample in dithiothreitol. Quantification was performed at 525 nm against an external ascorbate standard calibration curve in the range of 5 – 100 μmol mL^{-1} and the results were expressed as mg 100 g^{-1} fw.

4.2.7. Phenolic acids and flavonoids analysis

The leaf polyphenolic assay was performed analogously to Kyriacou and collaborators [27]. Briefly, plant extraction was carried out on 100 mg of lyophilized leaf

sample in 5 mL of a 60:40 *v*/*v* methanol/water solution. Phenolics separation was obtained via a UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 1.7 μm Biphenyl (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA). Mass spectrometry data were obtained via a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative ion mode (ESI-) for all the analyzed compounds was implemented. The accuracy and calibration of the Q Exactive Orbitrap LC–MS/MS was monitored daily via a reference standard mixture obtained from Thermo Fisher Scientific. Data analysis and processing were done using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific). All polyphenolic data are expressed as μg g^{-1} dw.

4.2.8. Untargeted Metabolomics Analysis and Data Processing

Lettuce leaves were harvested and freeze-dried to be analyzed by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) as previously reported [17]. Briefly, leaves samples were grounded with liquid nitrogen using a pestle and mortar and 2 g per sample were mechanically homogenized in 20 mL of 80% methanol acidified with 0.1% formic acid (*v*/*v*). Extracts were then centrifuged at 10,000 x *g* and filtered through 0.22-μm pore-size cellulose syringe filters before analysis through liquid chromatography quadrupole-time-of-flight mass spectrometry (UHPLC/QTOF-MS – 6550 iFuneel QTOF from Agilent Technologies, Santa Clara, CA, USA). A volume of 6 μL was injected into the analytical system and reverse-phase chromatography was applied for separation. A water-acetonitrile gradient elution (from 6 to 94% of acetonitrile in 33 min) on a Agilent Zorbax C18 column (15 cm x 2.1 mm, 1.7 μm particle size) was set. The mass spectrometer worked in SCAN mode (100–1200 *m*/*z* range) with a nominal resolution at 30,000 FWHM and in positive polarity. Two technical replicates were analysed for each sample, totaling 6 replicates per experimental group. Blank samples were injected at the beginning and the end of each randomized sequence run. Moreover, Quality Control (QC) samples (a pool of aliquots from each analyzed sample) were analyzed every 10 samples from the beginning of the run. QC samples were analyzed in data-dependent MS/MS mode (10 precursors per cycle, 1 Hz, 50–1200 *m*/*z*, positive polarity, active exclusion after 2 spectra), at specific collision energies (10, 20, and 40 eV) [17].

Raw mass data were processed by the Agilent Profinder B.07 (Agilent Technologies) software applying the "find-by-formula" algorithm. Therein, monoisotopic accurate mass and isotopic pattern (i.e., isotope spacing and ratio), with a mass accuracy tolerance of 5 ppm were applied following mass and retention time alignment. As referred by COSMOS Metabolomics Standards Initiative[29], a putative Level 2 annotation was achieved. Annotated features were filtered by frequency to retain compounds in at least 75% of replicates within at least one treatment.

4.2.9. Statistical Analysis

Data from the morpho-physiological and biochemical parameters were analyzed with the SPSS 28 software package (IBM, Armonk, NY, USA) and are presented as mean ± standard error, n = 3. The mean effects were subjected to two-way ANOVA (Nitrogen level × biostimulant) . A t-test was employed to compare the mean effect of nitrogen, and Tukey's HSD test was performed to separate both the biostimulant mean effect and salinity × biostimulant interaction. All tests were deemed significant at $p = 0.05$.

The Mass Profiler Professional B12.6 software tool (Agilent Technologies, Santa Clara, CA, USA) was used to elaborate the metabolomics dataset as previously reported by Benjamin and collaborators [30]. Compounds abundance was log2-transformed, normalized at the 75th percentile and baselined against the median of all samples. According to their metabolic profile, the similarities and/or differences among samples were reported through unsupervised hierarchical cluster analysis (HCA - Euclidean distance, Ward's linkage) based on fold change (FC) heat maps. From this clustering, two separate datasets were then obtained depending on the nitrogen level, providing distinctive interpretations for optimal and N-depleted conditions. A two-way analysis of variance (ANOVA) was further performed to detect the statistically significant compounds among treatments, setting a significance level of $\alpha = 0.05$ (Tukey's *post hoc* test; Bonferroni multiple testing correction). Both datasets were separately imported in SIMCA 16 (Umetrics, Malmo, Sweden), to perform a supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) multivariate modelling. The obtained model was successively cross-validated (CV-ANOVA; $p < 0.05$), inspected for outliers (Hotelling's T2), and the model's goodness parameters (goodness-of-fit and goodness-of-prediction) were checked. Model overfitting was then excluded by permutation testing (n=200). Metabolites with variable importance in projection (VIP) score > 1.2 were extrapolated. Statistically significant (p-value < 0.05 in at least one of the treatments) VIP compounds were selected for further interpretation on PlantCyc Pathway Tools software (Plant Metabolic Network, http://www.plantcyc.org, accessed on May 2022) [31].

4.3. Results

4.3.1. Leaf number and area, growth index, shoot fresh and dry weight, leaf dry matter

The biometric data of lettuce plants studied in this work are provided in Table 1. Among the assessed parameters, leaf number plant-1 and leaf dry matter percentage were only dictated by nitrogen level in the nutrient solution (NS). They were 1.7-fold lower and 1.3-fold higher, respectively, in low N. As for leaf area, growth index and shoot fresh weight (Table 1), an interaction among the factors tested (N and biostimulants (Bs)) was present; In the low N solution, the application of both protein hydrolysates caused, on average, an increase of 12.16, 11.25, and 7.93% compared to the

untreated Control, but it was deemed non-significant. On the contrary, in optimal N conditions, the application of the legume-derived (LPH) and Malvaceae-derived protein hydrolysates (CPH) caused a significant increase for all the previous parameters, except for leaf area where only LPH had a significant effect. LPH application provided a higher leaf area and growth index promotion than CPH, causing an increase of 41.30 and 10.97%, respectively, compared to the optimal N Control. Regarding shoot dry weight, both protein hydrolysates (PHs) caused an average increase of 6.51%, when averaged across the NS. Noting that shoot fresh weight was 4.62-fold higher in optimal N compared to low N in the NS, when averaged among the Biostimulant treatments.

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Table 1. Biometric parameters of *Lactuca sativa* L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	Leaf number (no. plant^{-1})	Leaf area (cm^2)	Growth Index (cm^3 plant^{-1})	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Leaf dry matter (%)
Nitrogen (N)						
Optimal (O)	33.62 ± 0.33 a	3521 ± 79 a	44012 ± 2342 a	283.72 ± 4.97 a	13.9 ± 0.18 a	4.9 ± 0.05 b
Low (L)	19.76 ± 0.32 b	799.1 ± 18 b	8344 ± 270 b	61.39 ± 0.95 b	3.88 ± 0.07 b	6.32 ± 0.06 a
t-test	***	***	***	***	***	***
Biostimulant (B)						
Control	26.14 ± 3.08	2064 ± 594 b	22116 ± 6518 c	162.5 ± 46.66 b	8.52 ± 2.15 b	5.67 ± 0.3
Malvaceae-derived PH (CPH)	27.42 ± 3.09	2117 ± 571 b	26082 ± 8050 b	175.9 ± 50.41 a	9.04 ± 2.3 a	5.53 ± 0.27
Legume-derived PH (LPH)	26.50 ± 3.18	2300 ± 665 a	30337 ± 9493 a	179.26 ± 52.21 a	9.11 ± 2.27 a	5.63 ± 0.38
	n.s.	**	***	**	*	n.s.
N × B						
O*Control	33.01 ± 0.58	3388 ± 111 b	36470 ± 2509 c	266.7 ± 5.14 b	13.33 ± 0.09	5.01 ± 0.09 b
O*CPH	34.28 ± 0.49	3390 ± 94.6 b	44049 ± 1098 b	288.48 ± 5.56 a	14.18 ± 0.26	4.92 ± 0.03 b
O*LPH	33.56 ± 0.59	3786 ± 37.2 a	51517.97 ± 1310 a	295.97 ± 2.87 a	14.18 ± 0.25	4.79 ± 0.11 b
L*Control	19.27 ± 0.11	739.2 ± 1.23 c	7761.84 ± 351.68 d	58.29 ± 1.31 c	3.7 ± 0.12	6.34 ± 0.09 a
L*CPH	20.56 ± 0.62	844.3 ± 20.5 c	8114.9 ± 96.85 d	63.32 ± 0.81 c	3.89 ± 0.07	6.14 ± 0.05 a
L*LPH	19.44 ± 0.59	813.8 ± 25.1 c	9155.3 ± 466.62 d	62.55 ± 1.08 c	4.05 ± 0.1	6.47 ± 0.07 a
	n.s.	*	***	*	n.s.	*

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

4.3.2. SPAD index, Fv/Fm, A_{CO2}, g_s, E and WUEi

Table 2 unveiled the physiological parameters that were assessed in this study. Although SPAD index was influenced by the interaction of N × B, it was not significantly increased by the PHs application, whereas it was affected by the concentration of N in the NS, with a reduction of 1.24-fold in low N treatment when treated with PHs. Similarly, stomatal conductance (g_s), transpiration rate (E) and instantaneous water use efficiency (WUEi) were significantly reduced under low N by 1.36-, 1.18- and 1.43-fold, respectively. On the other hand, fluorescence (Fv/Fm ratio) and net photosynthesis (A_{CO2}), were influenced by the interaction N × B. Significant increases were only noticed for Fv/Fm ratio under LPH treatment, referring to a 5.22% increase compared to the untreated optimal control. As for A_{CO2}, a significant increase was only stated under optimal N conditions when sprayed with both PHs, with an average increase of 26.39%.

Table 2. Physiological parameters of *Lactuca sativa* L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	SPAD index	Fluorescence F_v/F_m ratio	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	g_s (mmol H_2O m^{-2} s^{-1})	E (mol H_2O m^{-2} s^{-1})	WUEi (μmol CO_2 mol H_2O^{-1})
Nitrogen (N)						
Optimal (O)	31.24 ± 0.44	0.82 ± 0.01	18.20 ± 0.75	0.55 ± 0.02	11.2 ± 0.29	1.63 ± 0.05
Low (L)	25.85 ± 0.46	0.83 ± 0.01	10.77 ± 0.48	0.39 ± 0.02	9.48 ± 0.30	1.14 ± 0.03
t-test	***	ns	***	***	***	***
Biostimulant (B)						
Control	28.45 ± 0.82	0.81 ± 0.01 b	13.24 ± 1.17 b	0.48 ± 0.04	10.23 ± 0.45	1.29 ± 0.09
Malvaceae-derived PH (CPH)	28.84 ± 1.49	0.83 ± 0.01 a	14.74 ± 2.27 ab	0.45 ± 0.05	10.09 ± 0.68	1.42 ± 0.14
Legume-derived PH (LPH)	28.34 ± 1.55	0.84 ± 0.01 a	15.48 ± 1.78 a	0.49 ± 0.03	10.68 ± 0.39	1.43 ± 0.12
	ns	**	*	ns	ns	ns
N × B						
O × Control	29.91 ± 0.47 ab	0.80 ± 0.01 b	15.48 ± 0.60 b	0.56 ± 0.04	10.71 ± 0.77	1.45 ± 0.07
O × CPH	32.06 ± 0.66 a	0.82 ± 0.01 ab	19.74 ± 0.70 a	0.56 ± 0.04	11.45 ± 0.31	1.73 ± 0.08
O × LPH	31.74 ± 0.56 a	0.85 ± 0.00 a	19.39 ± 0.60 a	0.55 ± 0.03	11.43 ± 0.32	1.70 ± 0.03
L × Control	26.99 ± 1.00 bc	0.82 ± 0.01 ab	10.99 ± 1.20 c	0.41 ± 0.05	9.75 ± 0.43	1.12 ± 0.09
L × CPH	25.62 ± 0.50 c	0.83 ± 0.01 ab	9.74 ± 0.48 c	0.35 ± 0.03	8.74 ± 0.61	1.12 ± 0.02
L × LPH	24.94 ± 0.40 c	0.84 ± 0.01 ab	11.58 ± 0.52 c	0.43 ± 0.02	9.93 ± 0.28	1.17 ± 0.06
	*	*	**	ns	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD (p = 0.05). A_{CO2}: net photosynthesis, g_s: stomatal conductance, E: transpiration and WUEi: instantaneous water use efficiency.

4.3.3. Total nitrogen and minerals (P, K, S, Ca and Mg)

Among all the minerals analyzed in this work, only K exhibited a significant variation in response to the interaction between the factors tested (**Table 3**). PHs application caused an increase in K concentration under optimal N conditions, being significant with respect to the optimal control in the case of CPH, whereas no significant differences were noted under low N. **Table 3** demonstrated that the rest of the elements were dictated by the mean effect of N level like total N and P, whereas Mg was affected by both factors independently and Ca was only influenced by the mean effect of the biostimulants treatment. In contrast, S content was not significantly altered in any condition. Total N percentage, P and Mg had lower concentrations under low N treatment by 1.86, 1.24 and 1.34-fold, respectively, compared to the optimal N condition. As for K, the concentration was 1.13-fold higher under low N conditions. When averaged across N level, CPH significantly increased Ca and Mg contents in treated lettuce plants by 33.74 and 28.20% compared to control.

Table 3. Total nitrogen and mineral analysis of *Lactuca sativa* L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	Total N (%)	P (mg g^{-1} dw)	K (mg g^{-1} dw)	S (mg g^{-1} dw)	Ca (mg g^{-1} dw)	Mg (mg g^{-1} dw)
Nitrogen (N)						
Optimal (O)	2.90 ± 0.04	3.82 ± 0.1	53.0 ± 3.25	0.76 ± 0.09	6.58 ± 0.58	3.81 ± 0.27
Low (L)	1.56 ± 0.05	3.06 ± 0.13	59.7 ± 2.43	0.58 ± 0.04	7.80 ± 0.62	2.84 ± 0.14
t-test	***	**	*	ns	ns	**
Biostimulant (B)						
Control	2.26 ± 0.34	3.62 ± 0.13	51.4 ± 4.52 b	0.6 ± 0.05	6.58 ± 0.52 b	3.05 ± 0.09 b
Malvaceae-derived PH (CPH)	2.19 ± 0.30	3.46 ± 0.24	63.6 ± 2.77 a	0.83 ± 0.13	8.80 ± 0.75 a	3.91 ± 0.38 a
Legume-derived PH (LPH)	2.23 ± 0.28	3.24 ± 0.24	54.0 ± 1.27 b	0.58 ± 0.06	6.18 ± 0.54 b	3.02 ± 0.33 b
	ns	ns	**	ns	*	**
N × B						
O × Control	3.00 ± 0.08	3.88 ± 0.14	42.0 ± 3.65 b	0.62 ± 0.06	5.61 ± 0.50	3.16 ± 0.12
O × CPH	2.85 ± 0.08	3.85 ± 0.25	62.3 ± 2.50 a	0.99 ± 0.25	7.74 ± 1.24	4.62 ± 0.43
O × LPH	2.86 ± 0.06	3.74 ± 0.15	54.5 ± 1.67 ab	0.69 ± 0.07	6.39 ± 1.02	3.66 ± 0.34
L × Control	1.52 ± 0.07	3.37 ± 0.06	60.8 ± 1.16 a	0.58 ± 0.08	7.56 ± 0.41	2.95 ± 0.11
L × CPH	1.54 ± 0.13	3.07 ± 0.29	65.0 ± 5.52 a	0.67 ± 0.05	9.86 ± 0.42	3.20 ± 0.13
L × LPH	1.61 ± 0.06	2.74 ± 0.15	53.4 ± 2.25 ab	0.48 ± 0.02	5.97 ± 0.63	2.38 ± 0.17
	ns	ns	*	ns	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). dw: dry weight

4.3.4. MDA, H2O2, CAT and APX

As reported in **Table 4,** among the different biochemical compounds tested, only MDA was significantly affected by the interaction N × B, as CPH caused a slight non-significant increase under optimal N condition, while under low N condition, no significant increase was observed for any PH. In addition, MDA was 1.5-fold higher under low N conditions, which was also the case for H_2O_2 (2.22-fold) and APX (4.78-

fold). The application of both PHs provoked a significant decrease in H_2O_2, about 17.42% on average. Concerning CAT activity, it was strongly increased by biostimulants application, reaching an increase of 136.21% for CPH and 216.42% for LPH, whereas it was unaffected significantly by N supply.

Table 4. Biochemical parameters of *Lactuca sativa* L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	MDA (nmol g^{-1} fw)	H_2O_2 (µmol g^{-1} fw)	CAT (µmol H_2O_2 mg^{-1} protein min^{-1})	APX (µmol ascorbate mg^{-1} protein min^{-1})
Nitrogen (N)				
Optimal (O)	0.97 ± 0.05	0.49 ± 0.03	38.9 ± 7.21	0.09 ± 0.01
Low (L)	1.44 ± 0.07	1.09 ± 0.04	41.4 ± 5.39	0.43 ± 0.04
t-test	***	***	ns	***
Biostimulant (B)				
Control	1.26 ± 0.16	0.89 ± 0.13 a	18.4 ± 2.52 c	0.31 ± 0.07
Malvaceae-derived PH (CPH)	1.16 ± 0.1	0.7 ± 0.13 b	43.6 ± 3.22 b	0.29 ± 0.10
Legume-derived PH (LPH)	1.2 ± 0.13	0.77 ± 0.15 b	58.4 ± 3.97 a	0.18 ± 0.06
	ns	**	***	ns
N × B				
O × Control	0.92 ± 0.08 c	0.61 ± 0.03	13.2 ± 1.20	0.14 ± 0.01
O × CPH	1.06 ± 0.11 bc	0.42 ± 0.01	42.4 ± 2.61	0.08 ± 0.01
O × LPH	0.92 ± 0 c	0.43 ± 0	61.1 ± 5.71	0.05 ± 0.01
L × Control	1.6 ± 0.02 a	1.18 ± 0.02	23.7 ± 1.59	0.47 ± 0.04
L × CPH	1.25 ± 0.17 abc	0.98 ± 0.07	44.8 ± 6.59	0.5 0± 0.11
L × LPH	1.48 ± 0.06 ab	1.11 ± 0.05	55.6 ± 6.22	0.31 ± 0.02
	*	ns	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). MDA: malondialdehyde, H_2O_2: hydrogen peroxide , CAT: catalase and APX: ascorbate peroxidase activity. fw: fresh weight

4.3.5. Carotenoids and total ascorbic acid

Lutein, β-carotene and total ascorbic acid (TAA) data are presented in **Table 5**. The interaction N × B was found significant for all the mentioned parameters. Remarkably, the interaction O × CPH decreased lutein and β **-**carotene contents compared with both control and LPH. In low N conditions, both parameters exhibited no significant difference under any PHs application. As for TAA (**Table 5**), the applications of PHs did not cause any significant changes.

Table 5. Carotenoids and total ascorbic acid (TAA) of *Lactuca sativa* L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	Lutein (μg g-1 dw)	β-carotene (μg g-1 dw)	AA (mg 100 g-1 fw)
Nitrogen (N)			
Optimal (O)	218 ± 35.5	244 ± 33.0	135 ± 7.77
Low (L)	345 ± 22.0	328 ± 14.4	94.2 ± 6.16
t-test	***	***	***
Biostimulant (B)			
Control	262 ± 15.4 b	275 ± 14.1 b	119 ± 6.19
Malvaceae-derived PH (CPH)	249 ± 63.6 b	225 ± 43.3 c	107 ± 16.6
Legume-derived PH (LPH)	356 ± 22.51 a	358 ± 17.5 a	118 ± 12.3
	*	***	Ns
N × B			
O × Control	240 ± 12.1 b	251 ± 3.7 b	123 ± 13.1 ab
O × CPH	111 ± 10.5 c	130 ± 15.6 c	140 ± 17.2 a
O × LPH	345 ± 19.4 ab	352 ± 22.0 a	142 ± 12.4 a
L × Control	284 ± 23.4 ab	300 ± 19.8 ab	115 ± 0.77 ab
L × CPH	388 ± 27.9 a	321 ± 3.58 ab	73.8 ± 1.75 b
L × LPH	363 ± 38.7 a	364 ± 31.7 a	94.3 ± 6.01 ab
	**	**	*

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

4.3.6. Phenolic acids and flavonoids

In **table 6** all the detected phenolic acids did not show a significant variation due to the interaction N × B. When averaged across the biostimulants applications, all the phenolic acids had higher concentrations under low N conditions, except for coumaroyl-diglucoside and ferulic acid, which were non-variant among both N conditions. In general, chlorogenic acid was the most abundant phenolic acid, accounting for around 92.18% of total phenolic acids, followed by ferulic acid, synapoyl-hexose, coumaroyl-diglucoside and finally, disinapoylgentobiose. When averaged across the N conditions, the application of LPH significantly increased the concentration of chlorogenic acid (11.23%) and total phenolic acids (12.03%), while for disinapoylgentobiose and ferulic acid, both PHs provoked a significant increase of about 15.65% and 27.00% on average, respectively, compared to the control.

As for flavonoids **(Table 7)**, only isorhamnetin-3-rutinoside and kaempferol 3-hydroxyferuloyl-sophorotrioside-7-glucoside demonstrated a significant interaction among the factors tested (N × B). The first one significantly increased its concentration in optimal N × CPH, while no significant differences were noted in the low N condition. The second flavonoid exhibited a significant increase in both N conditions and under both PHs. As for the rest of the flavonoids, they were only dominated by the mean effect of N conditions, with all the flavonoids and total flavonoids being more concentrated

under low N conditions, around 5.68, 2.80, 5.08, 5.87 and 4.81% compared to optimal N condition for kaempferol 3,7-diglucoside, kaempferol 3-glucoside, quercetin-3-glucoside, rutin and total flavonoids, respectively. Noting that quercetin-3-glucoside was the most abundant flavonoid accounting for 90.70% of total flavonoids under low N conditions, followed by rutin, kaempferol 3,7-diglucoside, kaempferol 3-glucoside, isorhamnetin-3-rutinoside and kaempferol 3-hydroxyferuloyl-sophorotrioside-7-glucoside.

Table 6. Phenolic acids of Lactuca sativa L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	Chlorogenic acid (µg g-1 dw)	Coumaroyl-diglucoside (µg g-1 dw)	Disinapoylgentobiose (µg g-1 dw)	Ferulic acid (µg g-1 dw)	Synapoyl-hexose (µg g-1 dw)	Total Phenolic Acids (µg g-1 dw)
Nitrogen (N)						
Optimal (O)	1741.06 ± 55.69	2.69 ± 0.25	0.574 ± 0.028	148.54 ± 7.96	6.96 ± 0.23	1899.82 ± 57.12
Low (L)	1922.48 ± 51.71	1.96 ± 0.15	0.649 ± 0.028	135.71 ± 8.44	12.93 ± 0.97	2073.73 ± 53.58
t-test	*	ns	***	ns	***	*
Biostimulant (B)						
Control	1774.92 ± 75.52 b	2.42 ± 0.34	0.507 ± 0.017 b	120.46 ± 5.84 b	10.29 ± 1.29	1908.59 ± 77.72 b
Malvaceae-derived PH (CPH)	1746.13 ± 74.13 ab	2.18 ± 0.26	0.642 ± 0.018 a	155.06 ± 10.73 a	9.59 ± 1.36	1913.6 ± 70.12 b
Legume-derived PH (LPH)	1974.26 ± 37.73 a	2.38 ± 0.3	0.685 ± 0.021 a	150.86 ± 7.47 a	9.95 ± 2.05	2138.13 ± 37.73 a
	*	ns	***	*	ns	*
N × B						
O × Control	1644.66 ± 69.22	2.72 ± 0.67	0.47 ± 0.012	119.79 ± 3.29	7.65 ± 0.33	1775.29 ± 68.52
O × CPH	1674.44 ± 101.47	2.5 ± 0.43	0.604 ± 0.014	171.11 ± 7.82	6.76 ± 0.39	1855.41 ± 98.02
O × LPH	1904.08 ± 44.39	2.86 ± 0.24	0.648 ± 0.022	154.72 ± 1.26	6.45 ± 0.08	2068.76 ± 42.92
L× Control	1905.18 ± 82.22	2.12 ± 0.19	0.543 ± 0.004	121.13 ± 12.61	12.92 ± 1.11	2041.88 ± 87.99
L × CPH	1817.82 ± 109.73	1.86 ± 0.21	0.681 ± 0.006	139.01 ± 16.04	12.42 ± 1.06	1971.79 ± 107.67
L × LPH	2044.45 ± 14.83	1.89 ± 0.4	0.722 ± 0.021	147 ± 16.2	13.45 ± 2.95	2207.51 ± 21.48
	ns	ns	ns	ns	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, *** non-significant or significant at $p \leq 0.05$ and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). dw: dry weight

Table 7. Flavonoids of Lactuca sativa L. grown under two levels of nitrogen and sprayed with two different protein hydrolysates

Source of variance	Isorhamnetin 3-rutinoside	Km 3,7-diglucoside	Km 3-glucoside	Km 3-hydroxyferuloyl-sophorotrioside-7-glucoside	Quercetin-3-glucoside	Rutin	Total Flavonoids
	(μg kg^{-1} dw)	(μg kg^{-1} dw)	(μg kg^{-1} dw)	(μg kg^{-1} dw)	(μg kg^{-1} dw)	(μg kg^{-1} dw)	(μg kg^{-1} dw)
Nitrogen (N)							
Optimal (O)	0.65 ± 0.03	0.38 ± 0.04	0.7 ± 0.06	0.19 ± 0.03	13.94 ± 0.93	0.38 ± 0.04	16.25 ± 0.99
Low (L)	0.66 ± 0.02	2.16 ± 0.36	1.96 ± 0.13	0.26 ± 0.03	70.82 ± 3.98	2.23 ± 0.39	78.09 ± 4.32
t-test	ns	***	***	***	***	***	***
Biostimulant (B)							
Control	0.58 ± 0.01 b	1.64 ± 0.74	1.4 ± 0.3	0.12 ± 0.01 c	48.38 ± 15.21	1.73 ± 0.79	53.86 ± 16.69
Malvaceae-derived PH (CPH)	0.72 ± 0.02 a	1.09 ± 0.29	1.33 ± 0.28	0.22 ± 0.01 b	38.83 ± 11.02	1.09 ± 0.29	43.28 ± 11.83
Legume-derived PH (LPH)	0.67 ± 0.02 a	1.08 ± 0.34	1.26 ± 0.35	0.34 ± 0.02 a	39.94 ± 12.72	1.08 ± 0.34	44.37 ± 13.74
	***	ns	ns	***	ns	ns	ns
N × B							
O × Control	0.56 ± 0.01 c	0.29 ± 0.03	0.8 ± 0.16	0.1 ± 0.01 d	14.87 ± 2.75	0.29 ± 0.03	16.91 ± 2.97
O × CPH	0.76 ± 0.01 a	0.47 ± 0.12	0.73 ± 0.09	0.19 ± 0 c	14.37 ± 0.75	0.46 ± 0.12	16.97 ± 0.91
O × LPH	0.65 ± 0.04 bc	0.38 ± 0.03	0.57 ± 0.05	0.29 ± 0.02 b	12.59 ± 0.85	0.38 ± 0.03	14.86 ± 0.75
L × Control	0.61 ± 0.02 bc	2.99 ± 0.98	2.01 ± 0.21	0.14 ± 0.01 cd	81.88 ± 5.12	3.17 ± 1.03	90.8 ± 4.36
L × CPH	0.68 ± 0.01 ab	1.72 ± 0.03	1.93 ± 0.17	0.24 ± 0.01 b	63.28 ± 2.87	1.72 ± 0.03	69.58 ± 2.7
L × LPH	0.7 ± 0.02 ab	1.78 ± 0.28	1.95 ± 0.35	0.38 ± 0 a	67.3 ± 7.73	1.78 ± 0.28	73.89 ± 8.5
	**	ns	ns	*	ns	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). km: Kaempferol, dw: dry weight

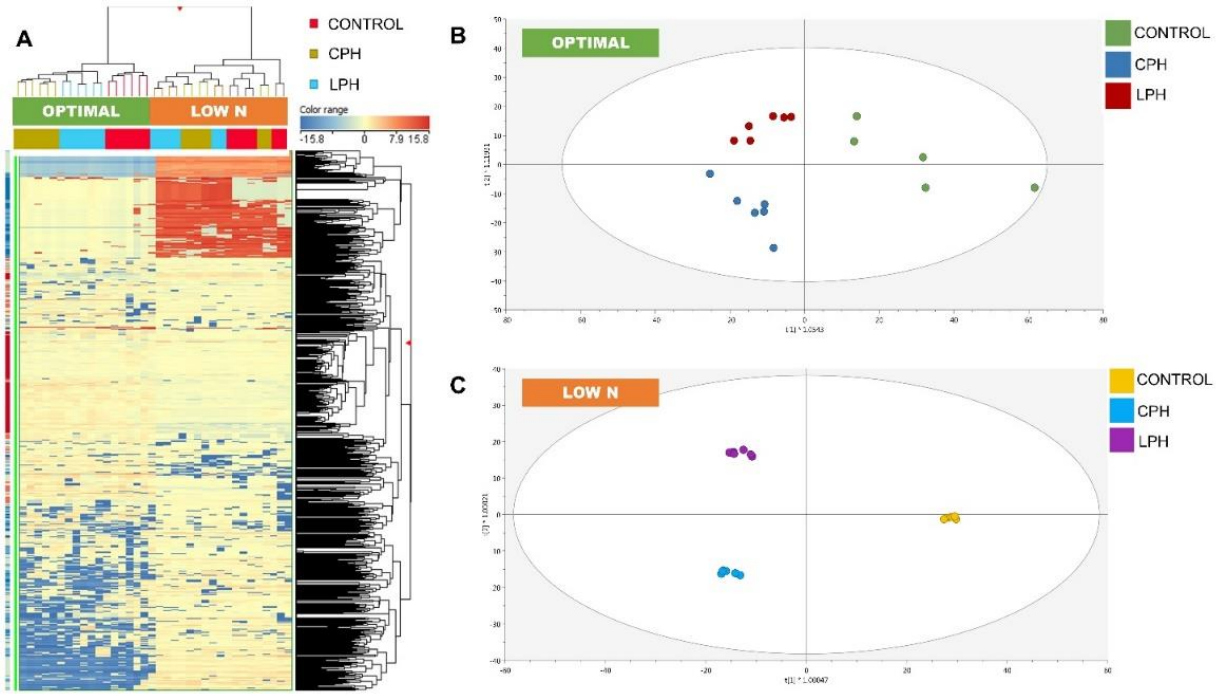
4.3.7. Untargeted metabolomic profile

To better understand the effect of protein hydrolysates under either optimal or depleted nitrogen supply on lettuce, a UHPLC-QTOF-MS analysis on lettuce leaf extracts was performed. The untargeted approach allowed us to annotate a total of 2626 compounds. Firstly, an unsupervised FC-based HCA was performed to evaluate the effect of both factors (N supply and biostimulants) on the metabolic profile of lettuce plants (Figure 1A). The obtained results highlight that N supply is the primary clustering factor. Biostimulants play a specific role under optimal N conditions, being both PHs clustered apart from the control. In contrast, under low N supply, such discrimination was not achieved since CPH played an intermediate effect between the control and LPH (Figure 1A).

Due to the hierarchically prevalent role of N supply on the metabolome of lettuce plants, two separate supervised OPLS-DA models were performed for each condition (Figure 1B and 1C). In both cases, the OPLS-DA models reported that PHs administration involved distinctively profiles with respect to control, according to the primary latent vector, whereas the specific effect of each biostimulant was reported according to the secondary latent vector, either under optimal or low N conditions (Figures 1B and 2C, respectively). In all cases, models presented adequate scores regarding the goodness of fit (R^2Y) and prediction ability (Q^2Y), being $R^2Y = 0.997$ and $Q^2Y = 0.776$ for the low N condition and $R^2Y = 0.880$ and $Q^2Y = 0.428$ for the optimal condition. Based on OPLS-DA discriminating models, a Variable Important in the Projection (VIP) approach was performed to identify the compounds with the highest discriminant power (VIP score threshold = 1.2, $p < 0.05$).

For optimal Nitrogen supply, 107 compounds were considered for interpretations on the Pathway Tool Omics dashboard as significantly modulated by the PH application. Overall, PHs promoted a generalized down-regulation of the biosynthetic metabolism, which was more evident in the secondary metabolism, followed by hormone biosynthesis and other classes with a lower modulation (Figure 2A). Concerning the secondary metabolism (Figure 2B), three major biosynthetic pathways were strongly modulated: terpenoids, phenylpropanoids, and N-containing compounds. In the case of terpenoids, PH-mediated modulation was more evident in the case of terpenes that showed a harsh down-accumulation, mainly in the case of CPH. Among the relevant terpenoids, they were generally represented by phytoalexins. Gypsogenate 28-β-D-glucoside exhibited the strongest down- accumulation by both PHs (logFC = -4.0 in both cases, Table S2). In contrast, the biosynthesis of other terpenoid phytoalexins was found to be differentially regulated depending on the PH, as was the case of hemigossypol 6-methyl ether, whose biosynthesis was down-regulated by LPH (logFC = -4.0), whereas

Figure 1. Chemometric results on the untargeted metabolomic profile of lettuce plants. A. Hierarchical cluster analysis (HCA) based on fold change



heatmap (Euclidean distance, Ward's linkage rule). B. Orthogonal Projection to Latent Structures (OPLS) model for discriminating metabolomic profiling of lettuce plants treated with PHs under optimal N condition. C. Orthogonal Projection to Latent Structures (OPLS) model for discriminating metabolomic profiling of lettuce plants treated with PHs under low N conditions

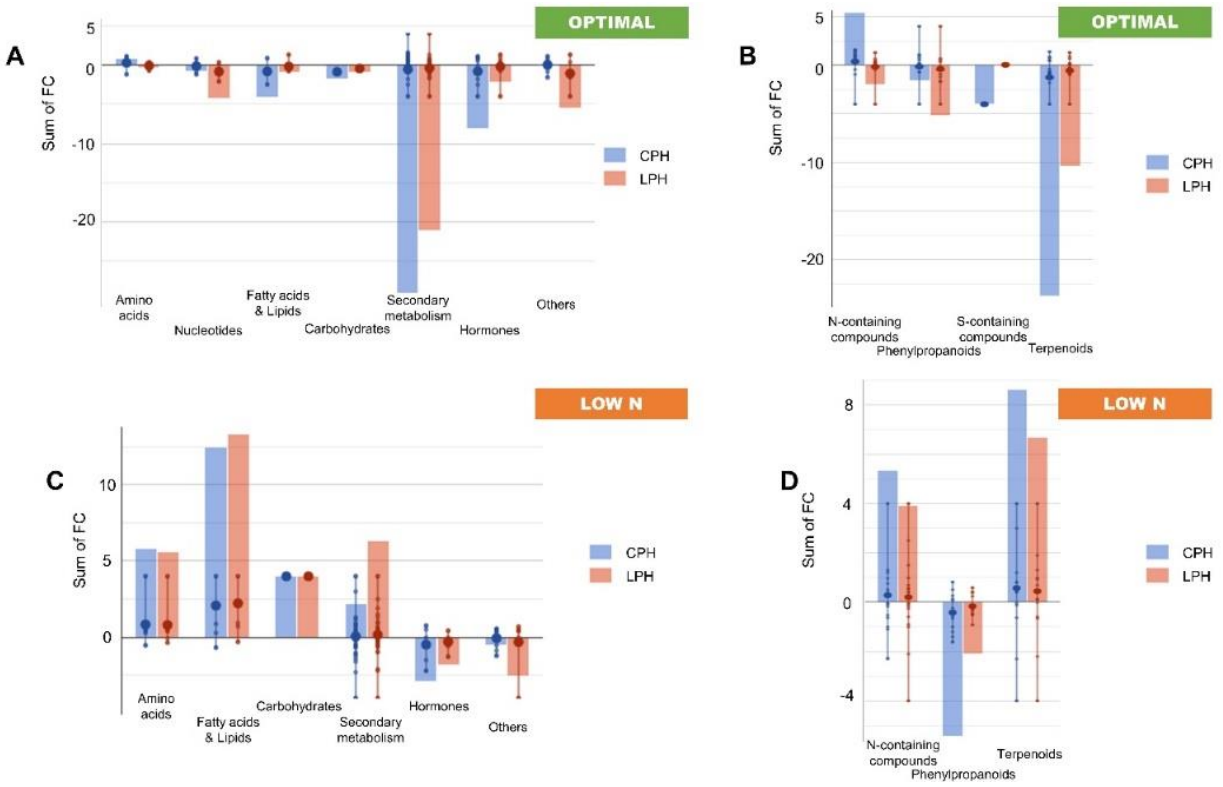


Figure 2. General biosynthesis and secondary metabolite biosynthesis of lettuce plants under optimal N conditions (A and B, respectively) and low N conditions (C and D, respectively) treated with PHs. The dataset containing the compounds meeting the inclusion criteria (VIP score > 1.2 and $p < 0.05$) was uploaded into the PlantCyc pathway tool. Sum of FC: sum of fold-change values of all metabolites included in each biosynthetic category.

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that of 3-hydroxylubimin was mostly down-regulated by CPH (logFC = -4.0). As shown for terpenoids, phenylpropanoids biosynthesis was also down-accumulated, but to a lesser extent, being LPH the biostimulant showing the strongest contribution (Figure 2B). Essentially, flavonoids and phenolic acids were reported within this biosynthetic pathway. Interestingly, the biosynthesis of the isoflavonoid (-)-medicarpin-3-O-glucoside was highly induced by both PHs (logFC = 4.0), whereas those of other isoflavonoids like isoformononetin and afrormosin-7-O-glucoside-6″-O-malate was slightly repressed (logFC < 1.0 in all cases). Concerning phenolic acids, their biosynthesis showed a PH-dependent regulation modulation since CPH drove a harsh down-regulation of ferulate (a hydroxycinnamic acid derivative, logFC = -4.0), and LPH caused the same effect on 1-O-sinapoyl-β-D-glucose (logFC = -4.0). Finally, the biosynthesis of N-containing compounds was generally up-regulated in the presence of CPH. In contrast, it was repressed when applying LPH as biostimulants, mainly represented by alkaloids and, secondarily, glucosinolates (Figure 2B). In particular, CPH promoted a slight accumulation of alkaloids like cinchonidine, 13-[(E)-2-methylcrotonyl]oxylupanine and 12-hydroxydihydrochelirubine (logFC = 0.86 – 1.54), whereas LPH promoted a strong down-accumulation of dehydrochelirubine (logFC = 4.0). Interestingly, CPH caused a strong down-regulation of 7-(methylsulfanyl)heptyl-desulfoglucosinolate biosynthesis and it was mildly induced by LPH (logFC = -4.0 and 0.19, respectively).

Besides secondary metabolism, PHs application under optimal N conditions also negatively modulated hormones biosynthesis, especially those belonging to the families of gibberellins and cytokinins and, secondarily, stress-related hormones like jasmonates and the phytosterol campestanol (Figure 2A). In detail, the biosynthesis of the gibberellins A_{24} and A_{38} was found to be harshly repressed by CPH (logFC = -4.0), where it was slightly induced by LPH (logFC = 0.19 and 0.92, respectively). In contrast, the cytokinin *trans*-zeatin was up-accumulated by both PHs, whereas its derivative dihydrozeatin was down-accumulated. Moreover, the biosynthesis of stress-related hormones *trans*-tuberonic acid (a jasmonate analogue) and campestanol were found down- accumulated in all cases. Finally, among other compounds (Figure 2A), it is worth to note that the chlorophyll biosynthetic precursors 71-hydroxychlorophyllide a and 3,8-divinyl protochlorophyllide a were decreased in response to both biostimulants application (Figure 2A; Table S2).

In contrast, for N-depleted lettuce plants, the results from the untargeted foliar profile included a total of 86 compounds (VIP score >1.2, $p < 0.05$; Table S3). The results from the PlantCyc pathway tool indicated a completely different effect of biostimulants under low N supply (Figure 2C), as both promoted a clear induction of primary metabolism, involving mainly fatty acids and amino acids, while promoting a mild down-accumulation of hormones biosynthesis. In contrast, a differential effect was

found for secondary metabolism, as it was generally up- accumulated by LPH administration while slightly down-regulated by CPH (Figure 2C). Considering fatty acids biosynthesis, polyunsaturated fatty acids (PUFAs) were significantly induced by both PHs, as shown by the accumulation of linoleoyl-CoA and (5Z, 8Z, 11Z, 14Z, 17Z)-icosapentaenoyl-CoA (logFC = 4.0 in both cases), followed by that of the monounsaturated oleate (logFC = 0.87 for CPH and 0.93 for LPH), whereas the saturated lauroyl-CoA was found down-regulated (logFC = -0.68 for CPH and -0.31 for LPH; Table S3). The same coordinated behavior of PHs was found in amino acids biosynthesis, as they played a similar effect on the induction of N-acetylglutamyl phosphate, Phe, and Tyr biosynthesis (logFC = 0.27 – 0.52) and a slight repression of L-cystathionine (logFC = -0.53 and -0.36 for CPH and LPH, respectively). In the case of hormones, both PHs promoted a depletion in the biosynthesis of the jasmonate precursors 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-butanoyl-CoA (logFC = -0.48 and -0.29 for CPH and LPH, respectively) and 3-oxo-2-(cis-2'-pentenyl)-cyclopentane-1-hexanoyl-CoA (logFC = -0.48 and -0.29 for CPH and LPH, respectively). In the same way, dihydrozeatin-7-N-glucoside was found down-accumulated in response to both biostimulants (logFC = -0.03 for CPH and -0.58 for LPH).

Conversely to the results for primary metabolism, biostimulants played a differential effect on the secondary metabolism of N-depleted lettuce plants (Figure 2D). Among the families of secondary metabolism, terpenoids biosynthesis was strongly induced by CPH and LPH, motivated by the up-accumulation of the carotenoid bixin (logFC = 3.03 and 1.88, respectively;), the indole-terpenoid alkaloid vellosimin (logFC = 4.0 in both cases) and kauralexin A2 (logFC = 0.79 for CPH and 1.26 for LPH). In contrast, CPH promoted a harsh down-accumulation of the triterpenoid phytoalexin gypsogenate 28-β-D-glucoside (logFC = -4.0). To a lesser extent, both biostimulants elicited the accumulation of N-containing compounds biosynthesis, involving glucosinolate and alkaloid accumulation. The PH-mediated accumulation of homomethionine derivatives, such as N,N-dihydroxy-tetrahomomethionine and N-hydroxy-tetrahomomethionine (logFC = 0.96 – 2.48 for both PHs), together with that of (R)-4-hydroxymandelonitrile (logFC = 0.41 for CPH and 0.56 for LPH) and the down-accumulation of 4-benzoyloxybutylglucosinolate (logFC = -1.14 for CPH and -1.05 for LPH), suggests an intense mobilization of glucosinolates upon PH administration under low N supply. In contrast, phenylpropanoids were decreased in response to PHs application, in particular following CPH application (Figure 2D). Thus, several families of polyphenols were affected, including chalcones like licodione and phlorizin, lignans such as 6-malonylpodophyllotoxin, diphyllin, and (+)-sesaminol 2-O-β-D-gentiotrioside, as well as flavonoids, such as quercetin glycosides, (-)-maackiain, afrormosin-7-O-glucoside, and malonyldaidzin (all of them presenting logFC < 0).

4.4 Discussion

The increase in shoot fresh weight upon the application of PHs of vegetal origin is in line with several previous research in the literature [13,14,32,33]. Cristofano and collaborators [14] reported similar results, where in shallow nitrogen conditions (1 mM NO_3^-), a legume-derived PH increased lettuce fresh weight but not enough to be significant, whereas in the optimal condition of nitrogen (8 mM NO_3^-), the increase was significant. Such a result is predictable in soilless cultivation, where the only source of nutrition is administrated via fertigation, and biostimulant application cannot compensate for the severe depletion of nitrogen. On the other hand, Ottaiano and coworkers [13] registered an increase of 21.14% in lettuce marketable yield after the application of a PH deriving from Fabaceae tissues enzymatic hydrolysis under sub-optimal conditions, in addition to the lettuce head diameter increase that is in line with the increment of the growth index that was registered in our research. As for the discrepancy regarding leaf number $plant^{-1}$ and leaf dry matter percentage between the latter research and ours, it can be explained by the difference in the cultivation technique (soil vs. quartz sand) and the duration of the growing cycle.

In line with our results, a decrease in A_{CO2} **(Table 2)** was also noted by Miras-Moreno and collaborators [34], when soilless lettuce was grown under reduced strength nutrient solution. The decrease in net photosynthesis was represented by a reduction in shoot fresh and dry weight observed in lettuce. Similarly, applying a legume-derived PH on greenhouse-grown lettuce at a similar dose did not significantly increase the SPAD index [1]. These authors revealed partially similar results regarding the physiological parameters tested (A_{CO2}, E and WUEi), where A_{CO2} increase was not significant, in addition to E and WUEi upon the PHs applications. Giordano and collaborators [33] equally demonstrated a non-significant increase in E and WUEi when a 'Salanova' lettuce was sprayed with PH, whereas for A_{CO2} and SPAD index, the increase was deemed significant. Moreover, Cristofano and collaborators [35] grew the same 'Canasta' cultivar in a raft floating system and applied a legume-derived PH in different modalities. They found similar non-significant results regarding SPAD index and E when applied via foliar application. However, the same authors revealed significant increases in all the physiological parameters when an additional application of the PH was added in the NS other than the foliar spray. As noted by Rouphael and collaborators [36], plant biostimulants trigger in optimal and sub-optimal conditions an array of mechanisms (molecular and physiological) like photosynthesis enhancement, nutrient uptake and translocation, in addition to phytochemicals production, as reported by the untargeted metabolomic approach developed. Nardi and collaborators [37] stated that an alfalfa-derived PH ameliorated shoot production and nitrogen assimilation of hydroponically grown plants. This latter biostimulant raised the activity of three enzymes involved in the tricarboxylic acid cycle and five enzymes concerned with N

reduction and assimilation. Indeed, the latter is among the mechanisms proposed regarding the action of PHs, along with the hormone-like activities and improved nutrient uptake [7]. The last authors presumed that PHs contain bioactive compounds able to reach specific receptors of the target cells, such as 'root hair promoting peptide' made of 12 amino acids. Equally, other amino acids like tryptophan, glutamic and aspartic acids and soluble peptides can trigger a signal transduction pathway by eliciting endogenous phytohormone synthesis[3].

The increase of MDA and H_2O_2 (**Table 4**) under nitrogen depletion is in line with the results of Francesca and collaborators [38] in tomato grown under abiotic stress. The same authors also demonstrated a reduction in these two biochemicals under biostimulant application, which is partially in harmony with our results. MDA is usually regarded as an indicator of membrane lipid peroxidation[38]. These levels were relatively decreased in our research under the application of PH, but not significantly. As explained by Vasconcelos and collaborators[39], a robust antioxidant system made of non-enzymatic and enzymatic antioxidants like superoxide dismutase (SOD), CAT and APX protect plant cells from the oxidative burst driven by stress conditions, where APX breaks down H_2O_2 efficiently by using ascorbate as substrate. This explains the increase of APX and CAT activities in low nitrogen levels as opposed to a H2O2 rise. The increase in CAT observed in our research is expected, since catalase constitutes an intracellular enzyme participating in toxic H_2O_2 decomposition [4].

The results for mineral composition obtained, where total N, P, S, Mg concentrations **(Table 3)** did not manifest any significant changes, are in harmony with those by Carillo and coworkers [1], who applied a PH at a similar dose on lettuce. Likewise, the results presented by Cristofano and collaborators [14] assessing a legume-derived PH in optimal and low N levels. In contrast, K and Ca concentrations increased when lettuce plants were sprayed with a PH [1], which is the case in our research when the plants were sprayed with CPH. Usually, when plants are treated with biostimulants, the density and length of root hair increase and assist the uptake of nutrients through an increment of absorptive surface area, other than modulating the gene expression and activity of enzymes related to plant metabolism [3,37].

Lutein and β-carotene increase under nitrogen reduction **(Table 5)** are partially in line with the results obtained by Cristofano and collaborators[14], who reported a significant rise in these carotenoids when similar levels of N were applied. The same authors denoted a decent increase of these carotenoids under the application of LPH, which was the case in our low level of N. However, under optimal N level, CPH a decreased the amounts of these compounds. PHs have been demonstrated to not solely ameliorate plant nutrition status but as well the qualitative aspects of vegetables regarding phytochemicals such as carotenoids, flavonoids, and other polyphenols [5].

These plant secondary metabolites are equally stimulated under N deprivation conditions [36], as reported by the UHPLC/QTOF-MS profiling reported in the present study (Figure 2).

Soil nitrogen affects anthocyanin and flavonoid content, and generally, a higher polyphenolic content is observed when less nitrogen fertilizer is added to the soil [40]. Equally, Miras-Moreno and collaborators [34] found an increase in secondary metabolites when lettuce was soilless-grown in reduced strength nutrient solution. Analogous increases in the concentrations of chlorogenic acid in lettuce were noted by both Qadir, Becker and their collaborators [41,42] in lettuce grown under downsized N levels. Furthermore, El-Nakhel and collaborators [43] mentioned an increase in total phenolic acids and anthocyanins in lettuce when grown hydroponically in a reduced-strength nutrient solution. Such results entail that the deficiency of N is a major proxy of phenolic acid accumulation [44], especially since abiotic stress such as nitrogen depletion activate the phenylalanine ammonia-lyase gene that is concerned with phenolics biosynthesis [42,43]. Notably, phenolic compounds are often used as a quality parameter influenced by nutrient availability [34] or simply as an interaction between the environment, nutrients availability, and the genetic background [16]. Total phenolic acids increase upon the application of PHs is in line with the results by Giordano and collaborators [33], as for total flavonoids, the response to PH was cultivar dependent. In our research, total flavonoids were not significantly modified by the biostimulant application, which was the case mentioned in a similar work with lettuce grown under different levels of N and subjected to PH application [14]. In our research, only LPH had a significant effect on total phenolic acids. It is well known that the differential composition of plant-based biostimulants drives a diverse outcome at the metabolomic level [7,14]. In this sense, PHs triggered the accumulation of antioxidant compounds, and induce the enzymatic systems involved in the oxidative stress defense due to an enhancement of intercellular transduction, especially implicated in reactive oxygen species (ROS) signaling network with an increase of phenolics and carotenoids or the production of stress-averting antioxidant molecules such as ascorbic acid, tocopherols, and antioxidant enzyme activities [6].

The application of untargeted metabolomics shed light on the mode of action of the selected biostimulants in lettuce, indicating that both CPH and LPH promoted a contrasting effect at a metabolic level, primarily depending on N supply. In general, the biochemical reprogramming was more evident in secondary metabolism (Figures 2A and 2B). The decrease of triterpenoid phytoalexins and the PH-mediated decrease of glucosinolates and stress-related hormones like jasmonates and phytosterols, suggest that the PH promoted a metabolic modulation from secondary to primary metabolism. In contrast, both terpenoid phytoalexins and glucosinolate-derived precursors and activation products were boosted in N-depleted lettuce plants (Figures 2C and 2D),

suggesting their implication in plant stress mitigation. The shift in metabolomics signatures is following previously existing literature, where vegetal-derived PHs induced the biosynthesis of glucosinolates and terpenoids in lettuce plants under different stress conditions, such as salinity [17] and drought [45]. This growth-promoting response reported under optimal conditions is reinforced by the PH-driven induction of the biosynthesis of growth-related hormones like the cytokinin *trans*-zeatin and the down-accumulation of chlorophyll catabolites (Figure 2A), such as chlorophyllide derivatives. These results align with those reported in faba bean, where vegetal extracts were used as biostimulants promoting the biosynthesis of zeatin and increasing chlorophyll levels accumulation [46]. Considering the metabolite-based antioxidant response to stress, the metabolic profiling showed that phenylpropanoids were mainly down-regulated in stressed plants, while PUFA accumulated thanks to the application of both biostimulants (Figure 2C). This PUFA-based antioxidant response is in line with the results reported in biostimulant-treated tomato plants under water stress [47], and is also in accordance with the results reported for MDA determination. Moreover, the accumulation of amino acids in PH-treated stressed plants, like Glu, Tyr and Phe (Figure 2C), is suggested to develop an efficient induction of antioxidant enzyme systems, as discussed earlier [33].

4.5. Chapter 4 Conclusions

The results illustrated in this research suggest that Malvaceae-derived protein hydrolysates had a similar effect to the legume-derived in terms of boosting growth index, shoot fresh weight and net photosynthetic rate under optimal nitrogen. In addition, it increased the concentration of Ca and Mg but was unable to increase lutein, β-carotene and phenolic acids like the legume-derive protein hydrolysate, nevertheless it equally reduced H_2O_2 levels and increased CAT levels to a lesser extent in the same condition. Both protein hydrolysates could not cope significantly with the severe depletion of nitrogen, confirming the complement action of biostimulants to nitrogen fertilization strategies rather than representing a complete substitute. The performance of untargeted metabolomics shed light on the mechanism of action of both PHs, reflecting a strong dependence on N supply based on the management of metabolic resources. Under optimal conditions, both biostimulants caused a decrease in stress-related metabolites, like jasmonate derivatives and glucosinolates, whereas a coordinated response involving both primary and secondary metabolites was attributed to PHs to mitigate the effects ascribed to N depletion. Noteworthy, distinct metabolomic responses could be observed under either optimal or limiting N conditions, thus strengthening the concept that tailored biostimulant strategies should be defined within a broader set of agronomic practices.

4.6 References for Chapter 4

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Chapter 5

Tracking the Biostimulatory Effect of Fractions from a Commercial Plant Protein Hydrolysate in Greenhouse-Grown Lettuce

Abstract: Protein hydrolysate biostimulants are environmentally friendly options for the reduction of nitrogen input, but still their plant growth-promoting mechanisms are not completely unveiled. Here, to put the "signaling peptide theory" to the test, a greenhouse experiment was undertaken using low (1 mM) and optimal (8 mM)-NO3 treated butterhead lettuce and three molecular fractions [PH1 (>10 kDa), PH2 (1–10 kDa) and PH3 (<10 kDa) fractions] in addition to the whole product Vegamin®: PH, in a randomized block design. PH1 and PH3 significantly increased fresh yield (+8%) under optimal (lighter leaves) but not under low (darker leaves) NO_3 condition. Total ascorbic acid, lutein and β-Carotene increased with PH3, and disinapoylgentobiose and kaempferol-3-hydroxyferuloyl-sophorosie-7-glucoside content increased with PH (whole/fractions) treatments, particularly under low NO_3 condition. The complete hydrolysate and analyzed peptide fractions have differential bio stimulatory effects, enhancing the growth and nutritional quality of lettuce.

5.1. Introduction

Nitrogen is a critical nutrient for plants, as plants rely on it for an insurmountable number of tasks, from nucleic acid building to enzymes and proteins. Furthermore, nitrogen is a critical element for photosynthesis, to which it provides the building blocks for chlorophyll and light-harvesting complexes [1]. Due to its importance at every level of plant physiology, farmers supply excessive amounts of this element to plants in the hope of obtaining better plant growth; however, this can increase nitrate concentrations in edible plant matter, the main dietary source for human consumption [2]. Moreover, by leaching into the soil, nitrate can also reach the water table, polluting drinking water sources [3].

One of the most cutting-edge solutions currently available for the reduction of nitrogen inputs is the use of plant biostimulants. As 2019's EU regulation 1009 points out, plant biostimulants (PBs) "act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates" [4]. Furthermore, PBs also pose themselves as a straightforward solution to increase plant functional quality parameters, which stem from the increase in secondary plant metabolites of known health-improving qualities, such as antioxidants and polyphenols [5]. Protein hydrolysate (PH) biostimulants are now a staple in the biostimulant scenario, and literature shows that the use of such products can alleviate some of the yield losses due to deficient nitrogen supply and/or improve the nitrogen uptake efficiency in many

greenhouse vegetable species such as lettuce, spinach and rocket [6–8]. Whilst a partial explanation of their effect may stem from the presence of amino acids, which are the building blocks for most plant tissues, research has postulated the role of the so-called signaling peptides to be essential in their effectiveness. Signaling peptides are short chains of amino acids (between 2 and 50) which induce hormone-simile responses at very low concentrations [9], and most PH literature point to the root hair-promoting peptide, a compound which has been found in widely available commercial formulations such as 'Trainer' as proof of this theory [10].

However, as plant source materials vary in their aminoacidic content and protein makeup, it is to be expected that biostimulants made from different protein sources may vary in the content of such peptides, and thus, effectiveness; research found this to be the case, as vegetal products from various botanical families exert distinct effects on either the growth or metabolism of plants, even when the same extraction process is performed [11]. One of the latest strategies to garner the best understanding of the inner workings of this biostimulant grouping has been molecular fractionation. Lucini and collaborators [9] found that the < 1 kDa fractionation—around nine amino acid residues—of the PH 'Trainer' elicited the best root growth performance through IBA-like effects seen in the metabolomic data. This proof-of-concept work shows that the next generation of biostimulants can be assayed based on the potency of their single fractions and marketed accordingly.

On these bases, the aim of this work is to verify the influence of a newly developed PH biostimulant based on vegetal sources on the growth and plant phytochemical profile of lettuce plants in both optimal and low nitrogen conditions. To further prove the effectiveness of the low-molecular-weight peptides, the biostimulant was subjected to molecular fractionation in order to obtain the < 1 kDa, 1–10 kDa and >10 kDa formulations. The experiment is meant to scale up previous lab work to greenhouse conditions, and to prove new biostimulant-making technologies for the industry, since the pressure on producers to find new and innovative products is stronger than ever, as nitrogen fertilizer is becoming both economically and environmentally unsustainable.

5.2. Materials and Methods

5.2.1. Growth Conditions, Experimental Design and Plant Material

A greenhouse experiment was carried out from October 2, 2020 (day after transplant 1, or DAT 1) to November 12, 2020 (DAT 42) in an unheated and passively ventilated greenhouse situated in the "Parco Gussone" area of the Department of Agricultural Sciences of the University of Naples "Federico II", 40° 48′ N, 14° 20′ E, 29 m.s.l. Seedlings of *Lactuca sativa* L. cv. 'Maravilla De Verano Canasta', herereby defined as 'Canasta' (Pagano Domenico e Figli, Scafati, Salerno, Italy), a butterhead-type lettuce were transplanted at the three-true leaves stage on October 2, 2020. Plants were

transplanted into 1.6-L plastic pots containing growing substrate which comprised of a mixture of 90:10 (*v*/*v*) 3 mm quartz sand (Vaga, Sabbie e Ghiaie Silicee, Località Sostegno—SP199 27010 Costa de'Nobili (PV) Italy) and perlite, respectively. The experimental setup consisted of four double rows with an inter and intra-row distance of 35 and 20 cm which represented a planting density of 14 plants m^{-2}. A split-plot experimental design was employed, whereby the main factor consisted of the nutrient solution (NS) nitrogen dosage which was deemed either optimal (O) or low (L). The sub-factor consisted of four biostimulant (B) treatments and an untreated control which were arranged in a randomized complete block design with three replicates. In total, the design employed 30 experimental units, each consisting of five lettuce plants.

The base nutrient solution had the following composition: 1.5 mM phosphorus, 4 mM potassium, 2.5 mM sulfur, 1.25 mM magnesium, 1 mM sodium, 1 mM chloride, 20 μM iron, 9 μM manganese, 0.3 μM cupper, 1.6 μM zinc, 20 μM boron and 0.3 μM molybdenum. To this solution, two differential amounts of nitrogen (calcium nitrate) were added in order to provide for two nitrogen treatments: O, corresponding to 8 mM nitrate and 4mM calcium, and L, corresponding to 1 mM nitrate and 0.5 mM calcium. To ensure equal calcium concentration and guarantee iso-osmosis across NS treatments, the low nitrogen treatment was supplied with calcium chloride. The electrical conductivity (EC) of the resulting solutions was 1.6 ± 0.5. The pH of the solutions was monitored and kept at 5.8 ± 0.2 with a portable pH meter (HI 991301, Hanna Instruments Italia S.R.L., Ronchi di Villafranca Padovana (PD), Italy).

5.2.2. Biostimulant Characteristics

The commercially available protein hydrolysate Vegamin ® (Hello Nature Italia S.R.L., Rivoli Veronese (VR), Italy), hereby referred to as PH, made from vegetal sources, was the biostimulant chosen for this trial. Quantitative analysis of this biostimulant, obtained analogously to Sorrentino and collaborators [12], shows carbon and nitrogen contents of 25.6 and 17.1%, respectively. The aminogram of the product, expressed in g kg^{-1} was determined as: Ala (12), Arg (19), Asp (33), Cys (4), Glu (54), Gly (13), His (8), Ile (12), Leu (24), Lys (19), Met (4), Phe (16), Pro (15), Ser (17), Thr (11), Trp (4), Tyr (13), and Val (16).

The ferric-reducing antioxidant power (FRAP) and the total phenolic and flavonoid contents, measured analogously to Paul and collaborators [13], were as follows: 1.32 mM Fe^{2+} g^{-1}, 8.94 mM gallic acid eq. g^{-1} and 770.3 μM quercitin eq. g^{-1}. The elemental composition was determined as (g kg^{-1} biostimulant): N (50.0), P (0.9), K (41.1), Ca (10.9), Mg (0.5), Fe (0.024), Zn (0.010), Mn (0.001), B (0.005), and Cu (0.001).

Vegamin ® does not contain phytohormones as the analysis conducted by Sorrentino and collaborators [12] shows. PH fractionation and nitrogen content analysis were carried out according to the methodology employed by Lucini and collaborators [9]. The

fractionation process consisted of two steps. First, the >10 kDa and <10 kDa fractions were obtained via the use of centrifuge filtering tubes (Amicon Ultra 15, Merck KGaA, Darmstadt, Germany). Second, after the use of 0.5-1 molecular cut-off (MWCO) cellulose acetate membranes (VWR, Milan, Italy),the <1kDa and >1kDa <10kDa fractions were obtained. To sum up, biostimulants were separated in three fractions: <1 kDa, hereby called PH3, 1–10 kDa or PH2 and >10k Da or PH1. Due to the use of water as the fractionation medium, dilution incurred and nitrogen contents of the obtained fractioned were subsequently determined as 0.11% (PH1), 0.16% (PH2) and 0.06% (PH3).

5.2.3. Biostimulant Treatments

Biostimulant treatments consisted of 4 levels of application of the Vegamin formulate and its fractions. The whole product, i.e., PH was applied at the manufacturer's suggested rate of 3 mL biostimulant L^{-1} solution or 2.38 g biostimulant L^{-1}. Due to dilution effects inherent to the fractionation process, the PH1, PH2 and PH3 treatment dosage rates were adjusted to provide plants with equal amounts of nitrogen to the unfractionated formulate; thus, dosage rates were 348.2 g L^{-1} for PH1, 251.74 g L^{-1} for PH2 and 659.0 g L^{-1} for PH3.

Treatments were administered to plants via foliar application using 10 L steel-bottle sprayers of the same model, which were tested for spraying volume accuracy using a graduated cylinder. Products were sprayed on lettuce plants until a uniform coverage was guaranteed, and polystyrene panels were used to avoid drift between different treatments. A total of five treatments were administered throughout the course of the trial, starting at DAT 13 and every seven days.

5.2.4. Yield, Growth Assessment, Leaf Colorimetric Measurement and Sampling

At the end of the experiment (DAT 42), three plants per experimental unit were randomly selected for fresh weight measurements. Dry plant matter was obtained upon desiccation of the fresh matter using a forced-air drying oven at 60 °C until a constant weight was reached.

Colorimetric measurements were carried out using a Minolta CR-300 Croma Meter (Minolta Camera Co. Ltd., Osaka, Japan) which was calibrated prior to use against a standard white control. Leaf color was sampled on the adaxial side of six fully expanded leaves per experimental unit. Measurements are expressed in the CIELAB color space, comprising of L* (lightness), a* and b* (chromatic information). Visual color appearance of the plants was also validated using the CIEDE2000 indicator developed by the CIE Technical committee [14]. To provide more succinct colorimetric information, values were also converted to chroma (Chroma = $((a^*)^2 + (b^*)^2)^{0.5}$) and hue (Hue = ((Arctan $(b^*/a^*)/2\pi) \times 360) + 180$).

The remaining two plants per experimental plot were chosen for the quality assays, and immediately transferred to a laboratory setting for further sampling. A set of fresh

leaf samples was immediately used for the determination of leaf chlorophyl and total ascorbic acid contents. A further set of leaf samples was harvested and immediately transferred into liquid nitrogen. Samples were later stored at −80 °C for quality assays, and an aliquot was lyophilized using Martin Christ Alpha 1–4 freeze-drying equipment (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

5.2.5. Carotenoids and Total Ascorbic Acid Determination

Chlorophyll Leaf pigment content was determined using 1 g of fresh leaf samples, which were extracted in pure acetone and kept in darkness for 15 min and subsequently centrifuged at 3000×*g* for five minutes. Pigment contents were determined using a Hach DR 2000 UV-Vis spectrophotometer (Hach Company, Loveland, CO, USA) by measuring their absorbance at 662 and 645 nm for chlorophyll a and b, respectively. Chlorophyll pigment quantification was made using the extinction coefficients found in Lichtenthaler and Buschmann's work [15] and determined as mg 100 g^{-1} fresh weight (fw).

Leaf β-carotene and lutein analysis were carried out on 100 mg of lyophilized leaf matter. Extraction was first performed analogously to what is described by Kyriacou and collaborators [16]. In brief, sample material was firstly extracted in 6 mL of ethanol—0.1% butylated hydroxytoluene (BHT) mixture, then potassium hydroxide was added for saponification. Pigments were then extracted using hexane and later dried using nitrogen gas; 1 mL of chloroform was added to this residue and separated using Shimadzu Model LC 10 chromatography equipment (Shimadzu, Osaka, Japan) using a reverse phase 250 × 4.6 mm, 5 μm Gemini C18 column (Phenomenex, Torrance, CA, USA) as described by Kyriacou and collaborators [17]. Carotenoid contents were quantified as mg 100 g^{-1} fw.

The total ascorbic acid (TAA) assay was performed on fresh leaf tissue by way of the Kampfenkel [18] method, which determines the sum of ascorbic and dehydroascorbic acids by measuring sample absorbance at 525 nm against an ascorbic acid standard calibration curve. All measures were undertaken using the Hach DR 2000 UV-Vis spectrophotometer (Hach Company, Loveland, CO, USA) and expressed as mg AA 100 g^{-1} fw.

5.2.6. Leaf Mineral Analysis

All mineral content analyses were conducted on dried samples which were processed using a model MF10.1 grinding mill (IKA-Werke GmbH & Co. KG, Staufen, Germany). Leaf mineral (NO3, P, K, Ca, S, Mg) and organic acid (citrate and malate) composition was determined using ICS-3000 ion chromatography equipment (Dionex, Sunnyvale, CA, USA). Anionic and cationic separations were obtained via the IonPac AS11-HC and IonPac CS12A analytical columns and quantified against chromatography standards using electrical conductivity detectors (Dionex, Sunnyvale, CA, USA) as

mentioned in detail by Rouphael and collaborators [19]. All mineral contents are quantified as mg 100 g−1 fw.

5.2.7. Leaf Polyphenolic Content

Polyphenols extraction was performed using 100 mg of freeze-dried leaf samples and 5 mL of a methanol/water (60:40 *v*/*v*) solution, according to Kyriacou and collaborators [16]. Briefly, qualitative, and quantitative profiling of the compounds was also performed analogously to the previously mentioned paper using an Ultra High Pressure Liquid Chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) which employed a 1.7 μm Biphenyl (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA). Later mass spectrometry analysis was carried out using a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). The targeted acquisition of polyphenolic compounds was carried out on parallel reaction monitoring (PRM) mode. This modality of acquisition allows a targeted MS/MS analysis using the mass inclusion list and expected retention times of the target analytes, with a 30 s time window, with the Orbitrap spectrometer operating in negative mode at 17,500 FWHM (*m*/*z* 200). The AGC target was set to 2e5, with the maximum injection time of 20 ms. The precursor ions in the inclusion list were filtered by the quadrupole at an isolation window of *m*/*z* 2 and fragmented in an HCD collision cell set at 30 Kv. Polyphenols quantification was done using calibration curves from authentical standards when available, otherwise based on calibration curves of standard compounds belonging to the same chemical group and with a similar response. In particular, authentical standards were used for quantitative analysis of chlorogenic acid, ferulic acid, isorhamnetin-rutinoside, kaempferol diglucoside, quercetin glucoside and rutin whereas semi-quantitative determination was carried out for coumaroyl-diglucoside (coumaric acid used as standard), disinapoylgentobiose (sinapic acid used as standard), synapoyl-hexose (sinapic acid used as standard) and for kaempferol 3-hydroxyferuloyl-sophorotrioside-7-glucoside (kaempferol-diglucoside used as standard). A mass tolerance of 5 ppm was employed. The instrument calibration was checked daily using a reference standard mixture obtained from Thermo Fisher Scientific.

Leaf polyphenolics contents were quantified as μg/g dw and then expressed μg 100 g^{-1} fw based on the samples dry matter percentage.

5.2.8. Statistical Analysis, Cluster Analysis and Heatmap

Experimental data were subjected to bifactorial (nitrogen level × biostimulant) analysis of variance using SPSS 28 software package (IBM, Armonk, NY, USA). Nitrogen dosage mean effect was compared by *t*-test. Biostimulants mean effect and factors interaction were separated by Tukey's HSD test performed at $p \leqslant 0.05$.

A hierarchical cluster analysis (HC) on the quality and phytochemical composition of lettuce leaves was performed, and a heatmap was generated using the ClustVis online

tool [20]. Matrix values were normalized as ln (x + 1), with Euclidean distance and complete linkage.

5.3. Results and Discussion

5.3.1. Lettuce Fresh Yield, Leaf Colorimetry Indices.

The results obtained by the greenhouse trial show two distinct outcomes in the case of the optimal and low nitrogen NS treatments (Figure 1; Supplementary Material Table S1). The commercial yield of lettuce was significantly -and greatly- affected by the nitrogen NS treatment, which decreased shoot fresh weights 4.5-fold in the low treatment.

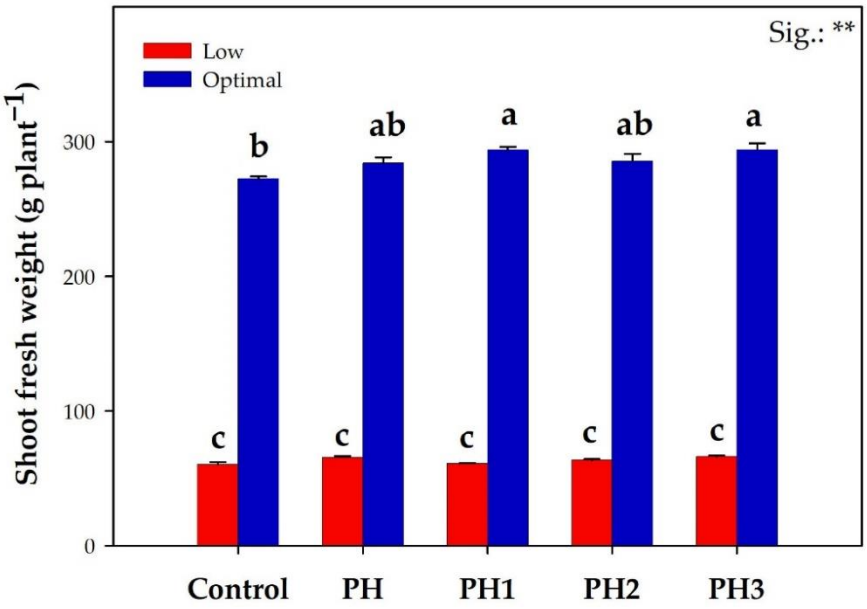

Figure 1. The commercial yield of lettuce plants as affected by nitrogen dosage and biostimulant application. All data are expressed as mean ± standard error, n = 3. Interaction data were deemed significant at $p \leq 0.01$ (**). Different letters above the bars indicate significant differences according to Tukey's HSD test, performed at $p \leq 0.05$. PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa). Nitrogen dosage: Optimal = 8 mM NO3, Low = 1 mM NO3.

Furthermore, significant biostimulant × nitrogen dosage effects were also recorded. In the optimal nitrogen group, both the H1 and H3 treatments were the best performing and recorded the highest shoot fresh weight, which translated into a 7.5% mean increase compared to their untreated control. Although PH and PH3 increased the shoot fresh weight by 9.0% compared to the control, the differences were not deemed significant in the suboptimal nitrogen group.

The marketable fresh weights increase in the optimal NS treatment is in line with the currently available literature on PH use in leafy vegetables, including lettuce, spinach and rocket which see marketable yield increases after the formulates were applied [5,6,21,22]. However, this was the first research instance where the used treatments are molecular fractions deriving from the same biostimulant matrix, but also one where the widely available working theory behind them is not backed up by experimental data.

Current PH literature agrees upon the role of signaling peptides as one if not the principal driving factor behind plant growth in stress and non-stress condition [23]. Evidence furthering this hypothesis comes from previous work by Lucini and collaborators [9] which showed that the PH3-equivalent fraction of the 'Trainer' PH biostimulant showed the best root growth when compared to both the other tested fractionates and an auxin hormone control. However, when analyzing the metabolic response to the PH3-equivalent action and the commercial formulate they found that while, again, the former better fit an auxin-like footprint, the latter induced an accumulation of gibberellins and a down-accumulation of brassinosteroids, cytokinins and jasmonates [9].

Results also show that pitted against low nitrogen availability, the employed PH biostimulants could not ameliorate the overly deficient -one eighth of the optimal- NS nitrogen conditions. This phenomenon is readily explained as nitrogen is critical to plant life as a constituent in both plant tissue and the photosynthetic machinery driving plant growth. C3 plants, which lettuce and many vegetable species are part of, allocate almost 24% of leaf nitrogen to thylakoids, and a large part of that nitrogen is employed for light-harvesting proteins [1]. Moreover, sustained nitrogen deficiency induces the breakdown of nucleic acids and enzymes, especially Rubisco, which irreversibly impairs photosynthesis and ultimately plant growth [24]. The conducted mineral analysis indisputably proves the point of an insufficient mineral amount to conduct basic plant metabolism, as nitrate, which plants use as nitrogen storage [25], was found to be depleted in plants from the low nitrogen NS group, which also explains the decrease in chlorophyll content found in this trial.

The significant shift in the color indices (Supplementary Material Table S2) is also a tell-tale sign of the low nitrogen stress. Average leaf color shows a CIE DE2000 of 6.80 which was noticeable by the naked eye. More in depth, compared to the O nitrogen treatment, L treated plants showed darker leaf color (L*, −3.5%), of substantially higher redness (a*, +203.8%) and blueness (b*, −22.6%) coloration, in addition to lower color saturation (Chroma, −23.9%). Such a change in leaf color attributes indirectly reveals the production of anthocyanins, which has been previously described in literature on red pigmented lettuce as a response to nitrogen deficiency stress, or nutrient solution deprivation, as shown for the same lettuce cultivar in a research conducted by Ciriello and

collaborators [26]. In particular, Becker and collaborators [27], consistently with this work, found an increase in anthocyanins in nitrogen-starved red lettuce as expressed as the cyanidin-derived cyanidin-3-O-(600-O-malonyl)-glucoside, a red pigmented molecule. Anthocyanins are desirable phytochemicals in vegetables, even when their low bioavailability is considered, as research shows in vivo and in vitro cardio-vascular and cancer disease-preventing effects [28].

5.3.2. Leaf Mineral and Organic Acid Contents

The effects of the nitrogen dosage and biostimulant treatments are shown in **Table 1**. Nitrogen concentration effects were deemed significant across all studied leaf mineral parameters, whilst significant biostimulant effects were recorded in the case of phosphorous, potassium, sulfur, calcium, and magnesium.

Table 1. Mineral and organic acids analysis of lettuce plants as affected by nitrogen dosage and biostimulant application.

Source of Variance	NO3 (mg kg−1 fw)	P (mg 100 g−1 fw)	K (mg 100g −1 fw)	S (mg 100g −1 fw)	Ca (mg 100g −1 fw)	Mg (mg 100g −1 fw)	Malate (mg 100g −1 fw)	Citrate (mg 100g −1 fw)
Nutrient Solution (NS)								
Optimal N (O)	1060 ± 46	20.5 ± 0.5	264 ± 11	3.51 ± 0.12	33.6 ± 1.4	18.7 ± 0.6	192 ± 6	19.6 ± 1.1
Low N (L)	11.3 ± 0.6	22.1 ± 0.6	407 ± 9	3.87 ± 0.15	57.7 ± 3.1	20.1 ± 0.5	283 ± 7	60.0 ± 2.4
t-test	***	*	***	*	***	*	***	***
Biostimulant (B)								
Control	534 ± 242	19.3 ± 0.9 b	291 ± 44 b	3.28 ± 0.23 b	37.0 ± 4.7 c	16.8 ± 0.7 c	211 ± 25 c	35.9 ± 9.3 b
PH	543 ± 243	20.6 ± 0.8 b	336 ± 30 ab	3.5 ± 0.19 ab	41.0 ± 4.1 bc	18.7 ± 0.4 bc	234 ± 22 abc	37.3 ± 8.6 b
PH1	526 ± 233	21.0 ± 0.3 ab	368 ± 32 a	4.2 ± 0.21 a	54.9 ± 9.1 a	21.5 ± 0.9 a	225 ± 21 bc	48.6 ± 10.8 a
PH2	505 ± 229	21.9 ± 0.8 ab	332 ± 30 ab	3.74 ± 0.17 ab	50.0 ± 6.5 ab	20.2 ± 0.8 ab	255 ± 17 ab	40.0 ± 9.7 ab
PH3	568 ± 254	23.5 ± 0.5 a	352 ± 32 a	3.73 ± 0.16 ab	45.3 ± 5.0 abc	20.0 ± 0.5 ab	263 ± 23 a	37.2 ± 8.1 b
	n.s.	**	**	*	**	***	**	*
NS × B								
O×Control	1058 ± 139	18.1 ± 1.0	194 ± 15	2.96 ± 0.28	27.2 ± 3.1 e	15.4 ± 0.5	161 ± 9	15.4 ± 1.1
O×PH	1074 ± 121	20.4 ± 1.2	275 ± 11	3.44 ± 0.05	32.7 ± 1.0 de	18.3 ± 0.4	184 ± 1	18.4 ± 1.1
O×PH1	1041 ± 77	20.4 ± 0.4	301 ± 17	3.82 ± 0.26	35.1 ± 3.4 de	19.9 ± 1.2	183 ± 17	24.8 ± 3.4
O×PH2	999 ± 139	20.9 ± 0.6	269 ± 15	3.56 ± 0.18	37.0 ± 3.0 cde	19.4 ± 1.	216 ± 6	20.1 ± 1.1
O×PH3	1127 ± 106	22.5 ± 0.6	280 ± 10	3.77 ± 0.21	35.8 ± 1.9 de	20.7 ± 0.6	213 ± 1	19.3 ± 0.5
L×Control	10.7 ± 2.0	20.5 ± 1.3	387 ± 15	3.61 ± 0.27	46.8 ± 1.5 bcd	18.1 ± 0.3	260 ± 25	56.4 ± 3.2
L×PH	12.9 ± 1.8	20.8 ± 1.2	397 ± 27	3.56 ± 0.43	49.4 ± 3.7 bcd	19.1 ± 0.8	284 ± 7	56.3 ± 2.5
L×PH1	11.4 ± 0.6	21.6 ± 0.1	435 ± 19	4.57 ± 0.14	74.6 ± 3.6 a	23.1 ± 0.5	267 ± 15	72.4 ± 1.5
L×PH2	11.4 ± 1.9	22.8 ± 1.4	394 ± 18	3.92 ± 0.27	62.9 ± 5.6 ab	21.0 ± 1.1	293 ± 5	59.9 ± 8.8
L×PH3	10.0 ± 1.0	24.6 ± 0.2	423 ± 9	3.69 ± 0.29	54.7 ± 5.5 bc	19.3 ± 0.6	313 ± 11	55.2 ± 1.4
	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.

All data are expressed as mean ± standard error, $n = 3$. n.s., *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen dosage means (O = 8 mM NO_3, L = 1 mM NO_3) were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa).

Such fresh weight mineral increases in the low nitrogen treatment probably stem from the concentration effect due to the elevated leaf dry matter recorded (data not shown). Leaf dry matter increases are compatible with previous literature and could indicate an accumulation of carbon in the form of photosynthesis-derived starches, which however cannot be processed for amino acid assimilation due to the nitrogen-limiting conditions [29,30]. The highest increase in potassium contents may further this theory, as it serves as the regulatory ion for the transport of photosynthesis products and has been found to increase also in soybean leaves under severe nitrogen stress [31].

However, the most egregious result in the recorded reduction is, indeed, the leaf nitrate concentration, as the L nitrogen treatment showed nitrate levels that were almost 100-fold less than the O treatment. Whilst the two-magnitude order reduction in nitrate contents denoted in L treatment indeed proves the nitrogen treatment as being too low, it may represent a favorable outcome when considering that the majority of the daily human nitrate intake comes from vegetables [25]. Methemoglobinemia, a biochemical anemia which results from nitrate exposure, is largely reported as the most common nitrate-derived human illness, and excess nitrate and nitrite consumption has been further linked to neoplasiae like gastric cancer [32]. The consumption of nitrate-deficient vegetables may entail all the benefits of this food group, which include a proven reduction in the risk of chronic disease and premature mortality, whilst balancing the intake derived from other nitrate-rich sources like cured meats and drinking water [32,33].

Nevertheless, it is also imperative to note that all recorded nitrate values in both optimal and low conditions were below the threshold imposed by the EU Regulation 1258/2011 for lettuce grown in protected culture.

Averaged across nitrogen treatments, phosphorous and potassium contents were significantly increased in comparison to the untreated controls by the PH3 treatment, which recorded 21.6% and 20.9% higher uptake. The PH1 treatment also recorded significantly higher leaf K (26.6%), S (21.9%) and Mg (28.2%) contents when compared to the untreated control.

Delving into the interaction data, the L*PH1 plants are characterized by significant higher leaf calcium, compared to both O (+174.3%) and L (+59.4%) controls. Our results do not contrast previous literature which shows the potential of PH biostimulants to increase the use efficiency of supplied nutrients in greenhouse-grown leafy vegetables. Both Cristofano, Rouphael and their collaborators [5,34] described the effect as due to the 'nutrient acquisition response', which is the sum of increased carbon and nitrogen metabolism, root growth, and gene expression for macronutrient transporters.

These results also prove that biostimulant supplementation to plants can improve their nutrient content, which can be especially useful to those populations exposed to nutrient deficiencies. The 2015–2020 dietary guides of Americans pit calcium and potassium as nutrients of a public health concern due to under consumption by the populace [35].

However, the results show some pointers which can be addressed to discriminate PH1 and PH3.

First, the result obtained by the PH1 treatment in regard to calcium concentration shows a modulation of calcium influx to the shoot tissue. When all conditions are equal,

shoot calcium concentration largely depends on element availability and transpirational water flux [36], which is impeded in nitrogen-limiting conditions as stomatal conductance decreases with decreasing nutrient supply [1].

In a second instance, both investigated organic acids were found to be differently modulated by the biostimulant treatments. When averaged across nitrogen NS concentration the H3 treatment yielded a significantly higher concentration of malate which increased by 24.7% compared to the untreated control. Citrate concentration was most affected by the H1 treatment which determined an increase of 35.2% when compared to the control average. Similarly to the results obtained in this trial, previous literature show that malate and citrate were increased by the application of a different vegetal PH applied to lettuce [37]. Moreover, previous research has shown that PH biostimulants stimulate carbon metabolism, as gene expression relative to enzymes in the tricarboxylic acid (TCA) cycle was found to be upregulated after application [38,39]. However, such distinctive behavior between treatments underpins their dissimilarity which could be due to the distinct modulation of the carbon metabolism cycle. Organic acids are crucial at the plant cell level, taking part in energy production, amino acids biosynthesis and adaptation to environmental changes, but most importantly contribute to human health due to their antioxidative role [40].

5.3.3. Leaf Pigments and Total Ascorbic Acid Content

Table 2 shows the effect of the biostimulant treatments on leaf pigments and total ascorbic acid content. Save for leaf total ascorbic acid, the NS treatments induced significant differences in the studied parameters, while the biostimulant treatments influenced all the parameters, with β-carotene being influenced by NS x B interactions.

Table 2. Chlorophyll, auxiliary pigments, total ascorbic acid content of lettuce plants as affected by nitrogen dosage and biostimulant application.

Source of Variance	Total Chlorophylls (mg 100 g^{-1} fw)	TAA (mg AA 100 g^{-1} fw)	β-Carotene (mg 100 g^{-1} fw)	Lutein (mg 100 g^{-1} fw)
Nutrient Solution (NS)				
Optimal N (O)	95.0 ± 3.4	139 ± 6.00	1.54 ± 0.11	1.54 ± 0.1
Low N (L)	58.5 ± 3.4	132 ± 7.43	2.47 ± 0.12	2.30 ± 0.06
t-test	***	n.s.	***	***
Biostimulant (B)				
Control	71.9 ± 7.0 ab	121 ± 6 bc	1.53 ± 0.16 c	1.69 ± 0.18 b
PH	64.8 ± 11.0 b	112 ± 3 c	1.95 ± 0.24 bc	1.91 ± 0.23 ab
PH1	79.0 ± 11.7 ab	131 ± 6 bc	2.12 ± 0.25 ab	1.99 ± 0.13 ab
PH2	80.7 ± 8.8 ab	141 ± 10 b	1.96 ± 0.35 b	1.72 ± 0.28 b
PH3	87.5 ± 6.8 a	172 ± 5 a	2.48 ± 0.18 a	2.29 ± 0.15 a
	*	***	***	**
NS × B				
H × Control	86.9 ± 2.6	126 ± 13	1.23 ± 0.08 e	1.30 ± 0.01
H × PH	86.6 ± 9.8	117 ± 7	1.51 ± 0.08 de	1.45 ± 0.05
H × PH1	103 ± 8.2	136 ± 11	1.57 ± 0.09 cde	1.72 ± 0.12
H × PH2	98.8 ± 7.0	149 ± 8	1.23 ± 0.17 e	1.14 ± 0.08
H × PH3	99.4 ± 7.2	166 ± 11	2.17 ± 0.24 abcd	2.10 ± 0.25
L × Control	56.9 ± 3.4	117 ± 0	1.83 ± 0.17 bcde	2.09 ± 0.08
L × PH	43.1 ± 6.5	107 ± 2	2.39 ± 0.28 abc	2.38 ± 0.20
L × PH1	54.7 ± 5.4	125 ± 7	2.67 ± 0.07 ab	2.26 ± 0.05
L × PH2	62.5 ± 2.8	133 ± 20	2.7 ± 0.24 ab	2.30 ± 0.22
L × PH3	75.6 ± 6.0	177 ± 2	2.78 ± 0.15 a	2.48 ± 0.09
	n.s.	n.s.	*	n.s.

All data are expressed as mean ± standard error, n = 3. n.s., *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen dosage means (O = 8 mM NO3, L = 1 mM NO3) were compared by t-test. Different letters within each column indicate significant differences according to Tukey's HSD (p = 0.05). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa. TAA: total ascorbic acid.

When averaged across nitrogen treatments PH and PH3 manifested significant differences between each other (+34.9% in the former) regarding the amount of total chlorophylls. Significant leaf total ascorbic acid content variation was only found in respect of the biostimulant applications, and PH3 recorded the highest result with an increase of 41.5% when compared to the untreated control. Interaction data from the leaf β-carotene content shows the PH3 treatment recording the highest figures in both optimal (+76,4%, compared to the O*Control) and low (+51.9%, compared to the L*Control) nitrogen conditions. Lastly, when NS nitrogen treatments were averaged, lutein content was significantly increased (+35.5%) by the PH3 treatment when compared to the untreated control.

The collected data shows that across sustained nitrogen stress, plant response to biostimulant application revolves in part around the enzymatic and non-enzymatic

antioxidant pathways, as highlighted by previous PH research showing similar outcomes [23]. Ascorbic acid or Vitamin C, usually present in the anionic form ascorbate, is a key substrate of the ascorbate peroxidase-glutathione reductase (APX-GR) system, which serves to detoxify reactive oxidative species (ROS) in plant tissue, especially in the case of plant stress [41]. Plant carotenoids like β-carotene and lutein serve both as enzymatic and non-enzymatic photooxidative protection by scavenging ROS, dissipating excess light energy via non photochemical quenching (NPQ, via the xanthophyll cycle [42]) and protecting cellular membranes in the case of stress [43]. However, it is particularly telling that the tested products induced distinct modulations of the studied phytochemicals, especially in the case of PH3. Lutein and β-carotene are the most abundant carotenoids in chloroplasts (70–75% of the total amount) [44], which coupled with APX as the dominant chloroplast antioxidant system [45], show PH3 primed plants to work against photo-oxidative stress.

The enrichment of such phytochemicals is of interest in the context of human health improvement.

While uncommon in developed countries, vitamin A deficiency is said by the WHO to be a public health problem in half of all countries [46]. Provitamin A carotenoids, which β-carotene is part of, are important to preserve eyesight; furthermore, while evidence on supplemental (i.e. exogenous) dietary carotenoids may be lacking, high intake from fruits and vegetables has proven health benefits, including a lower risk of developing chronic diseases, which confirms the importance of the plant matrix for nutrient absorption [43].

The same considerations can be made for Vitamin C, as its marginal i.e., not scurvy-inducing deficiency can occur in up to 15% of the general population, a figure which is doubled in the case of cigarette smokers, and can lead to a higher risk of all-cause mortality [47]. The data further the case for the application of products like PH3 to induce the production of phytochemicals of interest; this can have a tangible and quick effect when compared to biotechnological practices which could be used to achieve the same result, due to the complex regulatory network surrounding their accumulation [48].

5.3.4. Leaf Polyphenolics

Tables 3 and **4** show the modulation of the leaf polyphenolic contents by the NS and biostimulant treatments. When the biostimulant treatments are considered, the total leaf phenolic acid concentration was significantly affected (Table 3). In particular, when averaged across nitrogen treatments, the H2 treatment gave rise to the highest (+24.0%) significant increase in this parameter when compared to the untreated control.

Table 3. Phenolic acids profile of lettuce plants as affected by nitrogen dosage and biostimulant application.

Source of variance	Chlorogenic Acid (μg 100 g^{-1} fw)	Coumaroyl-Diglucoside (μg 100 g^{-1} fw)	Disinapoylgentobiose (μg 100 g^{-1} fw)	Ferulic Acid (μg 100 g^{-1} fw)	Synapoyl-Hexose (μg 100 g^{-1} fw)	Total Phenolic Acids (μg 100 g^{-1} fw)
Nutrient Solution (NS)						
Optimal N (O)	9736 ± 245	13.1 ± 0.8	3.17 ± 0.12	753 ± 32	32.1 ± 1.3	10537 ± 255
Low N (L)	13431 ± 416	11.4 ± 1.0	4.26 ± 0.15	899 ± 39	80.3 ± 4.8	14426 ± 436
t-test	**	**	***	n.s.	***	**
Biostimulant (B)						
Control	10308 ± 907 b	13.8 ± 2.0	2.83 ± 0.21 d	685 ± 57 b	60.0 ± 11.1	11069 ± 955 b
PH	11545 ± 936 ab	13.4 ± 1.3	3.85 ± 0.19 bc	813 ± 37 ab	53.7 ± 11.6	12429 ± 980 ab
PH1	11990 ± 1046 ab	12.9 ± 1.6	3.68 ± 0.26 c	903 ± 81 a	61.1 ± 14.6	12971 ± 1123 ab
PH2	12847 ± 1043 a	11.6 ± 1.4	3.98 ± 0.27 b	809 ± 55 ab	50.8 ± 10.4	13722 ± 1070 a
PH3	11227 ± 680 ab	9.59 ± 0.6	4.25 ± 0.33 a	919 ± 46 a	55.5 ± 12.5	12216 ± 718 ab
	**	n.s.	***	*	n.s.	**
NS × B						
O × Control	8450 ± 403	13.7 ± 3.9	2.38 ± 0.09 d	610 ± 23	38.5 ± 2.5	9114 ± 418
O × PH	10017 ± 428	14.4 ± 1.1	3.43 ± 0.05 c	759 ± 15	32.9 ± 2.6	10827 ± 446
O × PH1	9959 ± 736	12.8 ± 1.2	3.12 ± 0.12 c	761 ± 44	28.5 ± 1.9	10765 ± 690
O × PH2	10521 ± 130	14.4 ± 0.4	3.40 ± 0.13 c	765 ± 100	30.2 ± 3.3	11335 ± 71
O × PH3	9732 ± 32	10.4 ± 0.8	3.54 ± 0.12 c	869 ± 75.	30.7 ± 1.8	10645 ± 106
L × Control	12166 ± 709	13.8 ± 1.9	3.28 ± 0.02 c	760 ± 100	81.5 ± 12.2	13024 ± 754
L × PH	13073 ± 1364	12.5 ± 2.5	4.27 ± 0.06 b	867 ± 61	74.6 ± 15.3	14031 ± 142
L × PH1	14022 ± 893	13.0 ± 3.3	4.23 ± 0.06 b	1045 ± 104	93.8 ± 0.9	15178 ± 980
L × PH2	15173 ± 118	8.74 ± 1.1	4.56 ± 0.05 ab	852 ± 56	71.4 ± 10.5	16109 ± 143
L × PH3	12722 ± 278	8.87 ± 0.5	4.96 ± 0.15 a	970 ± 46	80.4 ± 12.7	13786 ± 322
	n.s.	n.s.	*	n.s.	n.s.	n.s.

All data are expressed as mean ± standard error, *n.* = 3. n.s., *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen dosage means (O = 8 mM NO_3, L = 1 mM NO_3) were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa).

Table 4. Flavonoids profile of lettuce plants as affected by nitrogen dosage and biostimulant application.

Source of variance	Isorhamnetin 3-Rutinoside	Kaempferol 3,7-Diglucoside	Kaempferol 3-Glucoside	Kaempferol 3-Hydroxyferuloyl-Sophorotrioside-7-Glucoside	Quercetin 3-Glucoside	Rutin	Total Flavonoids
	(μg 100 g–1 fw)	(μg 100 g–1 fw)	(μg 100 g–1 fw)	(μg 100 g–1 fw)	(μg 100 g–1 fw)	(μg 100 g–1 fw)	(μg 100 g–1 fw)
Nutrient Solution (NS)							
Optimal N (O)	3.33 ± 0.12	0.89 ± 0.11	3.82 ± 0.30	1.55 ± 0.10	67.8 ± 4.5	1.78 ± 0.22	80.0 ± 4.8
Low N (L)	5.50 ± 0.42	6.59 ± 0.78	12.6 ± 0.70	1.81 ± 0.14	495 ± 15	13.8 ± 1.52	535 ± 16
t-test	***	***	***	***	***	***	***
Biostimulant (B)							
Control	3.12 ± 0.26 d	5.11 ± 2.58	8.60 ± 2.16	0.69 ± 0.09 d	295 ± 98	10.2 ± 5.16	323 ± 107
PH	4.08 ± 0.41 c	3.60 ± 1.08	9.25 ± 2.72	1.92 ± 0.17 a	279 ± 97	8.69 ± 2.70	307 ± 103
PH1	4.17 ± 0.36 bc	3.23 ± 1.25	7.75 ± 2.02	1.32 ± 0.13 c	294 ± 102	6.46 ± 2.50	316 ± 109
PH2	4.58 ± 0.47 b	3.41 ± 1.09	7.32 ± 1.32	1.63 ± 0.17 b	291 ± 90	6.82 ± 2.18	315 ± 95
PH3	6.11 ± 0.99 a	3.85 ± 1.28	8.25 ± 2.29	1.89 ± 0.18 a	268 ± 89	7.69 ± 2.55	294 ± 96
	***	n.s.	n.s.	***	n.s.	n.s.	n.s.
NS × B							
O × Control	2.56 ± 0.09 d	0.73 ± 0.10	4.06 ± 1.07	0.51 ± 0.03 g	77.1 ± 18.6	1.46 ± 0.20	86.5 ± 20.0
O × PH	3.18 ± 0.09 cd	1.40 ± 0.07	3.32 ± 0.78	1.55 ± 0.07 c	67.8 ± 7.6	2.79 ± 0.15	80.0 ± 8.2
O × PH1	3.41 ± 0.09 c	0.52 ± 0.12	3.50 ± 0.02	1.03 ± 0.06 ef	71.9 ± 5.0	1.05 ± 0.23	81.4 ± 4.9
O × PH2	3.59 ± 0.07 c	1.02 ± 0.31	4.62 ± 0.63	1.26 ± 0.02 de	91.5 ± 8.2	2.04 ± 0.63	104 ± 9.7
O × PH3	3.90 ± 0.04 c	0.74 ± 0.28	3.59 ± 0.60	1.49 ± 0.01 cd	72.0 ± 5.7	1.49 ± 0.57	82.5 ± 6.2
L × Control	3.68 ± 0.06 cd	9.48 ± 3.76	13.1 ± 1.22	0.88 ± 0.06 f	513 ± 19.8	19.0 ± 7.52	559 ± 30
L × PH	4.97 ± 0.11 b	5.80 ± 0.98	15.2 ± 1.10	2.30 ± 0.03 a	491 ± 42.7	14.6 ± 1.28	534 ± 45
L × PH1	4.93 ± 0.22 b	5.94 ± 0.70	12.0 ± 1.52	1.60 ± 0.07 c	515 ± 58.2	11.9 ± 1.41	552 ± 62
L × PH2	5.56 ± 0.34 b	5.79 ± 0.36	10.0 ± 1.02	1.99 ± 0.06 b	491 ± 20.5	11.6 ± 0.72	526 ± 22
L × PH3	8.33 ± 0.09 a	5.91 ± 0.22	12.9 ± 2.06	2.28 ± 0.03 a	464 ± 30.6	11.8 ± 0.45	506 ± 32
	***	n.s.	n.s.	**	n.s.	n.s.	n.s.

All data are expressed as mean ± standard error, $n = 3$. n.s., **, *** non-significant or significant at $p \leq 0.01$ and 0.001, respectively. Nitrogen dosage means (O = 8 mM NO_3, L = 1 mM NO_3) were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, < 10 kDa).

When broken down into the analyzed components, chlorogenic acid gave the highest contribution (92.8% averaged across all treatments) to the total amount, and thus was similarly affected by the biostimulant applications. In fact, the H2 treatment still provided higher figures compared to the control, with an increase of 24.6% when considered across nitrogen treatments. Ferulic acid content was the second most present compound and was also affected by the tested biostimulants, as both H1 and H3 showed significant increases over the untreated counterparts by 33.1% on average.

When speaking about tissue flavonoid contents (Table 4), NS nitrogen dosage proved to be the most impactful factor when considering total content, as no significant difference was denoted from B treatment or N × B interaction. In fact, the L nitrogen treatment increased this parameter 6.7-fold compared to the optimal regimen. The most impactful driver of this change was the 7.3-fold increase in Quercetin-3-glucoside in the L treatment: this compound accounted for 92.5% of the L treatment's flavonoid content. When the discrete compounds are considered, only Kaempferol 3-hydroxyferuloyl-sophorotrioside-7-glucoside and Isorhamnetin 3-rutinoside incurred in combinatory N × B interactions. In the former case, all biostimulants significantly increased leaf concentrations compared to their respective controls. However, both the L × H3 and L × PH proved to be the most effective by showing a 2.6 and a 4.5-fold increase compared to the optimal and low controls, respectively. When looking at the interaction data for Isorhamnetin 3-rutinoside it is shown that the H3 treatments, in the L group were the most successful in augmenting this parameter, while in the O group all the fractions were significantly effective when compared to their untreated control.

Isorhamnetin-3-rutinoside was 2.3-fold higher in the L × H3 treatments versus the L control and 2.1 and 3.3-fold compared to the O × H3 and O × Control treatments, respectively.

Polyphenolics are a class of molecules which stem from a common origin and serve as regulators of plant growth and as plant stress-response molecules. Starting from shikimate, they are the product of the differentiation of phenylalanine-derived cinnamate via the enzyme phenylalanine ammonia lyase (PAL), which starts the central phenylpropanoid pathway [49]. As the products of this pathway are extremely diverse, and contain polymers like lignin and suberin and pigments like anthocyanins, we have grouped them based on their structural similarity as phenolic acids i.e., phenolic compounds with one carboxylic group, and flavonoids i.e., compounds with a C6-C3-C6 ring structure [50,51]. However, irrespective of their structure, the evidence here obtained shows that under very low nitrogen conditions lettuce plants behave according to the hypotheses set out by Becker and collaborators, which translate into a shift in carbon metabolism due to a high C/N ratio, and nitrogen recycling via PAL, which leaves with carbon skeletons for the phenylpropanoid synthesis [27]; this is particularly

evident, as both the total phenolic and total flavonoid assays show marked increase due to the low nitrogen conditions.

Chlorogenic acid is widely reported as the most present phenolic acid in lettuce [52] and biotechnological efforts to increase its concentration in plant tissues are documented in the literature due to its anti-carcinogenic and atherosclerosis-preventing activity [53]. Again, this shows how different molecular weight biostimulants impact plant metabolism in differential ways, as this phenolic acid has been shown to work as a connector of cell wall polymers, mechanically strengthening tissues as a barrier for pathogen stresses [54].

The recorded increases in leaf flavonoid contents are compatible with what is available in current literature in lettuce grown in nitrogen-deficient media [27,52], and is a common response to stressful conditions. Becker et al. [27] found a general increase in the flavonoid contents of nitrogen-deprived lettuce plants, which is compatible with the results obtained in this trial. The production of Kaempferol and quercetin-derived flavonoid molecules, which are key intermediates of anthocyanin production as they represent part of the biosynthetic pathway [50], was found to be highly induced by the nitrogen treatment. However, such an increase can prove interesting when considering a diet rich in the compound is beneficial in many aspects of human health, from antidiabetic to anti-inflammatory effects, and cardio-vascular disease prevention [28]. However, the modulation of total phenolic acids and total flavonoids upon the different biostimulant treatments is partially in line with the results obtained by Giordano and collaborators [36], who applied a PH on two different cultivars of lettuce. In this previous work, total phenolic acids were significantly boosted in both cultivars, while total flavonoids were only significantly higher in one cultivar and steady in the second one, when subjected to PH treatment. The accumulation of antioxidant molecules, such as phenols and flavonoids, has been associated with the PH's biostimulant modification of plant primary and secondary metabolism [5,13,37]. Indeed, according to the previous authors, plant-based PHs, as action mode, trigger secondary metabolism via an increase in the expression of genes encoding phenylalanine an ammonia-lyase enzyme. Anyhow, PH biostimulants have a proven track record of increasing nutrient use efficiency, plant stress tolerance and produce quality, all in accordance with EU-regulation 1009/2019.

5.3.5. Cluster Analysis and Heatmap of the Accumulation of phytochemicals

To provide a visual representation of the changes in phytochemical contents after the application of the biostimulant treatments, we have performed a hierarchical clustering analysis coupled with a heatmap, which can be seen in Figure 2.

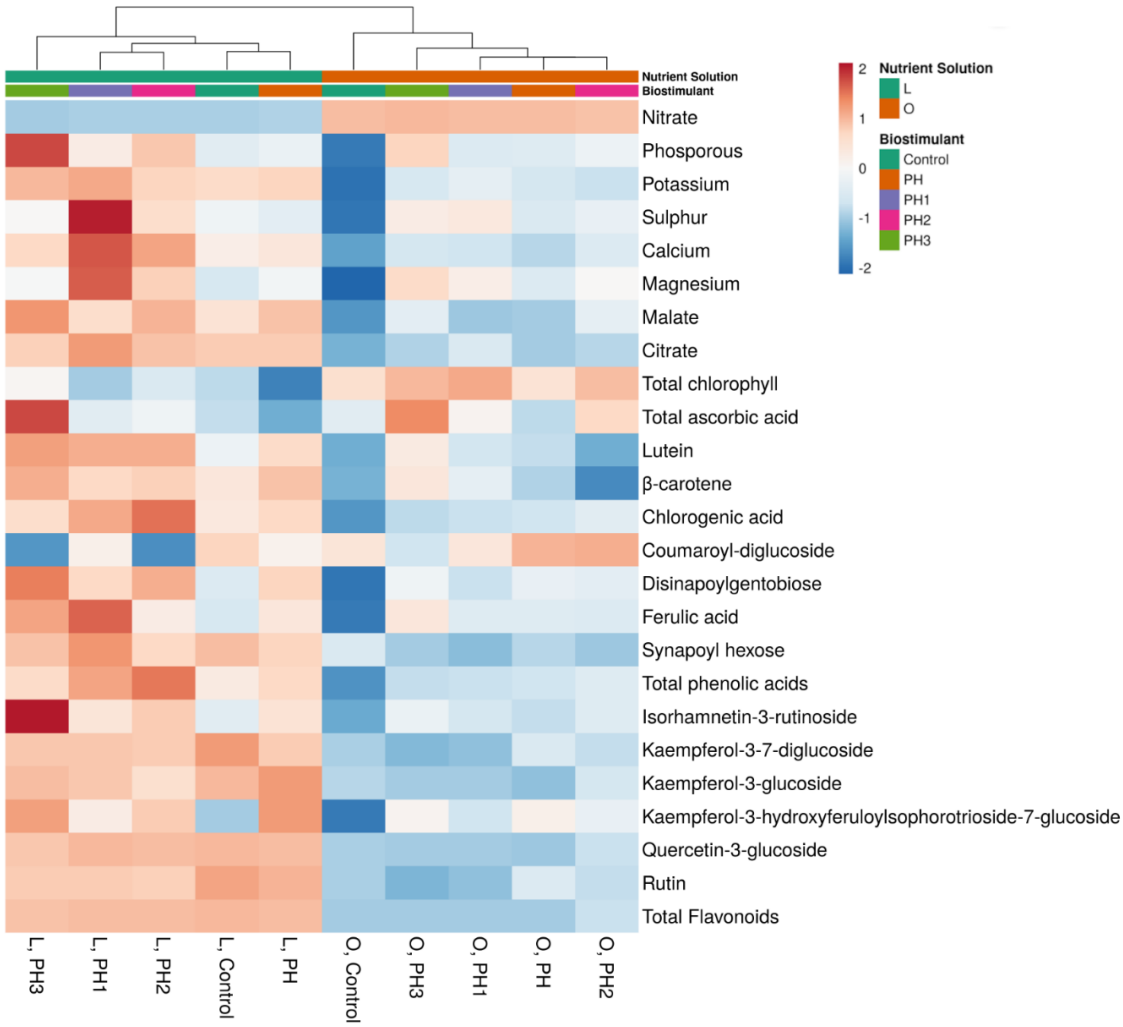

Figure 2. Cluster heat map analysis summarizing lettuce plants response to nitrogen dosage and biostimulant application**.** Original values are ln (x + 1)-transformed. Columns are clustered using Euclidean distance and complete linkage. PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, < 10 kDa).

The dendrogram provides for two main clusters, which are divided based on the NS nitrogen treatment. In low nitrogen conditions, represented by the left cluster, the PH3 treatment is clearly separated from the other biostimulants and the control due to the increases in phosphorous, total ascorbic acid and isorhamnetin-3-rutinoside, as well as the lower coumaroyl-diglucoside content. The PH1 and PH2 clusters are associated with higher sulphur, calcium, magnesium, but also with an increase in total phenolic acids.

In optimal nitrogen conditions, we find two clusters represented by the control which is separated by the biostimulant treatments. In this case, PH3 also separates from the remaining PH treatments due to the increases in phosphorous, total ascorbic acid, lutein, β-carotene and ferulic acid. PH, PH1, PH2 treatments are clustered together and associated with coumaroyl-diglucoside, but also reduced lutein, β-carotene and ferulic acid contents.

5.4 Chapter 5 Conclusions

The use of molecular fractionation is an adequate strategy to increase the potency of the PH-based products, and this trial represents a steppingstone in the lab-to-field journey. In optimal nitrogen conditions, both the PH1 and PH3 fractions successfully increased lettuce marketable yield by 7.9%, whereas in the low nitrogen conditions, biostimulants increases were not significant. However, across nitrogen conditions we found that the best performing products also incremented the produce nutritional quality in ways that underline their different mode of action. PH3 induced a significant increase in total ascorbic acid (+41.5%), lutein (+35.5%) and β-carotene in both optimal (+76,4% compared to the O*Control) and low (+51.9%) conditions which show that plants were primed to protect themselves from the oxidative stress by accumulating these compounds. The employed fractions also modulated the polyphenolic composition of the leaves in different, fraction-specific manners, as PH3 and PH1 induced a significantly higher accumulation of ferulic acid (+32.7%), when compared to total phenolic acid content, which was highest in the PH2 treatment (+24.6%). Again, whilst the limits of the study are found in a too low nitrogen concentration in the nitrogen stress group, it also successfully underlines the principle of PH biostimulants being a complex matter, as PH1 and PH3 provided for similar growing prowess, but modified plant secondary metabolites in a distinct way. However, the qualitative results here recorded also provide for a practical use-case of the fractions to improve the functional quality of produce.

5.5 Supplementary Material for Chapter 5

Table S1: Shoot fresh weight of lettuce plants as affected by nitrogen dosage and biostimulant application

Source of variance	Shoot fresh weight (g plant^{-1})
Nutrient Solution (NS)	
Optimal N (O)	286 ± 2.59
Low N (L)	63.5 ± 0.68
t-test	***
Biostimulant (B)	
Control	166 ± 47.4 b
PH	175 ± 48.9 ab
PH1	177 ± 52.0 a
PH2	175 ± 49.7 ab
PH3	180 ± 51.0 a
	**
NS × B	
O×Control	272 ± 1.75 b
O×PH	284 ± 4.13 ab
O×PH1	294 ± 2.16 a
O×PH2	286 ± 5.39 ab
O×PH3	294 ± 4.57 a
L×Control	60.6 ± 1.37 c
L×PH	65.8 ± 0.69 c
L×PH1	61.3 ± 0.14 c
L×PH2	63.7 ± 0.46 c
L×PH3	66.3 ± 0.61 c
	**

All data are expressed as mean ± standard error, n = 3. **, *** significant at $p \leq 0.01$ and 0.001, respectively. Nitrogen dosage means (O = 8mM NO3, L = 1mM NO3) were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa)

Table S2: Colorimetric measurements of lettuce plants as affected by nitrogen dosage and biostimulant application

Source of variance	L*	a*	b*	Chroma	Hue angle
Nutrient Solution (NS)					
Optimal N (O)	43.7 ± 0.36	-7.05 ± 0.43	26.6 ± 0.4	27.7 ± 0.43	107 ± 1.97
Low N (L)	42.2 ± 0.44	-2.32 ± 0.59	20.6 ± 0.86	21.1 ± 0.88	133 ± 9.87
t-test	**	***	***	***	*
Biostimulant (B)					
Control	42.3 ± 1.16	-3.62 ± 1.68	22.7 ± 2.12	23.4 ± 2.25	139 ± 17.6
PH	42.5 ± 0.36	-4.57 ± 1.44	22.1 ± 2.31	22.8 ± 2.45	118 ± 9.07
PH1	42.8 ± 0.70	-5.20 ± 0.93	24.9 ± 1.02	25.6 ± 1.13	119 ± 11.0
PH2	44.1 ± 0.45	-5.09 ± 0.97	24.7 ± 1.06	25.4 ± 1.20	107 ± 5.6
PH3	42.9 ± 0.56	-4.93 ± 1.52	23.9 ± 1.46	24.7 ± 1.63	118 ± 15
	n.s.	n.s.	n.s.	n.s.	n.s.
NS × B					
O×Control	44.3 ± 0.67	-6.21 ± 1.76	26.6 ± 1.85	27.5 ± 2.01	115 ± 9.25
O×PH	42.2 ± 0.52	-7.19 ± 0.85	25.7 ± 0.68	26.8 ± 0.86	105 ± 1.49
O×PH1	44.0 ± 0.61	-6.94 ± 1.01	26.9 ± 0.80	27.9 ± 0.83	104 ± 2.03
O×PH2	44.5 ± 0.86	-7.14 ± 0.66	27.0 ± 0.40	28.0 ± 0.39	105 ± 1.47
O×PH3	43.5 ± 1.02	-7.75 ± 0.68	27.0 ± 0.59	28.2 ± 0.57	106 ± 1.41
L×Control	40.3 ± 1.52	-1.02 ± 2.05	18.7 ± 1.91	19.2 ± 1.94	162 ± 30.0
L×*PH	42.9 ± 0.49	-1.96 ± 1.67	18.4 ± 3.59	18.8 ± 3.7	130 ± 15.9
L×PH1	41.6 ± 0.79	-3.45 ± 0.49	22.8 ± 0.58	23.3 ± 0.59	133 ± 19.7
L×PH2	43.7 ± 0.36	-3.04 ± 0.35	22.4 ± 0.53	22.8 ± 0.58	109 ± 12.2
L×PH3	42.2 ± 0.27	-2.12 ± 1.76	20.8 ± 0.86	21.2 ± 0.92	131 ± 31.5
	n.s.	n.s.	n.s.	n.s.	n.s.

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen dosage means (O = 8mM NO3, L = 1mM NO3) were compared by *t*-Test. Chroma = $((a^*)^2 + (b^*)^2)^{0.5}$), Hue = ((Arctan $(b^*/a^*)/2\pi)*360)+180$). Different letters within each column indicate significant differences according to Tukey's HSD (p = 0.05). PH: Protein Hydrolysate, molecular fractions PH1, PH2 and PH3 (>10 kDa, between 1 and 10 kDa, <10 kDa)

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Chapter 6

Modulation of Morpho-physiological and Metabolic Profiles of Lettuce Subjected to Salt-Stress and Treated with Two Vegetal Derived Biostimulants

Abstract: Salinity in water and soil is a critical issue for food production. Using biostimulants provides an effective strategy to protect crops from salinity-derived yield losses. The research supports the effectiveness of protein hydrolysate (PH) biostimulants based on their source material. A greenhouse experiment was performed on lettuce plants under control (0 mM NaCl) and high salinity conditions (30 mM NaCl) using the Trainer (T) and Vegamin (V) PH biostimulants. The recorded data included yield parameters, mineral contents, auxiliary pigments, and polyphenolics. The plant sample material was further analyzed to uncover the unique metabolomic trace of the two biostimulants. The results showed an increased yield (8.9/4.6%, T/V) and higher photosynthetic performance (14%) compared to control and salinity treatments. Increased yield in salinity condition by T compared to V was deemed significant due to the positive modulation in stress-protecting molecules having an oxidative stress relief effect such as lutein (39.9% 0 × T vs. 30 × V), β-carotene (23.4% vs. V overall), and flavonoids (27.7% vs. V). The effects of PH biostimulants on the physio-chemical and metabolic performance of lettuce plants are formulation dependent. However, they increased plant growth under stress conditions, which can prove profitable

6.1. Introduction

Soil and irrigation water salinity are outstanding problems threatening food security in a world with an ever-growing population. FAO estimates salt-affected soil area at more than 4.4% of total land area, with arid and semi-arid regions being the most affected [1]. Salinization is a double-faced problem in the Mediterranean region, as freshwater availability is becoming scarce due to climate change-induced droughts, and the use of saline water for irrigation can aggravate this issue, resulting in low-er-than-expected yields [2]. In plants, salt stress is described as the combination of an early onset drought-like condition due to the increase in osmotic pressure at the root level and a later toxic effect of both sodium and chloride ions. Osmotic stresses damage plant tissues by determining reactive oxygen species (ROS) formation due to limited water availability, which can gravely depress photosynthetic performance [3]. Fur-thermore, toxic sodium ions compete for the same cationic transporters and ion chan-nels as potassium, and this interchange can cause a further depression of the plants' metabolic performances [4].

Farmers have many strategies to cope with yield depression due to saline condi-tions, such as water and nutrient management, the switch to soilless agriculture and the use of salt resistant-genotypes [2,5,6]. A further addition to the farmer toolset is repre-sented by plant biostimulants (PBs), which are formulations proven to be particularly effective in alleviating yield losses due to suboptimal conditions [7]. Protein hydrolysate (PH) biostimulants, which are a mixture of amino acids and peptides obtained via the hydrolysis of protein matrices [8,9], can be rapidly deployed via foliar spray or substrate drench [10], and are a well-studied and well-proven category. A recent meta-analysis by Li and collaborators [7] found an average 16.5% marketable yield increase across the available PH literature, furthering their usefulness. Among this product category, veg-etal-derived PHs deriving from enzymatic hydrolysis are more environmentally friendly options compared to those derived from chemical hydrolysis. This is especially true when considering the plethora of waste biomass from crop cultivation that could be used for agrochemicals production [10,11]. Research has summarized the activity of PH biostimulants as the increase in root growth to the presence of an auxin-like action, thus providing for higher nutrient uptake, the stimulation of carbon and nitrogen metabo-lism and the priming effect of the antioxidant systems that fend off plant stresses [9]. This effect has been postulated to come from the presence of bioactive molecules, such as signaling peptides, of which the root hair promoting peptide is the most widely re-ported as being found in the Trainer biostimulant [10].

One of the techniques that has furthered the understanding of these products has been metabolomics, which offers a picture of the mode of action by identifying markers of the changes in plant metabolism. For instance, in drought-stressed tomato (Solanum lycopersicum L.) and salt-stressed lettuce (Lactuca sativa L.), the metabolic profile change to the application of the Trainer biostimulant has been described as the reprogramming of the phytohormone profile, which has resulted in improved resistance to oxidative stresses [12,13]. During salt stress, this leads to the hypothesis that the increase in root absorbing area and the priming of antioxidant-related defense mechanisms can lead to decreased toxic ion absorption and better oxidative status, thus increasing plant per-formance, as it has been found in tomato and spinach [14,15]. However, metabolomics has also provided evidence that not all vegetal-derived biostimulants are equal in their modulation of the metabolome. A recent study by Ceccarelli and collaborators [16], which tested vegetal PH biostimulants derived from five distinct protein matrices, found an accumulation of auxins and gibberellins in tomato root tissue on two of the tested formulations and an opposite behavior in the remaining three. This led them to consider that, due to the intrinsic variation in the protein makeup of the source matrix, a generalized approach to vegetal PHs cannot be taken [16].

There is an argument to be made about the composition of these products: their nitrogen content, as a proxy of the content of the nitrogen-containing active molecules, may explain the source of the variability seen in the literature. Recent research conducted on lettuce elucidated that foliar application rates of the Trainer biostimulant as high as double the normal rate increased both photosynthetic rate and nutrient uptake (P, S, K) compared to the base treatment [17]. While this may confirm that a higher supply may also entail an increase in active molecules supplied, it has also been found on lettuce that an overuse may cause growth regression, which has been found to be cultivar specific [18].

To test the validity of PH biostimulants in ameliorating salt stress tolerance, we set out a greenhouse experiment comparing two commercially available vegetal-derived PH biostimulants that differ in their composition (crucially, in the amount of nitrogen), and potentially, active ingredients content. We also tested both their salt stress and ameliorating effects via morpho-physiological and biochemical assays to confirm that the modulation of metabolic markers varies on a product-to-product basis. For this purpose, we also selected lettuce as the test crop, as it is a prime candidate for determining the stress-ameliorating power of biostimulants due to being widely cultivated and being a glycophyte, or moderately sensitive to salinity [18,19]. This study may shed further light on PH biostimulants and may further the argument for using such products in agriculture under suboptimal conditions.

6.2. Results

6.2.1. Lettuce growth and morphometric parameters

The impact of salinity and the biostimulant treatments on the growth of lettuce plants can be seen in **Table 1.** Overall, salinity significantly impacted all studied parameters save for the leaf number data. In particular, leaf area decreased by 14.0% and fresh weight by 23.3%, whereas leaf dry matter increased by 18.7%.

Biostimulant treatments significantly increased the shoot fresh weight when averaged across nutrient solution (NS) conditions. Data show the Trainer biostimulant being the most effective, as plants treated with this formulation recorded an 8.9% increase when compared to the untreated control, and 4.1% when compared to the Vegamin treatment; the latter also showed 4.6% higher figures compared to the control.

Leaf area was affected by the interaction between the biostimulant and the NS treatment; biostimulant-derived differences in the 0 mM NaCl group were deemed non-significant compared to the control. On the contrary, in salt conditions, both the Trainer and Vegamin treatments managed to increase this parameter by 5.3% on average.

Table 1. Yield and yield parameters of lettuce plants as affected by salinity and biostimulant application.

Source of variance	Leaf number (no. plant^{-1})	Leaf area (cm^2)	Shoot fresh weight (g plant^{-1})	Leaf dry matter (%)
Salinity (S; mM NaCl)				
0	34.7 ± 0.3	2357 ± 28 a	313.5 ± 3.3 a	5.29 ± 0.05 b
30	34.2 ± 0.4	2026 ± 37 b	240.5 ± 5.0 b	6.28 ± 0.03 a
t-test	ns	***	***	***
Biostimulant (B)				
Control	34.3 ± 0.5	2116 ± 72 b	265.1 ± 17.5 c	5.82 ± 0.22
Trainer	34.2 ± 0.1	2251 ± 60 a	288.7 ± 14.4 a	5.81 ± 0.24
Vegamin	34.8 ± 0.5	2207 ± 103 ab	277.2 ± 17.8 b	5.73 ± 0.22
	ns	*	***	ns
S × B				
0 × Control	35.3 ± 0.6	2276 ± 12 ab	303.1 ± 4.7	5.34 ± 0.13
0 × Trainer	34.0 ± 0.2	2356 ± 50 a	320.7 ± 3.1	5.28 ± 0.11
0 × Vegamin	34.8 ± 0.6	2438 ± 10 a	316.7 ± 3.9	5.24 ± 0.02
30 × Control	33.4 ± 0.2	1957 ± 27 e	227.1 ± 8.1	6.29 ± 0.04
30 × Trainer	34.3 ± 0.2	2147 ± 69 bc	256.7 ± 1.1	6.33 ± 0.02
30 × Vegamin	34.9 ± 0.9	1976 ± 7.8 cd	237.7 ± 3.8	6.23 ± 0.08
	ns	*	ns	ns

All data are expressed as mean ± standard error, n = 3. ns, *, *** non-significant or significant at $p \leq 0.05$ and 0.001, respectively. Nutrient solution dosage means were compared by t-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

6.2.2. Leaf photosynthetic and biochemical parameters

The impact of salinity and the biostimulant treatments on the studied photosynthetic and biochemical parameters of lettuce plants can be seen in **Table 2.1** and **Table 2.2**. Again, the impact of the salinity treatment was present across the board. Lower leaf stomatal conductance (-15,8%), transpiration (-13.9%) and thus higher intrinsic water use efficiency (18.7%) were denoted under salinity.

However, when biostimulants enter the picture, a S × B interaction was found only in the leaf CO2 assimilation rate. The application of the different biostimulants did not engender a significant increase in 0 mM NaCl condition, whereas both biostimulants under salinity (30 mM NaCl) increased A_{CO2} by 24.6% on average. When averaged across the NS, Vegamin application increased g_s by 18.8% and Trainer application increased WUEi by 13.5% compared to the untreated control (0 mM NaCl).

Table 2 Photosynthetic parameters of lettuce plants as affected by salinity and biostimulant application.

Source of Variance	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	g_s (mol H_2O m^{-2} s^{-1})	E (mol H_2O m^{-2} s^{-1})	WUEi (μmol CO_2 mol H_2O^{-1})	Proline (mM 100 g^{-1} FW)	MDA (μM 100 g^{-1} FW)	H_2O_2 (mM 100 g^{-1} FW)
Salinity (S; mM NaCl)							
0	17.73 ± 0.28	0.19 ± 0.00 a	3.96 ± 0.07 a	4.49 ± 0.12 b	19.4 ± 0.78 b	0.95 ± 0.04 b	4.66 ± 0.16 b
30	17.94 ± 0.74	0.16 ± 0.01 b	3.41 ± 0.14 b	5.33 ± 0.26 a	45.5 ± 2.28 a	1.05 ± 0.06 a	7.09 ± 0.22 a
t-test	ns	**	**	**	***	**	***
Biostimulant (B)							
Control	16.33 ± 0.44 b	0.16 ± 0.01 b	3.56 ± 0.23	4.66 ± 0.27 b	38.09 ± 7.26 a	1.17 ± 0.05 a	6.29 ± 0.66
Trainer	19.04 ± 0.38 a	0.17 ± 0.01 ab	3.65 ± 0.17	5.29 ± 0.33 a	29.29 ± 5.02 b	0.96 ± 0.04 b	5.66 ± 0.44
Vegamin	18.18 ± 0.43 a	0.19 ± 0.01 a	3.85 ± 0.13	4.66 ± 0.20 b	29.97 ± 5.35 b	0.87 ± 0.03 b	5.67 ± 0.62
	***	*	ns	*	***	***	ns
S × B							
0 × Control	17.13 ± 0.27 cd	0.18 ± 0.00	3.98 ± 0.06	4.31 ± 0.13	21.90 ± 0.94 c	1.08 ± 0.04 b	4.94 ± 0.28
0 × Trainer	18.57 ± 0.52 abc	0.19 ± 0.00	3.96 ± 0.12	4.71 ± 0.27	18.06 ± 0.38 c	0.88 ± 0.02 c	4.72 ± 0.21
0 × Vegamin	17.50 ± 0.18 bcd	0.19 ± 0.01	3.95 ± 0.19	4.45 ± 0.19	18.24 ± 1.22 c	0.89 ± 0.03 c	4.31 ± 0.27
30 × Control	15.53 ± 0.49 d	0.14 ± 0.00	3.15 ± 0.29	5.01 ± 0.47	54.29 ± 0.72 a	1.26 ± 0.03 a	7.64 ± 0.52
30 × Trainer	19.51 ± 0.49 a	0.16 ± 0.02	3.34 ± 0.17	5.87 ± 0.35	40.52 ± 0.11 b	1.04 ± 0.02 b	6.6 ± 0.19
30 × Vegamin	19.2 ± 0.01 ab	0.19 ± 0.02	3.74 ± 0.20	4.98 ± 0.33	41.70 ± 1.96 b	0.86 ± 0.05 c	7.04 ± 0.03
	**	Ns	ns	ns	**	*	ns

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nutrient solution dosage means were compared by t test. Biostimulant and S × B means were compared by two-way ANOVA. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). ACO2, CO2 net assimilation rate, gs, stomatal conductance, E, transpiration, WUEi, intrinsic water use efficiency.

All studied biochemical parameters significantly increased when lettuce plants were treated with the high salt NS treatment (Table 2). Our results showed higher proline (+134.5%), MDA (+10.5%) and H2O2 (52.1%) contents under salinity stress (30 mM NaCl).

Save for the peroxide content, our results denote how the S × B interaction modulated the content of these stress markers in lettuce leaves. When proline under high salinity is considered, both biostimulants significantly decreased its contents by 24.3% on average compared to the untreated high salt control. The results also show that MDA contents were significantly lowered in both NS conditions by the studied biostimulant treatments: on average, both Trainer and Vegamin decreased this membrane oxidation parameter by 18.1% in the control condition. When salinity condition is considered, Vegamin treatment lowered MDA contents by 31.7 and 17.3% when compared to both the untreated control and the Trainer biostimulant and brought this parameter down to 0 mM NaCl × biostimulants level.

6.2.3. Leaf mineral contents

The impact of salinity and the biostimulant treatments on the accumulation of leaf minerals can be seen in **Table 3.** Salinity condition impacted plant nutrient accumulation when sulphur (-7.9%), calcium (-23.1%) and magnesium (-13.8%) are considered. Sodium (+271.5%) and chloride (+125.7%) contents were understandably affected, particularly evident in the Na/K ratio, which increased three-fold due to sodium accumulation.

When considering the biostimulant treatments, Trainer effectively raised calcium and magnesium contents by 19.7 and 13.8%, respectively, when averaged across the NS treatments. When looking at S × B interactions, both biostimulants managed to decrease sodium accumulation compared to the salinity control by 37.2% on average, thus decreasing the Na/K ratio by 30.4%, whereas these values were unchanged in 0 mM NaCl condition.

Table 3. Mineral contents of lettuce leaves as affected by salinity and biostimulant application.

Source of variance	Total N (mg g^{-1} DW)	P (mg g^{-1} DW)	K (mg g^{-1} DW)	S (mg g^{-1} DW)	Ca (mg g^{-1} DW)	Mg (mg g^{-1} DW)	Na (mg g^{-1} DW)	Cl (mg g^{-1} DW)	Na/K ratio
Salinity (S; mM NaCl)									
0	32.85 ± 0.32	4.52 ± 0.12	49.12 ± 1.72	0.76 ± 0.01 a	2.86 ± 0.09 a	1.89 ± 0.05 a	2.67 ± 0.25 b	8.74 ± 0.22 b	0.06 ± 0.01 b
30	32.84 ± 0.34	4.81 ± 0.13	53.59 ± 1.53	0.70 ± 0.02 b	2.20 ± 0.12 b	1.63 ± 0.04 b	9.92 ± 0.90 a	19.73 ± 0.87 a	0.18 ± 0.01 a
t-test	ns	ns	ns	*	**	***	***	***	***
Biostimulant (B)									
Control	32.2 ± 0.45	4.52 ± 0.15	50.72 ± 2.81	0.70 ± 0.02	2.33 ± 0.21 b	1.67 ± 0.04 b	7.91 ± 2.37 a	15.13 ± 2.72	0.15 ± 0.04 a
Trainer	33.3 ± 0.36	4.89 ± 0.17	52.80 ± 1.68	0.75 ± 0.02	2.79 ± 0.16 a	1.90 ± 0.07 a	5.47 ± 1.43 b	14.41 ± 2.89	0.10 ± 0.03 b
Vegamin	33.04 ± 0.25	4.59 ± 0.12	50.84 ± 2.02	0.74 ± 0.03	2.38 ± 0.17 b	1.68 ± 0.09 b	6.08 ± 1.31 b	14.06 ± 2.14	0.12 ± 0.03 ab
	ns	ns	ns	ns	*	**	**	ns	**
S × B									
0 × Control	32.13 ± 0.85	4.25 ± 0.16	44.50 ± 0.65 b	0.73 ± 0.02	2.79 ± 0.02	1.76 ± 0.03	2.62 ± 0.27 c	9.15 ± 0.34	0.06 ± 0.01 c
0 × Trainer	33.27 ± 0.23	4.67 ± 0.21	51.57 ± 2.60 ab	0.78 ± 0.02	3.04 ± 0.11	2.03 ± 0.05	2.40 ± 0.63 c	8.26 ± 0.28	0.05 ± 0.01 c
0 × Vegamin	33.13 ± 0.26	4.70 ± 0.12	52.38 ± 3.15 ab	0.78 ± 0.03	2.68 ± 0.34	1.89 ± 0.07	3.14 ± 0.24 c	8.84 ± 0.39	0.06 ± 0.01 c
30 × Control	32.27 ± 0.52	4.78 ± 0.10	56.94 ± 0.47 a	0.66 ± 0.03	1.87 ± 0.11	1.58 ± 0.03	13.19 ± 0.46 a	21.11 ± 1.05	0.23 ± 0.01 a
30 × Trainer	33.32 ± 0.77	5.11 ± 0.24	54.03 ± 2.43 ab	0.72 ± 0.03	2.53 ± 0.23	1.76 ± 0.07	8.54 ± 0.66 b	20.55 ± 2.01	0.16 ± 0.01 b
30 × Vegamin	32.95 ± 0.47	4.53 ± 0.19	49.81 ± 3.01 ab	0.71 ± 0.03	2.19 ± 0.06	1.54 ± 0.03	8.04 ± 0.96 b	17.53 ± 0.37	0.16 ± 0.01 b
	ns	ns	*	ns	ns	ns	**	ns	**

All data are expressed as mean ± standard error, n = 3. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nutrient solution dosage means were compared by t-Test. Different letters within each column indicate significant differences according to Tukey's HSD (p = 0.05).

6.2.4. Leaf pigment content and antioxidant activity

The impact of salinity and the biostimulant treatments on the accumulation of leaf pigments and antioxidant activity can be seen in **Table 4.** A general across-the-board increase in β-carotene content and antioxidant activity was noted in salinity treatment.

However, when the biostimulants are considered, interaction data shows that the Trainer biostimulant was the only treatment which increased lutein content by 45.1% in the salinity condition compared to the untreated control. In contrast, in 0 mM NaCl condition no effect could be observed. The data showed no significant differences across NS treatments between the biostimulant treatments and the untreated control in the case of β-carotene. However, differences were deemed significant between Trainer and Vegamin (-19%). In addition, β-carotene increased under salinity by 30.3%.

When averaged across NS treatments, all the considered antioxidant assay data were significantly affected by the PH treatments, and the Trainer formulation yields the highest results in all cases. DPPH, ABTS and FRAP data showed increases by Trainer and Vegamin of 30.6, 32.5, 58,2% and 19.5, 32.5 and 29.5%, respectively, compared to the untreated control. These antioxidant assays were boosted by salinity stress (30 mM NaCl) by 9.31, 11.5 and 12.2% for DPPH, ABTS and FRAP, respectively.

Table 4. Auxiliary pigment content and antioxidant activity of lettuce leaves as affected by salinity and biostimulant application.

Source of variance	Lutein (mg kg^{-1} DW)	β-carotene (mg kg^{-1} DW)	DPPH (mmol Trolox kg^{-1} DW)	ABTS (mmol Trolox kg^{-1} DW)	FRAP (mmol Trolox kg^{-1} DW)
Salinity (S; mM NaCl)					
0	427.8 ± 11.2 b	219.5 ± 8.1 b	33.71 ± 1.58 b	43.60 ± 2.33 b	41.05 ± 2.80 b
30	595.6 ± 39.3 a	286.0 ± 11.3 a	36.85 ± 1.22 a	48.62 ± 3.68 a	46.05 ± 2.95 a
t-test	***	***	***	*	***
Biostimulant (B)					
Control	474.4 ± 19.7 b	254.3 ± 12.7 ab	30.23 ± 1.18 c	35.91 ± 1.31 c	33.70 ± 1.27 c
Trainer	585.9 ± 72.1 a	278.4 ± 20.5 a	39.49 ± 0.63 a	54.84 ± 2.70 a	53.31 ± 1.65 a
Vegamin	474.7 ± 31.4 b	225.6 ± 16.3 b	36.13 ± 0.71 b	47.57 ± 1.94 b	43.64 ± 0.87 b
	**	**	***	***	***
S × B					
0 × Control	436.5 ± 17.3 b	227.6 ± 7.7	27.83 ± 0.57	34.69 ± 0.69	30.89 ± 0.18
0 × Trainer	428.6 ± 27.0 b	234.9 ± 14.6	38.30 ± 0.36	49.77 ± 0.75	50.12 ± 0.54
0 × Vegamin	418.3 ± 20.1 b	196.1 ± 9.8	35.00 ± 0.99	46.32 ± 1.24	42.14 ± 0.91
30 × Control	512.3 ± 14.1 b	281.0 ± 6.2	32.63 ± 0.94	37.13 ± 2.58	36.50 ± 0.42
30 × Trainer	743.2 ± 22.5 a	321.8 ± 2.4	40.68 ± 0.67	59.91 ± 3.17	56.50 ± 1.78
30 × Vegamin	531.1 ± 36.8 b	255.2 ± 19.2	37.25 ± 0.56	48.82 ± 3.97	45.14 ± 0.83
	**	ns	ns	ns	ns

All data are expressed as mean ± standard error, $n = 3$. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nutrient solution dosage means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

6.2.5. Leaf Polyphenolic Contents

The impact of salinity and the biostimulant treatments on the modulation of leaf polyphenolic contents can be seen in **Table 5** and **Table 6.** The salinity treatment increased the concentration of the assayed phenolic acids and flavonoids.

Chlorogenic acid was largely the most represented phenolic acids, accounting for 93.9% of the total phenolic acids content when both nutrient solution conditions are averaged, and its concentration increased 12.6% in salt stress. Save for ferulic acid, which was unaffected by all treatments, all the studied phenolic acid compounds accumulated in response to biostimulant applications. Trainer and Vegamin increased chlorogenic acid concentration by 39.1% when averaged across NS treatments, and this increase was largely responsible for the differences in the total amount of phenolic acids. The second abundant components among the assayed phenolic acids were the sinapic acid conjugates (represented as synapoyl-hexose), which also showed an S × B interaction. In salinity condition, the 30 mM NaCl × Trainer treatment showed the highest concentration of these compounds when compared to both Vegamin (+24.5%) and the untreated control (+71.4%). In comparison, in 0 mM NaCl condition, both biostimulants engendered no significant effects when compared to the control. A similar trend was shown regarding coumaric acid esters (represented as coumaroyl-diglucoside) and disinapoylgentobiose, which the Trainer-treated plants accumulated the most across NS treatments, +69.6 and +50.0% when compared to the untreated control, respectively.

Flavonoids data showed very similar outcomes. When the S × B interaction data of the total flavonoids content is considered, Trainer induced the highest accumulation in both low salt (+136.2% and 33.1%) and salt conditions (+67.0% and +23.0%) compared to both the untreated control and Vegamin, respectively. The principal driver of the flavonoid profile was quercetin-3-glucoside, which accumulated at most in the leaves of Trainer-treated plants. Trainer induced a 2.46-fold accumulation in control conditions compared to the untread plants, and a 69.2% increase in stress conditions. When Vegamin is considered, the increases were 77.0% and 35.3%, respectively. Both isorhamnetin 3-rutinoside and kaempferol 3-glucoside showed S × B interactions; the data showed that biostimulants increased the amount of these compounds in both stress and non-stress conditions. The formulations yielded similarly higher concentrations in control conditions; however, Trainer elicited the highest accumulation in high salinity levels (59.5 vs 33.3%, and 53.2 vs 30.2%, Trainer vs Vegamin, respectively, compared to the untreated control). When averaged across NS treatments, Kaempferol 3,7-diglucoside was down-accumulated (-32.7%, on average) in biostimulant-treated plants, with no significant differences among the biostimulants. Lastly, strong, 4,3- and 1.3-fold increases in rutin concentration were noted as a mean effect of biostimulant and salinity treatments, respectively

Table 5. The modulation of the leaf content of phenolic acids as affected by salinity and biostimulant application.

Source of variance	Chlorogenic acid (mg kg^{-1} DW)	Coumaroyl-diglucoside (mg kg^{-1} DW)	Disinapoylgentobiose (mg kg^{-1} DW)	Ferulic acid (mg kg^{-1} DW)	Synapoyl-hexose (mg kg^{-1} DW)	Total Phenolic Acids (mg kg^{-1} DW)
Salinity (S; mM NaCl)						
0	1278 ± 86 b	1.51 ± 0.1 b	0.39 ± 0.02 b	37.67 ± 4.18	46.54 ± 1.79	1364 ± 89 b
30	1439 ± 77 a	1.88 ± 0.16 a	0.44 ± 0.03 a	39.87 ± 2.87	48.98 ± 3.82	1530 ± 80 a
t-test	*	***	**	ns	ns	*
Biostimulant (B)						
Control	1078 ± 57 b	1.25 ± 0.05 c	0.32 ± 0.02 c	33.59 ± 2.23	39.63 ± 2.63 c	1153 ± 57 b
Trainer	1551 ± 65 a	2.12 ± 0.15 a	0.48 ± 0.02 a	43.7 ± 4.87	56.17 ± 2.6 a	1654 ± 68 a
Vegamin	1447 ± 61 a	1.72 ± 0.09 b	0.43 ± 0.00 b	39.02 ± 4.84	47.48 ± 1.56 b	1536 ± 58 a
	***	***	***	ns	***	***
S × B						
0 × Control	953 ± 5.9	1.16 ± 0.05	0.30 ± 0.00	28.69 ± 0.65	43.35 ± 4.32 bc	1026 ± 3.6
0 × Trainer	1472 ± 53	1.81 ± 0.07	0.43 ± 0.00	45.53 ± 9.98	50.77 ± 1.98 ab	1571 ± 63.2
0 × Vegamin	1410 ± 72	1.57 ± 0.11	0.42 ± 0.00	38.78 ± 6.11	45.5 ± 1.04 bc	1497 ± 69.4
30 × Control	1203 ± 19	1.34 ± 0.05	0.34 ± 0.03	38.5 ± 0.63	35.91 ± 1.4 c	1280 ± 20.7
30 × Trainer	1631 ± 110	2.43 ± 0.06	0.53 ± 0.01	41.86 ± 3.95	61.56 ± 0.89 a	1737 ± 111
30 × Vegamin	1483 ± 111	1.86 ± 0.10	0.44 ± 0.00	39.26 ± 8.93	49.46 ± 2.69 b	1574 ± 102
	ns	ns	ns	ns	*	ns

All data are expressed as mean ± standard error, $n = 3$. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nutrient solution dosage means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

Table 6. The modulation of the leaf content of flavonoids as affected by salinity and biostimulant application.

Source of variance	Isorhamnetin 3-rutinoside (mg kg^{-1} DW)	Kaempferol 3,7-diglucoside (mg kg^{-1} DW)	Kaempferol 3-glucoside (mg kg^{-1} DW)	Quercetin 3-glucoside (mg kg^{-1} DW)	Rutin (mg kg^{-1} DW)	Total Flavonoids (mg kg^{-1} DW)
Salt (S; mM NaCl)						
0	0.37 ± 0.05 b	0.33 ± 0.02 b	1.21 ± 0.10 b	10.31 ± 1.27 b	0.55 ± 0.1 b	12.77 ± 1.49 b
30	0.55 ± 0.04 a	0.48 ± 0.04 a	1.39 ± 0.09 a	12.10 ± 0.93 a	0.72 ± 0.12 a	15.25 ± 1.12 a
t-test	***	***	***	***	***	***
Biostimulant (B)						
Control	0.29 ± 0.06 c	0.52 ± 0.05 a	0.95 ± 0.06 c	7.44 ± 0.72 c	0.20 ± 0.03 b	9.41 ± 0.9 c
Trainer	0.58 ± 0.04 a	0.38 ± 0.03 b	1.56 ± 0.05 a	14.88 ± 0.35 a	0.90 ± 0.05 a	18.3 ± 0.42 a
Vegamin	0.50 ± 0.03 b	0.32 ± 0.04 b	1.40 ± 0.03 b	11.3 ± 0.50 b	0.81 ± 0.06 a	14.33 ± 0.58 b
	***	***	***	***	***	***
S × B						
0 × Control	0.17 ± 0.04 d	0.41 ± 0.02	0.81 ± 0.03 d	5.91 ± 0.47 d	0.15 ± 0.01	7.46 ± 0.48 d
0 × Trainer	0.49 ± 0.01 bc	0.32 ± 0.01	1.45 ± 0.02 b	14.57 ± 0.39 a	0.79 ± 0.04	17.62 ± 0.37 a
0 × Vegamin	0.45 ± 0.02 c	0.25 ± 0.01	1.38 ± 0.04 b	10.46 ± 0.51 bc	0.70 ± 0.02	13.24 ± 0.44 c
30 × Control	0.42 ± 0.01 c	0.63 ± 0.02	1.09 ± 0.02 c	8.97 ± 0.09 c	0.25 ± 0.04	11.36 ± 0.14 c
30 × Trainer	0.67 ± 0.02 a	0.44 ± 0.01	1.67 ± 0.02 a	15.18 ± 0.6 a	1.01 ± 0.01	18.97 ± 0.56 a
30 × Vegamin	0.56 ± 0.02 b	0.39 ± 0.04	1.42 ± 0.04 b	12.14 ± 0.55 b	0.91 ± 0.09	15.42 ± 0.56 b
	*	ns	**	*	ns	*

All data are expressed as mean ± standard error, $n = 3$. ns, *, **, *** non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Nutrient solution dosage means were compared by *t*-Test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$).

6.2.6. Principal Component Analysis

To provide a summary of the changes in the morphological, physiological and metabolic traces left by the application of both the salt stress and PHs, a principal component analysis (PCA) was carried out, which separated the treatments based on the traits associated with them. The principal components (PCs) 1 and 2 (Figure 1) explained 84.7% of the total variance and were both associated with eigenvalues higher than 1. PC1 explained 50.5% of the total variance and was positively correlated with higher phosphorous, potassium and sodium chloride contents, salt-stress markers such as H_2O_2, MDA and proline, and auxiliary pigments such as β-carotene and lutein. PC1 was negatively correlated with shoot fresh weight (SFW), leaf area, photosynthetic parameters such as stomatal conductance (gs), transpiration (E), and sulfur, magnesium and calcium contents. The second principal component (PC2) explained 34.2% of the variance and was correlated with higher CO_2 assimilation (A_{CO2}), leaf nitrogen content and antioxidative markers including ABTS, FRAP, DPPH, and total flavonoids (TFLA), while being negatively correlated with the membrane oxidation parameter MDA. As it is visible from the PCA biplot, there is separation from the studied treatments based on both salt and biostimulant combination. In particular, the left quadrant shows the presence of both Trainer and Vegamin treatments in control conditions. These treatments were associated with higher fresh weight, sulfur calcium and magnesium contents. The upper right quadrant shows both biostimulant treatments in salt-stress conditions. In particular, the 30 × Trainer treatment was associated with the increase in antioxidant markers, auxiliary pigments and phosphorous and potassium contents. Both the control treatments sit opposite from their respective biostimulant counterparts, in the lower left and lower right quadrants.

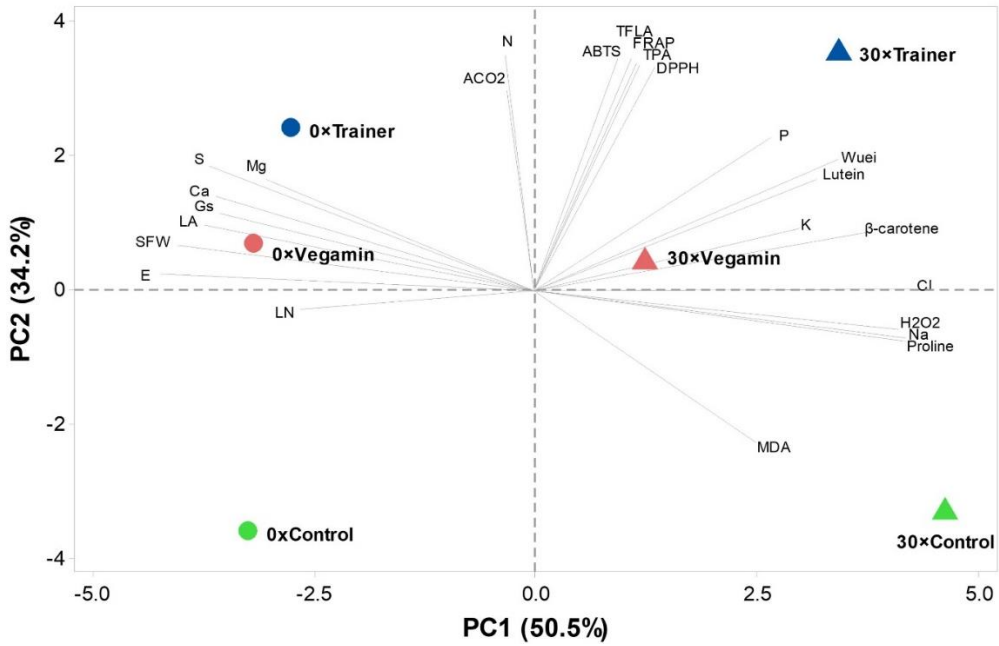

Figure 1. Principal component loading plot and scores of the principal component analysis (PCA) on biometric (leaf number (LN), leaf area (LA), shoot fresh weight (SFW)) physiological and biochemical (carbon dioxide accumulation (A_{CO2}), stomatal conductance (g_s), transpiration (E), proline, MDA and H_2O_2), mineral content (N, P, K, S, Ca, Mg, Na, Cl), auxiliary pigments (β-carotene and lutein), antioxidant activity (DPPH, ABTS, FRAP), total phenolic acids (TPA) and flavonoid contents (TFLA) of lettuce plants as modulated by salt (0, 30 mM) and biostimulant ("Trainer", "Vegamin") applications.

6.3. Discussion

This work aimed to test the performance of two vegetal-based PH biostimulants to provide an understanding of how they can manage to improve growth performance in salt-stressed lettuce plants. It also aimed to delineate their dissimilarities using mor-pho-physiological, biochemical measurements and metabolomics. We found the recorded growth increases across nutrient solution conditions to align with PH literature on leafy vegetables under stress (salt, low nutrient availability) and non-stress conditions [10]. In more practical terms, the 8.9 and 4.6% average increase in fresh weight recorded by the Trainer and Vegamin biostimulants in this work can be quantified in 3.3 and 1.7 T ha−1 of marketable lettuce biomass, which could prove to be economically advantageous.

However, as lettuce leaf area is a proxy for the whole plant growth, we found no difference between the two treatments in the control NS treatment. To explain the difference in marketable yield of the two biostimulants, we first need to preface that this study showed further confirmation that the leaf number parameter is under genotypical control and, at least for this cultivar, not steered by biostimulant effects as confirmed by

previous research [18]. Furthermore, we found on average that leaf dry matter percentage, and water content in turn, remained equal across nutrient solution treatments.

Based on the results of our research, we can confirm the different mechanisms through which the used biostimulants ameliorated plant stress and boosted plant growth in control conditions, which were highlighted in previous research [10,14,20]. Firstly, we found an increased photosynthetic output in stress and non-stress conditions; this has been described as the plant metabolism stimulation by bio-effectors in the products such as signaling peptides [9]. Due to hormone-like effects, these compounds effectively prime plants to perform better in stress and non-stress conditions by impacting enzymes related to both carbon and nitrogen metabolism [9,18]. Additionally, we found an across-the-board improvement in plant nutrition and cell homeostasis parameters, exemplified by the decrease in proline content, the decrease in sodium content, which translated into lower Na+/K+ ratios, and the Trainer-specific increase in leaf calcium and magnesium. The regulation of cellular osmotic balance is a key stress-averting strategy that plants employ to fight off salt stress [21]. To adjust to the increased osmotic pressure due to media salinity, plants produce a variety of molecules such as proline and soluble sugars, deemed compatible solutes, which accumulate in tissues. In general, the combination of the production of such solutes and the accumulation of cell-wall-strengthening molecules such as lignin in salt stress results in in-creased plant dry weights [22,23], consistent with what has been recorded in our trial.

Biostimulants are known to induce an accumulation of proline in tissues subjected to osmotic stresses such as drought and salinity as a way to favor osmotic homeostasis [10] and fight off oxidative stresses [24]. However, our results show an opposite trend, contrasting what was previously obtained on salt-stressed lettuce treated with the Trainer biostimulant [13]. In their study, Lucini and collaborators [13] found no significant differences in the amount of proline in the leaves of biostimulant-treated plants grown in a saline environment compared to the untreated control. Effectively, our results show a better adaptation to the saline environment by biostimulant-treated plants, as exemplified by the lower Na contents and Na+/K+ ratio, thus providing the lower proline content. This could be explained by various factors, including biostimulant-mediated root growth and genotype strategies for salt resistance.

PH biostimulants have a proven ability to increase root growth through an aux-in-like effect [13,16], and while root expansion was not evaluated in this trail, it is safe to assume that higher absorbing area in the lower substrate horizons could effectively decrease salt uptake, as higher concentrations tend to be in the upper layers due to evaporation/transpiration. However, this does not completely elucidate how sodium, and not chloride, was reduced in tissues of the treated plants. A further explanation may come from how plants reduce sodium accumulation in the shoot. Aside from vacuole

sequestering, which would have manifested in the mineral analyses, sodium ions can be transported to the phloem and later, to the roots, by high-affinity potassium transporters (HKTs), and then expelled to the substrate via the salt overly sensitive (SOS) pathway [25]. This hypothesis is validated by numerous instances of biostimulant studies in the literature, whereby after the application of these substances, there was an increase in the expression of HKTs and sodium antiporters in the SOS pathway [10,26,27]. Lastly, we did not find a decrease in leaf potassium contents, which can be expected due to the ionic affinity of K+ and Na+ [21], and has been found in a recent study on lettuce plants subjected to salt stress [22]. This may be due to genotype-specific strategies, which may minimize the leaking of potassium and decrease sodium import from the substrate via the endodermis [25]. While there is no confirming evidence of this phenomenon occurring, a previous study on the same lettuce cultivar showed high phenotypical plasticity in stress conditions, namely high irradiance and heat [28].

A third stress-ameliorating mechanism manifested in this trial was the biostimulant-mediated induction of oxidative-stress defense mechanisms. Reactive oxygen species, or ROS, are formed during photosynthesis due to the salinity-derived water stresses, leading to increased hydrogen peroxide and the peroxidation of membrane lipids, disrupting metabolism [29]. ROS-derived damage can be averted by combining enzymatic and non-enzymatic systems, including auxiliary pigments (carotenoids, anthocyanins) and polyphenolics. Plant carotenoids such as β-carotene and lutein are plastid-bound pigments that serve as auxiliary pigments, as they absorb light energy and then transfer it to chlorophylls. They also work by scavenging ROS, dissipating excess light energy by generating heat, and they protect cellular membranes by reacting with lipid peroxidation reaction products, thus ending oxidative chain reactions [24,30]. Our results showed that the Trainer biostimulant provided the highest lutein contents in saline conditions compared to the untreated control and the Vegamin treatment. Lutein is a key element to photosystem II protection via the xanthophyll cycle [31], which suggests better photo-oxidative protection after applying the Trainer formulation.

Polyphenolics are a class of molecules that stem from the central phenylpropanoid pathway and serve as plant growth regulators and stress-response molecules [32]. Their function, among others, is to donate electrons to peroxidases for H2O2 detoxification, thus acting like antioxidants, and are involved in the mechanical strengthening tissues to enhance the resistance to water deficit [33]. When looking at the phenolic acid assays, both Trainer and Vegamin biostimulants increased chlorogenic acid, which is the most present phenolic acid in lettuce, in accordance with previous studies in the literature, which found similar increases [34,35]. However, when the leaf flavonoids are considered, we found quercetin-3-glucoside or cyanidin 3-glucoside, and anthocyanin [36] contents to be increased in both control and salt conditions. Due to their antioxidant

activity, anthocyanins are particularly useful to plants in stressful conditions [37], and in this case, it furthers the case of Trainer being the most successful biostimulant in eliciting a plant-stress averting response.

The principal component analysis, other than providing a visual summary of the effect that the two products had on the studied features, is an effective tool to delineate their mode of action, as previous PH research shows [38]. In both control and salt conditions, the two tested biostimulants sit opposite their untreated counterparts, yet the Trainer biostimulant is associated with higher values, especially when photosynthetic and antioxidant activities are considered. This could be due to a variety of factors, but it could be safe to assume that they all stem from the product composition. As the Trainer biostimulant contains over double the nitrogen content, it could be inferred that some of the active ingredients may be actually more concentrated in this product, when com-pared to the Vegamin formulation. As further proof, recent research of the Vegamin biostimulant has shown that by splitting the product in its molecular fractions, especially in the <1 kDa molecular weight class, it is possible to increase some aspects related to their bioactivity, especially the ones related to oxidative stress defense [39]. This is in line with the theory surrounding the inner workings of PH biostimulants, which sees in the low molecular weight peptides the key to their action [10]. Furthermore, FRAP analysis conducted on the products shows stark distinction between the two in terms of the antioxidant power of the formulations, which may also suggest a higher capacity of the Trainer biostimulant in helping plants against the accumulation of ROS [40].

Overall, our results delineate a scenario of better protection against stresses provided by the Trainer biostimulant, which can compound to a better plant physiological state and thus the recorded higher shoot fresh weight.

6.4. Materials and Methods

6.4.1. Growth Conditions, Experimental Design and Plant Material

The greenhouse trial started on 22 March 2021 (DAT 1, or the day after transplant) and ended on 29 April 2021, for a total of 39 days. The experiment was carried out in an unheated greenhouse at the Department of Agricultural Sciences of the University of Naples "Federico II" (40°48′ N, 14°20′ E, 29 m.s.l.). Three-true leaf stage seedlings of *Lactuca sativa* L. cv. "Maravilla De Verano Canasta", or "Canasta", were transplanted into 1.6 L plastic pots containing a 90:10 (*v*/*v*) mixture of 3 mm quartz sand (Vaga, Sabbie e Ghiaie Silicee, Costa de'Nobili (PV) Italy) and perlite, respectively. The pots were arranged in a configuration consisting of four 35 × 20 cm double rows; thus, the planting density was set at 14 plants m^{-2}. The double rows were set at a 50 cm distance.

The experimental design consisted of a split plot system, whereby each of the two couples of double rows was assigned to a tank which contained a base nutrient solution (NS) or a NS to which sodium chloride was supplied.

The composition of the base NS was: 8 mM nitrate, 1.5 mM phosphorus, 4 mM potassium, 4 mM calcium, 2.5 mM sulfur, 1.25 mM magnesium, 20 μM iron, 9 μM manganese, 0.3 μM copper, 1.6 μM zinc, 20 μM boron and 0.3 μM molybdenum. The base NS had an electrical conductivity of 1.6 dS m^{-1}, whereas the addition of 30 mM of NaCl created the salinity NS treatment (4.4 dS m^{-1}). The pH of the solutions was monitored and kept at 5.8 ± 0.2 with a portable pH meter (HI 991301, Hanna Instruments Italia S.R.L., Ronchi di Villafranca Padovana (PD), Italy).

The biostimulant (B) subfactor consisted of two biostimulant treatments and an untreated control, which were arranged inside the NS plots in a randomized complete block system with three replicates. Each B replicate was composed of five lettuce plants, for a total of 15 plants per biostimulant treatment, per NS plot.

6.4.2. Biostimulant Treatments

The PH biostimulants chosen for this trial were Vegamin and Trainer (Hello Nature Italia S.R.L., Rivoli Veronese (VR), Italy), both made from vegetal sources, and consisting of mixtures of amino acids and soluble peptides [38]. Quantitative analysis of both products, in accordance to Sorrentino and collaborators [41], show carbon and nitrogen contents to be (carbon) 17.6 and 17.2%, and (nitrogen) 5 and 2.2% for Trainer and Vegamin, respectively.

The amino-acidic content of the Trainer formulation has been described in a previous work by Paul and collaborators [12] as (g kg−1 product): Ala (12), Arg (19), Asp (33), Cys (4), Glu (54), Gly (13), His (8), Ile (12), Leu (24), Lys (19), Met (4), Phe (16), Pro (15), Ser (17), Thr (11), Trp (4), Tyr (13), and Val (16). Analogous analyses were performed on the Vegamin biostimulant, which yielded an aminogram comprising (g kg−1 product): Ala (7), Arg (10), Asp (18), Cys (1), Glu (33), Gly (6), His (4), Ile (5), Leu (8), Lys (9), Met (1), Phe (6), Pro (9), Ser (5), Thr (6), Trp (1), Tyr (3) and Val (5).

Both products were also subjected to further analysis to determine the fer-ric-reducing antioxidant power (FRAP) and total phenolic and flavonoid contents in accordance to Paul and collaborators' [12] work, and were quantified as: (Trainer) 41.9 mmol Fe2+ g−1 f.w., 8.93 mg of gallic acid equivalent per gram of fresh product and 0.95 mg of quercetin equivalent per gram of fresh product, (Vegamin) 1.32 mM Fe2+ g−1 fresh products, 1.52 mg gallic acid equivalent g−1 fresh product and 0.23 mg quercetin equivalent g−1 fresh product.

Both biostimulants do not contain phytohormones, as previous research shows [41,42]. Foliar applications of the biostimulants, both at a rate of 3 mL formulation L−1

solution, were made using 10 L steel-bottle sprayers, which were tested for spraying volume consistency. Applications of the biostimulants were carried out in order to provide a uniform coat of the products on all leaf surfaces.

A total of five treatments were applied during the experiment, starting from the day after transplant (DAT) 10 and once per week.

4.3. Yield, Growth Assessment, Leaf Area Measurement and Sampling

At the end of the experiment (DAT 39), three plants per experimental unit were chosen for fresh weight measurements, which included leaf number, leaf area and shoot fresh weight, and later for dry matter analyses.

Leaf area measurements were carried out via leaf photography and later quantification using the ImageJ v1.52a software (U.S. National Institutes of Health, Bethesda, MD, USA) and expressed in cm^2.

After the fresh weight measurements, all plant matter was dried in a forced-convection oven at 60 °C until a constant weight was reached. After the drying step, leaf dry matter percentage (DM%) was quantified as:

$$\mathrm{DM\%} = \frac{\text{Leaf dry weight}}{\text{Leaf fresh weight}} \times 100 \quad (1)$$

The obtained dry matter was further processed using a grinding mill (MF10.1 model, IKA-Werke GmbH & Co. KG, Staufen, Germany) for leaf mineral content determination.

A pool of four leaves from two plants per experimental replica was immediately quenched in liquid nitrogen and later stored at −80 °C to determine leaf proline and oxidative stress markers (malondialdehyde or MDA, and hydrogen peroxide or H_2O_2). A further set of fresh samples were stored at −20 °C and later freeze-dried using a model Alpha 1–4 lyophilizer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), for the determination of leaf auxiliary pigments, antioxidant activity (DPPH, ABTS, FRAP), and polyphenolic contents.

6.4.4. Leaf gas exchange and biochemistry parameters

Leaf gas exchange measurements were carried out on 28 April 2021 (DAT 38) on healthy, young and fully expanded leaves, using an LCi T compact photosynthesis system (ADC Bioscientific Ltd., Herts, UK), equipped with a broad-leaf chamber and a programmable LED light. Photosyntetic photon flux density (PPFD) inside the chamber was set as 1000 μmol m^{-2} s^{-1}, and airflow as 200 mL s^{-1}; both relative humidity and CO_2 concentration were kept at ambient levels. The data recorded included CO_2 net assimilation rate (A_{CO2}; μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}) and transpiration (E; mmol H2O m^{-2} s^{-1}). A fourth derived measurement, instantaneous water use efficiency or WUEi was calculated as:

$$\mathrm{WUEi} = \frac{\mathrm{ACO2}}{\mathrm{E}}$$

Leaf proline, MDA and H_2O_2 measurements were carried out using analogous methods described by Kumar and collaborators in their previous research [36]. In brief, proline content was determined on 0.5 g of fresh tissue in three steps, namely a homogenization step in sulfosalicylic acid, a reaction in a mixture of 50:50 (*v*/*v*) acid-ninhydrin and glacial acetic acid, and final spectrophotometric determination of the toluene-extracted proline at 520 nm. Proline measurements were quantified as mg proline g^{-1} fresh weight (FW).

Leaf MDA concentration was also determined on fresh tissue after homogenization in 0.1% trichloroacetic acid (TCA), centrifugation and reaction with thiobarbituric acid to form a 532 nm chromophore. The absorbance was recorded at 532 and 600 nm and MDA concentration was calculated as the difference in absorbance values. MDA measurements are quantified as nmol MDA g^{-1} FW.

Lastly, hydrogen peroxide (H_2O_2) measurements were carried out on TCA-homogenized tissues after adding 10 mM K-phosphate buffer (pH 7.0) and 1 M potassium iodide. Absorbance was measured at 390 nm against an H_2O_2 standard, and the measurements were quantified as μmol H_2O_2 g^{-1} FW.

6.4.5. Leaf Total Nitrogen and Mineral Analysis

The total leaf nitrogen assay was conducted on dry leaf samples using the Kjeldahl method after mineralization with sulfuric acid and potassium sulfate - copper sulfate catalyst, as described in previous works [37,38].

A further set of minerals, namely P, K, S, Ca, Mg, Na and Cl contents were determined using the ICS-3000 ion chromatography system (Dionex, Sunnyvale, CA, USA) after water extraction of dry sample matter in an 80 °C heated bath for 10 minutes. After separation using the IonPac AS11-HC and IonPac CS12A analytical columns, the amount of minerals was quantified against analytical standards as described in previous work [15]. All leaf mineral contents are expressed as mg g^{-1} dry weight (DW).

6.4.6. Leaf Carotenoid Contents, Antioxidant Activity

Leaf lutein and β-carotene determinations assays were done using 100 mg of lyophilized leaf matter. As described by Kyriacou and collaborators [39], a first sample extraction was performed in ethanol – 0.1% BHT mixture, and a later saponification step was employed using KOH. Pigment extraction was carried out in n-hexane which was then evaporated in a nitrogen flow. Thereafter, 1ml of chloroform was added to the dry residue and the mixture was separated using a Shimadzu Model LC 10 chromatographer (Shimadzu, Osaka, Japan) equipped with a reverse phase 250 × 4.6 mm, 5 μm Gemini C18 column (Phenomenex, Torrance, CA, USA) as described by Kyriacou and collaborators [40]. Carotenoid contents were quantified as mg kg^{-1} DW.

The spectrophotometric determination of the DPPH, ABTS and FRAP antioxidant activities was obtained on lyophilized samples following the protocols described in detail by Formisano and collaborators [41]. For ABTS, 100μL from a 1:10 dilution of sample material in 70% methanol was added to 1mL of ABTS solution, and the 734 nm absorbance was recorded after 2.5 minutes. Similarly, DPPH results were obtained by adding 200 μL of the extract to 1mL of DPPH solution; samples were incubated at ambient temperature for 10 minutes and their 517 nm absorbance was recorded. Lastly, the ferric reducing antioxidant power (FRAP) data was obtained by mixing 150 μL of the methanolic extract with 2.85 mL of FRAP solution. Samples were incubated for 4 minutes after which the 593 nm absorbance was read. All antioxidant activity results were expressed as mmol Trolox equivalents kg^{-1} DW.

6.4.7. Leaf Polyphenolic Contents

The leaf polyphenolic assay was performed analogously to the protocol followed by Kyriacou and collaborators [39]. Extraction was carried out on 100 mg of lyophilized leaf sample in 5 mL of a 60:40 v/v methanol/water solution. Phenolics separation was obtained via UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA), equipped 1.7 μm Biphenyl (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA). Mass spectrometry data were obtained via a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). All polyphenolic data are expressed as mg kg^{-1} DW.

6.4.8. Statistical Analysis

Morpho-physiological and biochemical parameter data were analyzed with the SPSS 28 software package (IBM, Armonk, NY, USA) and are presented as mean ± standard error, n = 3. Data were first tested in order to meet the assumption of normality and homogeneity of variance using the Shapiro–Wilk and Levene tests, after which the mean effects were subjected to two-way (salinity level × biostimulant) ANOVA analy-sis. A t-test was employed to compare the salinity mean effect, and Tukey's HSD post hoc test was employed after a significant ANOVA test to separate both biostimulants mean effect and salinity × biostimulant interaction. All tests were deemed significant at $p = 0.05$. Principal component analysis (PCA) was performed on the studied parameters using the Minitab® 18 software (Minitab LLC, State College, PA, USA), and the PCA biplot was obtained through the same software.

6.5. Chapter 6 Conclusions

As we put two commercial PH biostimulants to the test against salt stress, we found an increase in yield of 8.9 and 4.6% by the Trainer and Vegamin biostimulants compared to the untreated control. We found that both biostimulants successfully managed to mitigate the salinity stress by modulating ion homeostasis parameters, which manifested in a decreased sodium accumulation and thus lower proline accumulation in the currently applied salt condition. This effect, coupled with the increase in photosynthesis parameters, has compounded the growth increases observed on the current genotype. In conclusion, we found confirmation that the effects of PH biostimulants on the physio-chemical and metabolic performance of lettuce plants are formulation-dependent, yet both the tested products provided increased plant growth in stress conditions, which can prove profitable in similar conditions. Deeper investigation on finer details of plant-stress response unveiled by this research in relation to the ap-plication of the PH biostimulants, such as increased root growth in salt stress conditions, root and shoot metabolic modulation and altered molecular pathways to fend off this particular stress, is warranted. A combined targeted metabolomic/transcriptomic approach may shed some more light on the inner workings of this product category.

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Chapter 7

A Graminaceae-derived Protein Hydrolysate and its Fractions Provide Differential Growth and Modulate Qualitative Traits in Lettuce Grown under Low and Mild Salinity Conditions

Abstract: The modulation of plant secondary metabolism by the manipulation of nutrient solution (NS) electrical conductivity and biostimulant application has the potential to increase crop growth and the content of bioactive compounds, thus ameliorating the qualitative attributes of food vegetables. A Graminaceae-derived protein hydrolysate (PH) and its molecular fractions PH1 (>10 kDa), PH2 (1 < x <10 kDa) and PH3 (<1 kDa) were applied on lettuce grown in a soilless system and irrigated with two levels of NaCl in the NS (0 and 30 mM). The different PH fractions provided distinct responses, with PH2 in saline conditions increasing lettuce fresh weight by 10.48%, and decreased Na, Cl, malate and citrate concentrations in the leaves by 37.24%, 15.45%,31.4% and 33.73% compared to control. Similar results were achieved by PH3. However, PH, PH2 and PH3 increased total flavonoids in salinity conditions, while PH and PH3 increased total phenolic acids in both salinity conditions. At the same time, the 3 fractions increased lutein in both conditions. PH fractions should be casted, under tailored conditions, to achieve the desired growth and modulation of qualitative attributes. Nonetheless, omic approaches should be further recommended to support and clarify the mechanisms of these fractions.

7.1 Introduction

Nutrition is a mechanism about how nutrients are utilized via an array of biochemical pathways with the purpose of growth, development, and sustenance. Food provides nourishment and derives consequentially from plants, of which numerous are considered vegetables, with more than 65% of the world population relying on a vegetarian diet [1]. Nowadays, purchasers are more conscious of fresh product consumption and decent health interrelationship [2,3] with fresh fruits and vegetables as a backbone of balanced nutrition [4] and providing minerals, fibers and phytochemicals [2,4,5] Vegetables enclose treasured food ingredients implemented to enhance and reconstruct the body. The diversity of phytochemicals present in vegetables includes various classes, among which we mention pigments (chlorophylls, carotenoids and beta alanine) and phenolic compounds [2,5]. These latter encompass phenolic acids, flavonoids, etc.., that correspond to almost 90% of the dietary polyphenols [5]. In particular, leafy vegetables boost antioxidant activity and guard against oxidative stress, with few studies suggesting the transformation of nitrate content of these vegetables into vasodilator-tissue protective secondary compounds that lower blood pressure, thus supporting cardiopulmonary function [2]. A strong correlation is found between fruits

and vegetables plentiful diets and decreased risk of chronic diseases [3,5,6], attributing this advantageous aftermath to plants' secondary metabolites [3]. Moreover, nutrient-rich vegetables consumption is a path to beat malnutrition [7].

Plant reaction to abiotic stresses is species-specific and can provoke modulations in the physiology and metabolism of plants, depending on the duration of the stress and its intensity and the phenological stage [8,9], in addition to manifesting a visual modification of the final product [8]. Abiotic stresses such as salinity, drought and abnormal temperatures can have impacts on crop production and its quality [10,11]. However, eustressors as stressful factors of chemical, physical or biological origin, when applied to plants, can trigger signaling pathways guiding to an increased content of bioactive compounds of plant products [8]. Such application of positive stress like mild or moderate salinity drift plant metabolism and trigger the biosynthesis of bioactive secondary metabolites, which in turn ameliorate the qualitative attributes of food vegetables [8,9,12]; thus considered a cost-effective approach to manipulate the phytochemical content and producing highly valued products [8]. Several studies on leafy vegetables subjected to salinity demonstrated an increase in secondary metabolites in red lettuce [12,13] and *Cichorium spinosum* [14].

Plant secondary metabolites, as a key component in the network of interaction between plants and the environment, are evidently engaged in plant response to abiotic stresses [15]. Their accumulation is stimulated by biostimulant application which helps to counteract stress negative effect by the increment of antioxidant compounds that assure a decrease in plant stress sensitivity [10,15,16] with considerable results registered amid the application of protein hydrolysates of vegetable origin on lettuce in counteracting salinity effects [15–17]. Biostimulants boost primary metabolism through increased photosynthetic activity, and more importantly, they boost the second metabolism by eliciting specific biosynthetic pathways and thus increasing the nutritional quality of the edible parts of plants [15]. Several mechanisms are induced when biostimulants are implicated in mitigating stress negative effects, such as physiological, biochemical, molecular, and anatomical alterations, other than accelerating the antioxidative machinery to scavenge reactive oxygen species (ROS) [11].

The Biostimulants market is expanding promptly, which necessitates innovative products that meet the evolving needs of agriculture, accompanied by development and research to make the products more effective and sustainable. Biostimulants can be generated from agro-industrial by-products and food waste that are abundant in bioactive compounds and are of great significance to agriculture [15,18,19]. Such implication of by-products to produce biostimulants illustrates an advanced circular economy strategy and reduces inadvertent disposal and leads to environmental-friendly solutions [18]. Different research has shed light on the use of plant biomass from

different botanical families (Graminaceae, Malvaceae, Brassicaceae, Fabaceae, etc.) as a protein source for the production of protein hydrolysates (PH) and their effectiveness in stress tolerance to abiotic stresses [20,21]. Notwithstanding, to our knowledge, very few research has dealt with the comprehension of the bioactive fractions involved in the biostimulant activity of plant PH, which can lead to significant improvements in the production of more effective plant biostimulants such as the research of Cristofano and collaborators [17] on Fabaceae-derived PH fractions applied on lettuce and Lucini and collaborators [22] who applied also used Fabaceae-derived PH fractions on tomato plants, both in different nitrogen conditions. Therefore, the aim of this current research is to shed light on a new promising source of PH such as the graminaceae-derived matrix, together with the derived molecular fractions. The biostimulant activity is tested in terms of the ability to cope with saline stress, including the activity of the different molecular weight fractions, using lettuce as a model crop for leafy vegetables.

7.2 Materials and Methods

7.2.1 Experimental setup and design

A greenhouse experiment was set up on 22 March 2021 for a total of 39 days. The experiment was carried out in an unheated greenhouse at the Department of Agricultural Sciences of the University of Naples Federico II. The seedlings of *Lactuca sativa* L. cv. "Maravilla De Verano Canasta", were transplanted into plastic pots (1.6 L) filled with a 90:10 (*v*/*v*) mixture of 3 mm quartz sand (Vaga, Sabbie e Ghiaie Silicee, Costa de'Nobili (PV) Italy) and perlite, respectively. The planting density consisted of 14 plants m^{-2}. A split-plot experimental design was adopted, with the level of salinity in the nutrient solution (NS) as the main factor (2 levels: 0 and 30 mM NaCl), and the sub-factor as the biostimulant treatment (5 levels: a Graminaceae-based protein hydrolysate (PH), its three fractions and an untreated Control). Inside the main blocks, the biostimulant treatments were organized in a randomized block design with 3 replicates. Each replicate consisted of five plants.

The NS consisted of the following macro and micronutrients: 8 mM nitrate, 1.5 mM phosphorus, 4 mM potassium, 4 mM calcium, 2.5 mM sulfur, 1.25 mM magnesium, 20 μM iron, 9 μM manganese, 0.3 μM copper, 1.6 μM zinc, 20 μM boron and 0.3 μM molybdenum. The NS exempt of NaCl had an electrical conductivity (EC) of 1.6 ± 0.1 dS m^{-1}, while the NS with 30 mM NaCl had an EC of 4.4 ± 0.1 dS m^{-1}. The pH of both NS was set at 5.8 ± 0.2. Each NS was prepared in a 500 L plastic tank containing (0.04 dS m^{-1}) osmotic water. The tanks were equipped with a submerged pump and a drip irrigation system (2 L/h dripper $plant^{-1}$).

7.2.2 Protein hydrolysates treatments

The vegetal-origin biostimulant chosen for this trial was a Graminaceae-derived PH consisting of a mixture of amino acids and soluble peptides. This PH was characterized

by 5.20% N (32.5% amino acids and peptides), 18.9% total carbon, and had an aminogram as follows: Ala (9.34), Arg (3.60), Asn (5.94), Asp (0.96), Gln (0.29), Glu (6.50), Gly (6.12), His (1.54), Ile (1.27), Leu (9.25), Lys (1.38), Met (1.16), Orn (2.97), Phe (3.29), Pro (5.97), Ser (17.54), Thr (2.92), Trp (0.29), Tyr (2.71), and Val (3.40); in addition, total amino acids (AA; 86.83), minor AA (27.90) and branched chained AA (13.93), expressed in μmol mL^{-1}. As for the minerals and organic acids analysis, it revealed the following: Na^+ (47.14), NH_4^+ (60.26) K^+ (12.39), Mg^{2+} (52.92), Ca^{2+} (29.36), Cl^- (10.37), NO_2^- (2.37), NO_3^- (0.66), SO_4^{2-} (83.38), acetate (118.84), and malate (0.65), expressed in μmol mL^{-1}. As recommended, the foliar application of the Graminaceae-derived Biostimulant (PH), was done at a rate of 3 mL L^{-1} solution via a steel-bottle sprayer. A total of five treatments were applied during the experiment. The first treatment was done 10 days after the transplant, and the following were repeated weekly. PH fractionation and nitrogen content analysis were carried out according to the methodology employed by **Lucini and coworkers (2020**). The fractionation process consisted of two steps. First, the >10 kDa and <10 kDa fractions were obtained via the use of centrifuge filtering tubes (Amicon Ultra 15, Merck KGaA, Darmstadt, Germany). Second, after the use of 0.5-1 molecular cut-off (MWCO) cellulose acetate membranes (VWR, Milan, Italy), the <1kDa and >1kDa <10kDa fractions were obtained. In brief, biostimulants were separated into three fractions: <1 kDa, hereby called PH3, 1–10 kDa or PH2, and >10k Da or PH1. The Fractions PH1, PH2, PH3 had the following N% 0.19, 0.12 and 0.05, respectively, and the following C% 0.73, 0.76 and 0.51, respectively. The fractions PH1, PH2 and PH3 were diluted in a way to meet the same N% of PH.

7.2.3 Sampling and biometric measurements

At the end of the experiment, three plants per experimental unit were chosen for fresh weight measurement. After the fresh weight measurements, all plant matter was dried in a forced-convection oven at 60°C until a constant weight was reached. The obtained dry matter was further processed using a grinding mill (MF10.1 model, IKA-Werke GmbH & Co. KG, Staufen, Germany) to be used for leaf mineral content determination and leaf organic acids.

A pool of eight leaves from two plants per experimental unit was immediately quenched in liquid nitrogen and later stored at -80 °C for the determination of carotenoids, chlorophylls, total ascorbic acid and polyphenols.

7.2.4 Leaf mineral and organic acids analysis

Leaf minerals, namely NO_3, P, K, S, Ca, Mg, Na and Cl, and leaf organic acids (malate and citrate) were determined using the ICS-3000 ion chromatography system (Dionex, Sunnyvale, CA, USA) after water extraction of 0.250 g dry sample matter in an 80 °C heated bath for 10 minutes. After separation using the IonPac AS11-HC and IonPac CS12A analytical columns, the minerals were quantified against analytical standards as

described in detail in the previous work of Rouphael and collaborators [23]. All leaf minerals and organic acids were expressed as mg 100 g^{-1} fresh weight (fw), based on the samples dry matter percentage.

7.2.5 Lutein, β-carotene, total chlorophylls and total ascorbic acid analysis

Leaf lutein and β-carotene determinations assays were done using 100 mg of lyophilized leaf matter. As described by Kyriacou and collaborators [24], a first sample extraction was performed in 0.1% BHT in ethanol, followed by a saponification step using KOH. Pigment extraction was performed in *n*-hexane, which was later evaporated under a nitrogen atmosphere. Afterwards, 1 mL of chloroform was added to this residue, and the mixture was separated through a reverse Phase-HPLC-DAD using a Shimadzu Model LC 10 chromatographer (Shimadzu, Osaka, Japan) equipped with a 250 × 4.6 mm, 5 μm Gemini C18 column (Phenomenex, Torrance, CA, USA). The absorbance of the eluent was measured at 450 nm. Authentic lutein and β-carotene were used to evaluate their quantity in the sample based on external calibration curves ranging 5–100 μg mL^{-1}, including a minimum of six levels of concentration. Lutein and β-carotene were quantified as μg g^{-1} dw and then expressed as mg 100 g^{-1} fw.

Total chlorophylls (chlorophylls a and b) were assessed spectrophotometrically on 0.5 g of fresh leaves, after extraction with ammoniacal acetone, according to the method described by Wellburn[25] at 662 and 647 nm. Total chlorophylls were expressed as mg 100 g^{-1} fw.

The total ascorbic acid, defined as the sum of ascorbic and dehydroascorbic acids, was assessed by spectrophotometric assays based on the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and the spectrophotometric detection of Fe^{2+} complexes with 2,2-dipyridyl [26]. Dehydroascorbate was first reduced to ascorbic acid by pre-incubating the sample in dithiothreitol. Quantification was performed at 525 nm against an external ascorbate standard calibration curve in the range of 5 – 100 μmol mL^{-1} and the results were expressed as mg 100 g^{-1} fw.

7.2.6 Phenolic acids and flavonoids analysis

The leaf polyphenolic assay was performed analogously to the protocol followed by Kyriacou and collaborators [24]. Briefly, plant extraction was carried out on 100 mg of lyophilized leaf sample in 5 mL of a 60:40 *v*/*v* methanol/water solution. Phenolics separation was obtained via a UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a 1.7 μm Biphenyl (100 × 2.1 mm) column (Phenomenex, Torrance, CA, USA). Mass spectrometry data were obtained via a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA). An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative ion mode (ESI-) for all the analyzed compounds was implemented. The accuracy and calibration of the Q Exactive Orbitrap LC–MS/MS were monitored daily via a manufacturer-recommended reference standard

mixture. Data analysis and processing were done using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific). The instrument calibration was checked daily using a reference standard mixture obtained from Thermo Fisher Scientific. All polyphenolic data were quantified as μg g^{-1} dw and then expressed as μg 100 g^{-1} fw.

7.2.7 Statistics and heatmap

The data were analyzed with the SPSS 28 software package (IBM, Armonk, NY, USA) and are presented as mean ± standard error, n = 3. The mean effects were subjected to two-way ANOVA analysis (Salinity level × biostimulant). A *t*-test was employed to compare the mean effect of the salinity level, and Tukey's HSD test was performed to separate both the biostimulant mean effect and salinity × biostimulant interaction. All tests were deemed significant at $p = 0.05$. A hierarchical cluster analysis (HC) on the mineral and phytochemical composition was performed, and a heatmap was generated using the ClustVis online tool [27]. Matrix values were normalized as ln (x + 1), with Euclidean distance and complete linkage.

7.3 Results and Discussion

7.3.1 Lettuce shoot fresh weight

Lettuce shoot fresh weight as illustrated in **Figure 1** was influenced by the interaction of the factors tested (Salinity × Biostimulant)**.** The application of the Graminaceae-derived Protein Hydrolysate (PH) and its fractions in 0 mM NaCl condition did not engender significant changes in lettuce shoot fresh weight when compared to the Control, whereas PH3 treated plants had a significant 5.35% higher weight than PH and PH1 (>10 kDa) treated plants. On the other hand, in 30 mM NaCl condition, the application of PH2 (1 < x <10 kDa) caused a significant 10.48% increase compared to the Control (30mM NaCl). In general, lettuce fresh weight was decreased by 26.42% when the salinity level of 30 mM of NaCl in the nutrient solution (NS) was taken into account in comparison to the absence of salinity.

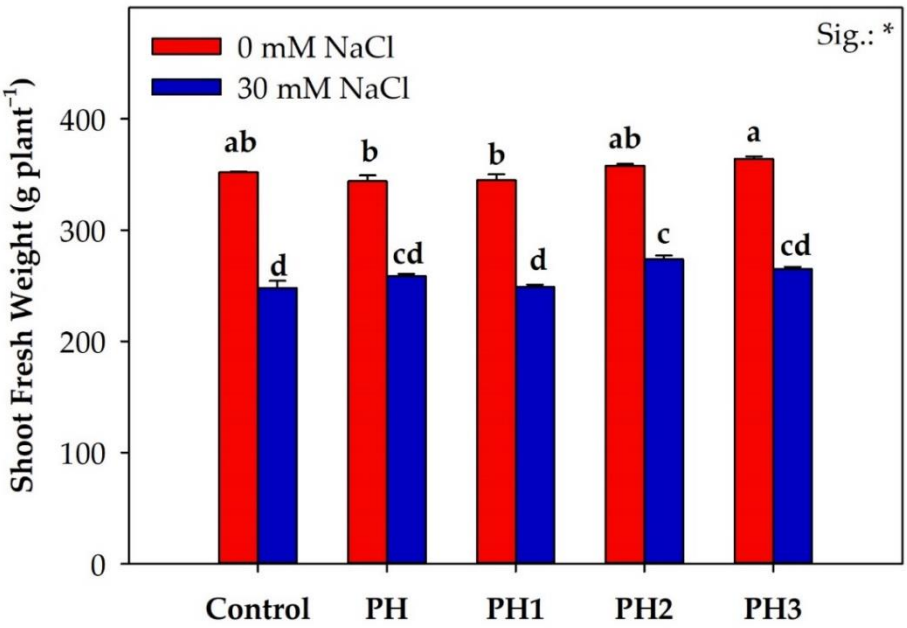

Figure 1. Lettuce shoot fresh weight as influenced by NaCl level (0 and 30 mM) in the nutrient solution and biostimulant application (PH, PH1, PH2 and PH3). All data are expressed as mean ± standard error, $n = 3$. Factors interaction significance $p \leq 0.05$ (*). Different letters above the bars indicate significant differences according to Tukey's HSD test, performed at $p = 0.05$. PH: Graminaceae-derived protein hydrolysate, molecular fraction PH1(>10 kDa), PH2 (1 < x <10 kDa) and PH3 (<1 kDa).

The decrease in lettuce fresh weight due to salt stress is in line with several research in the literature [3,17,28–31]. In fact, a decrease in biomass production in such conditions is common feedback of glycophytes [32] Dissolved salts close to plant's roots can limit growth and downsize biomass production since water uptake by plants is reduced as a result of the osmotic effect [9], in addition to ion toxicity and nutrient imbalance happening in cells cytoplasm [10]. Such excessive presence of salts in plant tissues other than altering nutrient balance can easily affect plant photosynthesis and thus growth [9]. On the other hand, biostimulants are known to have valuable outcomes on plant development, yield and stress resistance [16,33]. Indeed, Sabatino and collaborators [4], who sprayed a legume-derive protein hydrolysate on the same lettuce cultivar grown in a greenhouse, had a significant increase in head fresh weight. While under salinity conditions (25 and 40 mM NaCl, respectively), a decrease in growth suppression was noted on lettuce when Lucini and coworkers [32] applied through foliar and/or drench application and when Rouphael and coworkers [31] applied through substrate

incorporated legume-derived granules, which was similar to our case when lettuce plants were sprayed with Graminaceae-derived fraction PH2 in saline conditions. Although deemed non-significant, PH3 fraction increased fresh weight by 6.85% compared to the Control, and its effect was not significantly different than PH2. Whereas, in 0 mM NaCl conditions, the application of PH and its fractions did not engender significant increases. Such differences can be explained by the efficacy of biostimulants in sub-optimal conditions being more evident. Moreover, evident increases due to biostimulants were noticed in the last two experiments [31,32] that were done in the Autumn season, whereas our experiment was done in Spring, thus better light conditions for growth which could mask to a certain level the efficiency of biostimulants when applied.

7.3.2 Nitrate content, mineral profile and organic acids

The lettuce leaf mineral profile was assessed as listed in **table 1.** Leaf nitrate, together with leaf S and Ca, was only influenced by the main effect of the salinity in the NS. Under salinity stress, nitrate and S increased by 42.38 and 10.52%, respectively, while Ca decreased by 13.55%. Leaf P and K were both influenced by the main effect of the experimental factors. 30 mM NaCl in the NS increased P and K concentrations by 31.93 and 37.98%, respectively. PH3-treated plants had a significantly lower P concentration compared to Control and PH-treated plants, while PH2-treated plants had a lower K concentration in comparison to PH-treated plants. As for Leaf Na, Cl, malate and citrate, an interaction among the factors tested was obvious. While under 0 mM NaCl the application of the protein hydrolysates did not cause any significant differences among the means of the treatments, a different trend was noted under 30 mM NaCl in the NS. Indeed, Na and Cl concentrations were clearly reduced by PH2 and PH3, around 31.70 and 14.90% on average when compared to their respective Control. As for malate and citrate, only PH2 was able to reduce significantly by 31.40 and 33.73%, respectively, these concentrations in comparison to the Control.

Analyzed minerals in lettuce grown in saline conditions (25 mM NaCl) showed a decrease of all elements (N, P, K, Ca and Mg) when expressed on a dry weight basis [32]. Equally, lettuce in a different experiment in saline conditions (40 mM NaCl), exhibited a decrease in leaf elements (N, P, S, K, Ca and Mg[31]). K in a different experiment consisting of green and red lettuce cultivars with increasing salinity levels, showed constant concentration in the red cultivar, while the concentration decreased in the green one with salinity surpassing 20 mM NaCl [34]. Previously mentioned experiments were coupled with an expected increase in Na and Cl concentrations in leaves equally to our lettuce plants in similar saline conditions. Under high salinity levels, plants undergo osmotic stress negatively influencing their nutritional composition [11]. Although a reduction of undesired compounds like nitrate accounting in leafy vegetables is feasible under saline conditions [29], nitrate in our case increased under 30 mM NaCl. As

explained by Sa and collaborators [35], nitrogenous compounds accumulation is assumed to assist in osmoprotective processes, protect macromolecular protein structures and alleviate oxidative stress by scavenging reactive oxygen species. In addition, NO_3 uptake and nitrate reductase activity are promoted in certain plants upon NaCl exposure, and NO_3 nutrition can assist some species such as *Pisum sativum* and *Glycine max* adapt to saline conditions.

The lettuce cultivar used in this study indicates a kind of tolerant aspect to salinity since the net photosynthetic rate showed a significant decrease under 30 mM salinity, still the registered values were high (data not shown). Indeed, leaf P, K and S analysis illustrated an increase in these concentrations instead of an expected decrease. Wang and collaborators [36] indicated that saline stress could trigger plants to absorb P and lead to an excessive accumulation. On the other hand, the high K concentration at 30 mM can be explained by the ability of plants to maintain a high cytosolic K/Na ratio that seems to be crucial for salt tolerance, as explained by progresses in plant electrophysiology; This cytosolic ratio can be preserved by loss prevention of K or by accumulation restriction of Na [37]. The same authors suggested that the performance of crops in saline conditions can be enhanced by the exogenous application of compatible solutes via foliar spray, where these solutes can have an indirect adjustment of the cytosol via regulatory or osmoprotective functions. The decrease of Na and Cl under PH2 and PH3 fractions is similar to the result obtained on *Pisum sativum* grown in saline conditions and treated with licorice root extract [38]. These plants exhibited considerable plant biomass increase, coupled with lower Na and Cl accumulation. As a matter of fact, PH2 and PH3 treated lettuce plants resulted in higher fresh weight, with PH2 resulting significantly higher than 30 × Control plants. Concomitantly, leaf malate and citrate were significantly reduced in 30 × PH2 reaching values similar to the 0 mM NaCl treatments. Knowing that plants commonly accumulate soluble organic solutes like free amino acids, proline, organic acids and soluble sugars to maintain cell turgor and secure the activity conformation of enzyme molecules [36]. A list of organic acids like malate, tartrate and citrate may promote human health due to their antioxidative role as metal chelators [39]. In addition, lettuce is rich in macro-nutrients that could assist the human diet. Around 17 key minerals that partake in plant development are conveyed to human nutrition, where they play a role in averting disorders and maintaining body metabolism and homeostasis [40].

Table 1. Minerals and organic acids analysis of *Lactuca sativa* L. grown under two levels of salinity and sprayed with a Graminaceae-based protein hydrolysate (PH) and its three molecular fractions (PH1, PH2 and PH3). All data are expressed as mean ± standard error, n = 3

Source of variance	Leaf NO_3 (mg kg^{-1} fw)	Leaf P (mg 100 g^{-1} fw)	Leaf K (mg 100 g^{-1} fw)	Leaf S (mg 100 g^{-1} fw)	Leaf Ca (mg 100 g^{-1} fw)	Leaf Mg (mg 100 g^{-1} fw)	Leaf Na (mg 100 g^{-1} fw)	Leaf Cl (mg 100 g^{-1} fw)	Leaf malate (mg 100 g^{-1} fw)	Leaf citrate (mg 100 g^{-1} fw)
Nutrient Solution (NS)										
Low Salinity (L)	1109 ± 45.4 b	23.8 ± 0.52 b	258 ± 6.36 b	3.99 ± 0.11 b	15.5 ± 0.72 a	10.5 ± 0.30	12.2 ± 0.79 b	44.8 ± 0.9 b	194 ± 6.62 b	22.1 ± 0.82 b
High Salinity (H)	1579 ± 66.5 a	31.4 ± 0.84 a	356 ± 9.05 a	4.41 ± 0.15 a	13.4 ± 0.74 b	11.1 ± 0.39	68.1 ± 3.97 a	124 ± 3.92 a	228 ± 12.5 a	43.4 ± 2.53 a
t-test	***	***	***	*	*	n.s.	***	***	**	***
Biostimulant (B)										
Control	1375 ± 173	29.5 ± 2.02 a	319 ± 25.3 ab	4.45 ± 0.21	14.3 ± 1.02	11.5 ± 0.57	45.3 ± 14.5 ab	87.4 ± 17.9 a	227 ± 9.89 ab	36.3 ± 6.51 ab
PH	1532 ± 150	28.9 ± 2.62 a	328 ± 30.1 a	4.41 ± 0.29	16.1 ± 0.88	11.4 ± 0.57	47.3 ± 16.2 a	91.6 ± 22.0 a	237 ± 26.8 a	38.9 ± 7.12 a
PH1	1343 ± 112	28.3 ± 1.74 ab	313 ± 21.6 ab	4.24 ± 0.12	14.7 ± 1.84	10.8 ± 0.61	41.9 ± 14.7 ab	85.2 ± 18.4 a	212 ± 11.1 ab	31.1 ± 4.45 ab
PH2	1230 ± 76.3	26.3 ± 1.38 ab	280 ± 13.9 b	3.99 ± 0.08	12.6 ± 1.23	10.1 ± 0.32	33.6 ± 9.79 b	71.6 ± 15.7 b	183 ± 11.1 b	27.5 ± 3.14 b
PH3	1241 ± 133	25.0 ± 1.64 b	296 ± 26.8 ab	3.90 ± 0.32	14.5 ± 0.80	10.5 ± 0.61	36.4 ± 9.48 ab	68.1 ± 15.6 b	197 ± 15.5 ab	30.0 ± 4.79 ab
	n.s.	**	*	n.s.	n.s.	n.s.	**	***	*	**
NS × B										
L×Control	1011 ± 46.1	25.3 ± 1.48	264 ± 14.8	4.23 ± 0.33	15.8 ± 0.97	11.0 ± 0.71	13.0 ± 1.55 d	47.5 ± 2.19 d	211 ± 13.0 ab	22.1 ± 1.80 c
L×PH	1222 ± 114	23.2 ± 1.22	262 ± 13.0	3.81 ± 0.19	16.3 ± 0.52	10.7 ± 0.25	11.6 ± 0.86 d	42.6 ± 2.13 d	181 ± 5.90 ab	23.5 ± 0.29 c
L×PH1	1193 ± 143	24.6 ± 0.74	271 ± 22.6	4.25 ± 0.24	16.7 ± 3.59	10.8 ± 1.33	9.61 ± 0.20 d	44.4 ± 2.78 d	209 ± 23.7 ab	22.8 ± 3.82 c
L×PH2	1087 ± 37.3	23.6 ± 0.78	255 ± 5.12	3.94 ± 0.14	15.2 ± 0.76	10.5 ± 0.28	10.9 ± 1.15 d	46.2 ± 0.41 d	200 ± 10.9 ab	21.7 ± 0.72 c
L×PH3	1034 ± 132	22.4 ± 1.38	240 ± 14.7	3.70 ± 0.33	13.7 ± 0.69	9.65 ± 0.48	15.4 ± 2.43 d	43.2 ± 1.7 d	171 ± 3.59 ab	20.3 ± 1.52 c
H×Control	1740 ± 122	33.7 ± 0.76	373 ± 5.55	4.68 ± 0.26	12.8 ± 1.39	12.1 ± 0.91	77.6 ± 3.57 a	127 ± 3.73 a	242 ± 9.32 ab	50.4 ± 2.91 a
H×PH	1841 ± 64.0	34.6 ± 0.39	393 ± 8.63	5.01 ± 0.14	16.0 ± 1.88	12.1 ± 1.03	83.0 ± 5.90 a	141 ± 1.40 a	292 ± 21.4 a	54.4 ± 3.88 a
H×PH1	1492 ± 141	32.0 ± 0.97	355 ± 6.98	4.23 ± 0.11	12.7 ± 0.50	10.7 ± 0.33	74.1 ± 6.83 ab	126 ± 4.51 ab	215 ± 7.03 ab	39.3 ± 4.06 ab
H×PH2	1374 ± 84.2	29.1 ± 1.10	305 ± 17.3	4.04 ± 0.09	10.0 ± 0.54	9.61 ± 0.47	48.7 ± 5.88 c	110 ± 5.97 bc	166 ± 14.8 c	33.4 ± 3.84 bc
H×PH3	1447 ± 168	27.7 ± 2.13	351 ± 16.9	4.10 ± 0.61	15.4 ± 1.42	11.3 ± 0.96	57.3 ± 1.77 bc	106 ± 8.39 c	222 ± 23.0 abc	39.7 ± 4.35 ab
	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	***	**	*

ns, *, **, ***: non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Salinity level means were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). FW: fresh weight. Molecular fractions PH1, PH2 and PH3 (>10 kDa, 1 < x <10 kDa, <1 kDa). L: 0 mM NaCl, H: 30 mM NaCl.

7.3.3 Total chlorophylls, carotenoids, and total ascorbic acid

Total chlorophylls of lettuce leaves were assessed and listed in **Table 2**, showing that only the salinity stress factor (30 mM NaCl) influenced this parameter and increased it by 10.53%. As for lutein, it was influenced by the mean effect of both factors **(Table 2)**. When averaged among biostimulant treatments, lutein increased by 31.48% when 30 mM NaCl was applied in the NS, whereas when averaged among the NS treatments, lutein increased by 36.70% on average when treated with the 3 fractions of PH compared to the control. On the other hand, β-carotene and total ascorbic acid (TAA) were influenced by the interaction of the factors (B × NS). When the NS was exempted from NaCl, the application of the biostimulants did not engender any significant changes in β-carotene. At the same time, in 30 mM NaCl condition, the PH fractions increased it, but it was deemed a significant increase only in the case of PH1. The application of PH and its fractions significantly decreased TAA when no NaCl was added to the NS, whereas in the presence of NaCl, PH and PH3 application decreased it by 1.93-fold and 1.22-fold, respectively.

Table 2. Pigments (Total chlorophylls and carotenoids) and Total ascorbic acid (TAA) of *Lactuca sativa* L. grown under two levels of salinity and sprayed with a Graminaceae-based protein hydrolysate (PH) and its three molecular fractions (PH1, PH2 and PH3). All data are expressed as mean ± standard error, n = 3

Source of variance	Total chlorophylls (mg 100 g^{-1} fw)	TAA (mg 100 g^{-1} fw)	β-carotene (mg 100 g^{-1} fw)	Lutein (mg 100 g^{-1} fw)
Nutrient Solution (NS)				
Low Salinity (L)	119 ± 1.70 b	155 ± 7.39 a	1.37 ± 0.05 b	3.24 ± 0.16 b
High Salinity (H)	133 ± 2.30 a	138 ± 9.71 b	2.21 ± 0.08 a	4.26 ± 0.14 a
t-test	***	***	***	***
Biostimulant (B)				
Control	121 ± 4.49	177 ± 11.3 a	1.61 ± 0.14 bc	2.97 ± 0.23 b
PH	128 ± 4.81	115 ± 16.7 c	1.57 ± 0.15 c	3.57 ± 0.41 ab
PH1	129 ± 5.56	150 ± 7.35 b	1.96 ± 0.28 a	3.85 ± 0.28 a
PH2	130 ± 3.81	126 ± 3.63 c	1.91 ± 0.20 a	4.14 ± 0.22 a
PH3	124 ± 2.38	163 ± 10.9 ab	1.89 ± 0.22 ab	4.21 ± 0.18 a
	n.s.	***	**	***
NS × B				
L×Control	113 ± 3.87	201 ± 7.39 a	1.31 ± 0.04 d	2.46 ± 0.08
L×PH	120 ± 4.44	151 ± 8.30 c	1.25 ± 0.02 d	2.77 ± 0.27
L×PH1	118 ± 0.68	158 ± 10.3 bc	1.34 ± 0.08 d	3.30 ± 0.10
L×PH2	123 ± 2.77	124 ± 2.35 c	1.51 ± 0.17 cd	3.72 ± 0.24
L×PH3	124 ± 4.24	139 ± 4.45 c	1.43 ± 0.13 d	3.93 ± 0.19
H×Control	130 ± 3.26	152 ± 2.38 c	1.92 ± 0.08 bc	3.47 ± 0.13
H×PH	136 ± 5.73	78.7 ± 2.28 d	1.90 ± 0.09 bc	4.36 ± 0.36
H×PH1	141 ± 4.18	143 ± 10.7 c	2.58 ± 0.08 a	4.39 ± 0.30
H×PH2	136 ± 4.96	128 ± 7.57 c	2.30 ± 0.10 ab	4.57 ± 0.05
H×PH3	124 ± 3.22	186 ± 4.33 ab	2.36 ± 0.03 ab	4.5 ± 0.19
	n.s.	***	*	n.s.

ns, *, **, ***: non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Salinity level means were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). fw: fresh weight. Molecular fractions PH1, PH2 and PH3 (>10 kDa, 1 < x <10 kDa, <1 kDa). L: 0 mM NaCl, H: 30 mM NaCl.

TAA exhibited different trends in saline conditions, where in the same experiment in a green lettuce cultivar it remained unchanged, whereas it increased until 20 mM NaCl and then decreased again at 30 mM NaCl in a red cultivar [34]**.** Moreover**,** ascorbic acid was reduced under salinity stress (20 mM NaCl) in green baby lettuce, while remaining equal to the Control in red baby lettuce [41]. In another leafy vegetable (rocket), TAA was also reduced under salinity stress [42]. TAA was similarly reduced under protein hydrolysate application in spinach [43]**.** However, in our research, this metabolite increased only under PH3 fraction × salinity. The synthesis and accumulation of secondary metabolites like TAA and phenols, can be associated with the activity of key enzymes involved directly in phytochemical homeostasis [44]**.** Ascorbic acid is an

essential endogenous component and a water-soluble vitamin that diminishes immunosuppression in humans and scavenges reactive oxygen species, restoring the vascular function and counteracting lipid peroxidative damage [36]. Notwithstanding, ascorbic acid is a crucial detoxifying compound [41].

As for lutein and β-carotene, our results are in line with those registered by Ciriello and coworkers [45] on basil grown in hydroponics in saline conditions (60 mM NaCl) and those registered in Cristofano and collaborators' [17] work on the same lettuce cultivar grown in saline conditions (30 mM NaCl). It was mentioned that salinity might trigger carotenoids, chlorophylls, and tocopherols (lipophilic antioxidant molecules), which safeguard the photosynthetic apparatus of lettuce to dictating lettuce shelf life and nutritional profile [46]**.** Moreover, it was also stated that a high concentration of leaf pigments like carotenoids was noted in rocket, lettuce and endive upon biostimulant treatment [10].

7.3.4 Phenolic acids and Flavonoids

The phenolic acids profile of lettuce plants is listed in **Table 3**. Chlorogenic acid was the most abundant phenolic acid, accounting for 93.50% of the entire profile, followed by synapoyl-hexose and ferulic acid and then by coumaroyl-diglucoside and disinapoylgentiobiose. Phenolic acids were influenced by the interactions of the factors tested (B × NS), noting that both NS and biostimulant factors boosted the levels of the phenolic acids in a significant manner, where for example 30 × Control plants had 56.23% higher concentration of total phenolic acids compared to 0 × Control plants, reaching 8924 μg 100 g^{-1} fresh weight (fw). In the NS exempted from NaCl, the application of PH and its fractions PH1 and PH2 increased the total phenolic acids by 51.41% on average compared to Control. While in 30 mM NaCl condition, only PH and PH3 increased the total phenolic acids significantly by 26.60% on average, when compared to the Control. Noting that among each other the PH and its fractions effect was not significantly different, except 30 × PH1 and 30 × PH3.

Table 3. Phenolic acids of *Lactuca sativa* L. grown under two levels of salinity and sprayed with a Graminaceae-based protein hydrolysate (PH) and its three molecular fractions (PH1, PH2 and PH3). All data are expressed as mean ± standard error, n = 3

Source of variance	Chlorogenic acid (μg 100 g^{-1} fw)	coumaroyl-diglucoside (μg 100 g^{-1} fw)	Disinapoylgentobiose (μg 100 g^{-1} fw)	Ferulic Acid (μg 100 g^{-1} fw)	Synapoyl-hexose (μg 100 g^{-1} fw)	Total Phenolic Acids (μg 100 g^{-1} fw)
Nutrient Solution (NS)						
Low Salinity (L)	7191 ± 357 b	9.95 ± 0.46 b	2.18 ± 0.10 b	242 ± 12.1	242 ± 16.2 b	7688 ± 373 b
High Salinity (H)	9480 ± 304 a	13.5 ± 0.63 a	2.93 ± 0.11 a	278 ± 22.3	313 ± 12.1 a	10087 ± 323 a
t-test	***	***	***	n.s.	***	***
Biostimulant (B)						
Control	6847 ± 698 c	8.30 ± 0.64 b	2.18 ± 0.21 d	252 ± 12.6 ab	208 ± 29.0 d	7318 ± 726 d
PH	9419 ± 574 a	13.3 ± 0.89 a	3.06 ± 0.21 a	248 ± 23.8 ab	334 ± 13.3 a	10018 ± 586 a
PH1	8315 ± 199 ab	12.0 ± 0.51 a	2.49 ± 0.04 bc	236 ± 13.2 b	284 ± 6.63 b	8849 ± 188 bc
PH2	7850 ± 732 bc	12.1 ± 0.75 a	2.29 ± 0.20 cd	229 ± 20.1 b	247 ± 19.5 c	8340 ± 764 cd
PH3	9245 ± 752 a	12.9 ± 1.46 a	2.74 ± 0.25 b	336 ± 45.0 a	315 ± 24.5 a	9911 ± 798 ab
	***	***	***	*	***	***
NS × B						
L×Control	5300 ± 177 d	6.92 ± 0.38 f	1.71 ± 0.08 e	258 ± 25.9 b	145 ± 13.0 e	5712 ± 205 e
L×PH	8276 ± 410 bc	11.4 ± 0.44 cde	2.61 ± 0.08 bc	236 ± 13.4 b	306 ± 0.76 b	8833 ± 424 cd
L×PH1	8117 ± 393 bc	11.1 ± 0.22 de	2.51 ± 0.05 bc	256 ± 17.0 b	294 ± 7.87 bc	8680 ± 377 cd
L×PH2	6357 ± 610 cd	10.6 ± 0.44 de	1.86 ± 0.05 de	206 ± 38.0 b	206 ± 3.08 d	6781 ± 647 de
L×PH3	7904 ± 578 bc	9.74 ± 0.36 e	2.22 ± 0.08 cd	256 ± 39.2 b	260 ± 5.70 c	8432 ± 550 cd
H×Control	8394 ± 111 abc	9.69 ± 0.03 e	2.65 ± 0.04 b	246 ± 9.24 b	272 ± 4.15 bc	8924 ± 118 cd
H×PH	10562 ± 417 a	15.3 ± 0.25 ab	3.52 ± 0.07 a	259 ± 50.2 b	362 ± 10.3 a	11203 ± 366 ab
H×PH1	8513 ± 62.0 abc	12.8 ± 0.69 cd	2.47 ± 0.08 bc	216 ± 13.7 b	274 ± 7.67 bc	9018 ± 77.4 bcd
H×PH2	9343 ± 285 ab	13.5 ± 0.60 bc	2.73 ± 0.05 b	252 ± 6.63 b	288 ± 14.3 bc	9899 ± 266 abc
H×PH3	10586 ± 834 a	16.1 ± 0.70 a	3.27 ± 0.15 a	415 ± 47.3 a	369 ± 1.74 a	11390 ± 835 a
	*	**	***	*	***	**

ns, *, **, ***: non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Salinity level means were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). fw: fresh weight. Molecular fractions PH1, PH2 and PH3 (>10 kDa, 1 < x <10 kDa, <1 kDa). L: 0 mM NaCl, H: 30 mM NaCl.

The different flavonoids analyzed in lettuce leaves are listed in **Table 4**. Quercetin-3-glucoside was the most abundant flavonoid, followed by kaempferol-3-glucoside, rutin, isorhamnetin-3-rutinoside and kaempferol-3-7-diglucoside. All the listed flavonoids were influenced by the interaction NS × B, including total flavonoids. Total flavonoids were significantly increased by 30 mM NaCl application in the NS with 30 × Control causing a 74.55% increment compared to 0 × Control. In the NS exempted from NaCl, the application of PH and its fractions PH1 and PH3 significantly increased total flavonoids compared to the Control. In 30 mM NaCl treatment, PH, PH2 and PH3 application were responsible for the significant increase of total flavonoids compared to the Control. Similarly, saline stress increased phenolics and flavonoids of green and red lettuce grown under different NaCl concentrations [3], and red lettuce grown at 30 mM NaCl [17], as well as total phenolics, total phenolic acid derivatives and total flavonoid derivatives of basil grown in saline conditions (60 mM NaCl; Ciriello et al., 2022). Neocleous and coworkers [41] registered similarly enhanced phenolics under 20 mM NaCl in red baby lettuce, coupled with higher radical scavenging capacity. These authors also mentioned that an initial high concentration of antioxidants in red lettuce safeguards the plants from the detrimental effects of abiotic stresses like salinity. Phenolic compounds are normally produced during normal plant growth [12] and are over-synthesized as a stress response to salinity [3,12]. Such an increase in antioxidants production is triggered in response to salinity, to counterbalance the exalted ROS in cells induced by NaCl[41]. The antioxidative property of phenolics/polyphenols emanate from reactivity as electron or hydrogen donors and chain-breaking function; such an increase in vegetables is deemed of high nutraceutical weight, and the consumption of commodities rich in antioxidant compounds offers beneficial aftermath on human health [41].

On the other hand, the growing interest in PH biostimulants is due to their positive effect on crop performance under environmental stress conditions. This effect was attributed to their ability to activate the primary and secondary metabolisms of plants and the synthesis of the molecules responsible for defense and stress tolerance mechanisms, and many studies have shown that the application of PH increases the content of carotenoids, flavonoids, anthocyanins, and phenolic acids, thus indicating a plant-defense response [28]. Our data on increasing phenols such as chlorogenic acid under biostimulant application is in line with different research on lettuce using legume-derived biostimulants [17,43] and on pepper using alfalfa-derived and red-grape products based biostimulants [44]. Such bioactive compounds increase could be mediated via the modulation of key enzymes, such as chalcone isomerase, connected to flavonone precursors biosynthesis [47].

Table 4. Flavonoids of *Lactuca sativa* L. grown under two levels of salinity and sprayed with a Graminaceae-based protein hydrolysate (PH) and its three molecular fractions (PH1, PH2 and PH3). All data are expressed as mean ± standard error, n = 3

Source of variance	isorhamnetin-3-rutinoside (μg 100 g^{-1} fw)	kaempferol-3-7-diglucoside (μg 100 g^{-1} fw)	Kaempferol-3-glucoside (μg 100 g^{-1} fw)	Quercetin-3-glucoside (μg 100 g^{-1} fw)	Rutin (μg 100 g^{-1} fw)	Total Flavonoids (μg 100 g^{-1} fw)
Nutrient Solution (NS)						
Low Salinity (L)	2.24 ± 0.24 b	1.52 ± 0.1 b	7.00 ± 0.35 b	56.1 ± 3.90 b	3.50 ± 0.21 b	70.3 ± 4.74 b
High Salinity (H)	3.57 ± 0.11 a	2.25 ± 0.13 a	9.78 ± 0.52 a	79.4 ± 4.26 a	4.94 ± 0.22 a	99.9 ± 5.15 a
t-test	***	***	***	***	***	***
Biostimulant (B)						
Control	2.05 ± 0.48 d	1.35 ± 0.21 c	6.23 ± 0.58 d	48.1 ± 5.94 d	3.26 ± 0.42 c	61.0 ± 7.54 d
PH	3.66 ± 0.18 a	2.22 ± 0.18 a	10.4 ± 0.82 a	88.4 ± 4.99 a	5.29 ± 0.32 a	110 ± 6.43 a
PH1	2.98 ± 0.11 b	1.71 ± 0.04 b	7.73 ± 0.06 c	61.5 ± 2.19 c	4.01 ± 0.17 b	77.9 ± 2.45 c
PH2	2.58 ± 0.50 c	1.90 ± 0.26 b	8.07 ± 0.78 c	63.0 ± 7.56 c	3.7 ± 0.36 bc	79.3 ± 9.32 c
PH3	3.26 ± 0.27 b	2.25 ± 0.22 a	9.47 ± 0.97 b	77.6 ± 7.27 b	4.84 ± 0.43 a	97.4 ± 9.07 b
	***	***	***	***	***	***
NS × B						
L×Control	0.97 ± 0.06 f	0.89 ± 0.01 d	4.95 ± 0.12 f	35.2 ± 0.89 f	2.36 ± 0.04 e	44.4 ± 0.83 f
L×PH	3.3 ± 0.13 bcd	1.84 ± 0.01 b	8.69 ± 0.09 bc	77.9 ± 1.82 bc	4.62 ± 0.09 b	96.4 ± 1.84 bc
L×PH1	2.76 ± 0.06 de	1.74 ± 0.01 bc	7.66 ± 0.05 cd	57.8 ± 2.35 de	3.71 ± 0.17 cd	73.7 ± 2.29 de
L×PH2	1.46 ± 0.07 f	1.35 ± 0.06 cd	6.38 ± 0.27 e	47.4 ± 2.26 ef	2.91 ± 0.04 de	59.5 ± 2.26 ef
L×PH3	2.69 ± 0.02 e	1.76 ± 0.02 bc	7.34 ± 0.39 de	61.9 ± 2.45 de	3.89 ± 0.17 bc	77.6 ± 2.31 d
H×Control	3.12 ± 0.02 cde	1.81 ± 0.13 bc	7.51 ± 0.1 cde	60.9 ± 3.25 de	4.16 ± 0.29 bc	77.5 ± 2.97 d
H×PH	4.01 ± 0.16 a	2.59 ± 0.11 a	12.2 ± 0.5 a	98.8 ± 3.49 a	5.96 ± 0.26 a	123.6 ± 4.25 a
H×PH1	3.19 ± 0.13 cde	1.67 ± 0.07 bc	7.8 ± 0.11 cd	65.1 ± 2.30 cd	4.32 ± 0.18 bc	82.1 ± 2.69 cd
H×PH2	3.7 ± 0.12 abc	2.45 ± 0.22 a	9.77 ± 0.28 b	78.6 ± 6.1 bc	4.48 ± 0.15 bc	99.0 ± 6.25 b
H×PH3	3.83 ± 0.22 ab	2.73 ± 0.06 a	11.6 ± 0.06 a	93.3 ± 3.45 ab	5.78 ± 0.02 a	117 ± 3.55 a
	***	***	***	*	*	**

ns, *, **, ***: non-significant or significant at $p \leq 0.05$, 0.01 and 0.001, respectively. Salinity level means were compared by *t*-test. Different letters within each column indicate significant differences according to Tukey's HSD ($p = 0.05$). fw: fresh weight. Molecular fractions PH1, PH2 and PH3 (>10 kDa, 1 < x <10 kDa, <1 kDa). L: 0 mM NaCl, H: 30 mM NaCl.

7.4 Heatmap

In order to summarize the changes in the amount of leaf minerals and phytochemicals due to the application of the Graminaceae-derived biostimulant and its molecular fractions, we conducted a hierarchical clustering analysis (HCA) which has been coupled with a heatmap (Figure 2).

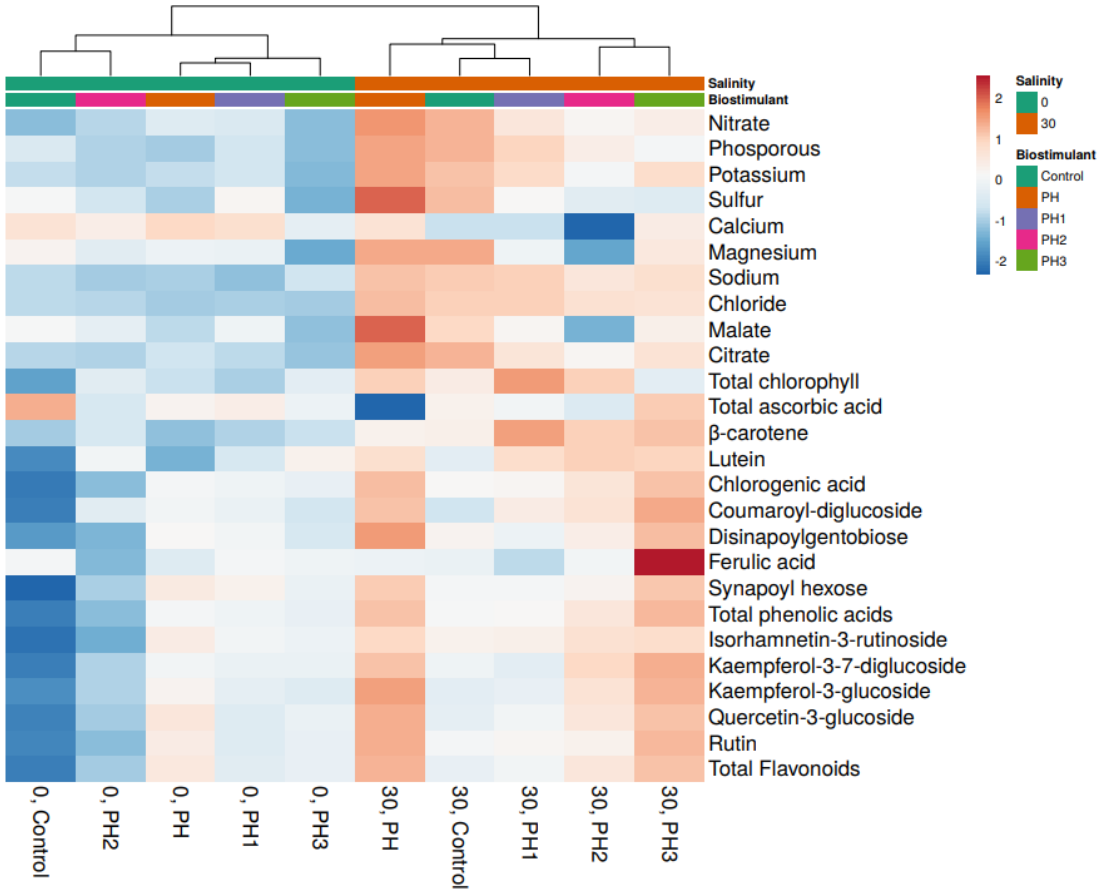

Figure 2. Cluster heat map analysis of the response of lettuce plants to salinity conditions (0 and 30 mM NaCl) and the application of biostimulants. Original values are ln (x + 1)-transformed. Columns are clustered using Euclidean distance and complete linkage. PH: Graminaceae-based protein hydrolysate; molecular fractions PH1, PH2 and PH3 (>10 kDa, 1 < x <10 kDa, <1 kDa).

The dendrogram hierarchically delineates two main clusters, which are separated due to the salinity treatment. In low (0 mM NaCl) salinity condition, represented by the left cluster where the untreated control and PH2 fraction are clustered together due to the lower concentration of polyphenolics. Treatments PH, PH1 and PH3 are clustered

and associated with higher polyphenolic contents (total phenolic acids and total flavonoids).

In mild salinity conditions (30 mM NaCl), we found two clusters, one composed of PH, PH1 and control treatments associated with higher mineral content (Na and Cl). However, the PH treatment is separated by the latter two due to its lower ascorbic acid and higher flavonoids and phenolic acids contents. The PH2 and PH3 cluster is associated with a lower concentration of Na and Cl and similar lutein and β-carotene content. PH2 showed the lowest malate content, and lower polyphenols (flavonoids and phenolic acids) than PH3.

On the other hand, Lucini and collaborators [22] found that fractions with low molecular weight (< 0.5–1 kDa) strongly affected phytohormones profile and induced changes of metabolomic nature with a trend similar to those caused by indole-butyric acid (IBA) that have auxin like activity. Thus, fractionation could enrich the products with components (small molecules oligopeptides) characterized by auxin-like activity. Moreover, they mentioned that other biostimulant substances under 3.5 kDa could attain plants plasmalemma, while when above 3.5 kDa it could act only on cell walls. Small peptides are major signaling compounds and command several aspects in plants stimulating key genes related to plant growth and stress response.

7.5 Chapter 7 Conclusions

Molecular fractionation of Graminaceae-derived protein hydrolysate by dialysis proved fruitful in discerning the action of the biostimulant tested and its different fractions in different salinity levels. The experiment proved that the different fractions had, for certain parameters, distinct behavior under different conditions. The fraction PH2 (1-10 kDa) was demonstrated to be effective in raising shoot fresh weight only in mild salinity conditions (30 mM NaCl) coupled with a significant decrease in Na, Cl and malate leaf content, while PH and PH3 (<1 kDa) improved total phenolic acids in the same conditions, and PH3 alone boosted total ascorbic acid. While in 0 mM NaCl condition, PH, PH1 and PH3 had a significant effect on phenolic acids but no effect on shoot fresh biomass. Such results help biostimulant producers to direct the application of their products in appropriate scenarios, to better address farmers' needs. Nevertheless, omics approaches such as transcriptomics and metabolomics should be integrated into future works of fractionation to get solid explanations of the accurate activity of the Graminaceae-based biostimulant and its fractions in different abiotic stresses of concern to modern agriculture.

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Chapter 8

Conclusions

Climate change and its related issues have driven agriculture scientists, specialists, and growers to find newly developed solutions to tackle the challenges for a sustainable future. With the advent of the biostimulants, we have found tools that can provide for help in many situations, by increasing the use efficiency of nutrients, providing the much need support to plants in stressful conditions, and augmenting functional quality.

In chapter 2 we provided an as-thorough-as-possible look at the state of the biostimulant literature and found that many of the commercial formulation stem from industrial waste or waste biomass, thus adhering to the principle set out by the EU in the matter of newly developed fertilizers. However, we also found out that the literature does not paint a black-and-white picture: there is a fair share of leeway in the growing systems, cultivar selection and what we have defined as *biostimulant management*, which can and will, as we later found out, determine a substantial amount of the outcome of biostimulant use.

Our works' crop Lettuce (*Lactuca sativa* L.), as we found out in chapter 3, represent a very diverse species which contains an incredible amount of variation in its cultivars, starting from head and leaf shape throughout all the growing stages, to the amount of pigmentation or lack thereof. The consulted literature very much agrees on the role of leaf physical and morphological characteristics for biostimulant absorption through the leaf surface, but also suggests that absorption via the root is mediated by the microbiome. This latter factor is very important but can prove very complex to study.

Our test results definitely show a cultivar-dependent effect of biostimulant treatments. To summarize, we have found that the green butterhead 'Ballerina' cultivar recorded its highest growth performance at the lowest nutrient solution biostimulant application rate, resulting in a much desirable yield increase of 29.4 tons ha^{-1}, whereas the red crisphead 'Canasta' showed the best yield and functional quality results at the highest combined biostimulant application, i.e. foliar plus higher nutrient solution treatment. This resulted in a yield increase of 25.1 tons ha^{-1}.

This increase in the effective functional quality of the product due to the induction of defense-related compounds in the red cultivar has been corroborated by multiple research instances on protein hydrolysate biostimulants on multiple crops and it further outlines one of the mechanisms through which they work.

However, research has also shown that not all protein-based biostimulants are the same. In fact, by pitting against one of the gold standards in protein hydrolysate literature i.e. the commercial product 'Trainer', to a newly formulated product based on

spent cotton biomass, we found similar performance in optimal conditions in the regards of increased growth index, shoot fresh weight and net photosynthetic rate under optimal nitrogen conditions. Furthermore, the cotton-based product recorded higher leaf calcium and magnesium contents, which could suggest higher absorption through the root tissue. However, importantly, we found that the legume-based commercial product determined an increase of lutein, β-carotene and phenolic acids which strongly suggests a product-by-product modulation of secondary metabolites.

The modulation of plant metabolism, both primary and secondary, has long being hypothesized as due to a variation of the bioactive molecules contained in the protein hydrolysate products. In particular, the signaling peptide theory is the long-standing theoretical framework that was put to the test in recent times in lab conditions, and found to be in accordance with phenotypical data. In chapter 5 we scaled up the experiments to greenhouse conditions and provided for a test of different molecular fractions of a commercial product in order to shed light on some of the phenomena we observed previously. What we found is that, compatibly with the theory, the smallest fractionate induced a significant increase in secondary metabolism products such as ascorbic acid, lutein and β-carotene in both optimal and low nitrogen conditions. This shows that effectively, what we called the PH3 (<1kDa) fraction possesses superior bioactivity in the respect of secondary metabolism.

However, both the biggest and smallest fractions (>10kDa and <1kDa) successfully increased lettuce marketable yield by 7.9% in optimal nitrogen conditions, which suggests that a one-size-fits-all explanation to this biostimulant category may not be advisable.

Chapter 4 and 5 also provide a very useful take-home message in the case of low nitrogen conditions: whilst in unfertilized or marginal soil PH can and has been proven to increase yield due to an increase in nutrient use efficiency (uptake, and utilization), this is not the case of carefully controlled conditions with high imposed nutrient scarcity. That is to say, it confirms that when sticking to the suggested treatment rates, biostimulant application is not fertilization, and it also underlines that biostimulant are not the silver bullet to solve all crop management problems: careful nutrient administration is still paramount to harness all positive effects.

In the case of salinity stress, to which chapter 6 and 7 are dedicated, we tried to shed some light on some of the strategies biostimulant-treated plants employ when fending off this particular condition. We found that the 'Trainer' and 'Vegamin' biostimulants successfully managed to mitigate the salinity stress by modulating ion homeostasis parameters, which manifested in a decreased sodium accumulation and lower proline accumulation in salt condition. Both biostimulants managed an across-the board increase in marketable fresh weights of 8.9 and 4.6% ('Trainer', 'Vegamin') but more

differences cropped up when considering, again, the modulation of metabolites. Lutein, β-carotene, and Quercetin-3-glucoside are all markers of secondary metabolism activation which suggest that, again, the 'Trainer' biostimulant might contain more bioactive molecules, which could be in the <1kDa range as exemplified in both our and previous research on the topic.

Again, in the case of the fractions of the Graminaceae-derived biostimulant, we found that varying results, as only the PH2 fraction (1-10 kDa) managed to be effective in raising shoot fresh weight in the salinity condition, a result which was coupled with a significant decrease in sodium and chloride contents. The unfractioned PH and PH3 (<1 kDa) improved total phenolic acids in the same conditions, and PH3 alone boosted total ascorbic acid. Overall, in control conditions, we did not find any appreciable differences in terms of yield between the tested products.

What these results add are pieces of the protein hydrolysate biostimulant puzzle: cultivar selection and application-mode, source protein matrix and molecular composition are what define their effectiveness. However proper crop management is still paramount in order to achieve the objectives that we are setting up for the future.

We are inching closer to getting a full understanding of this category of products, and further, multidisciplinary efforts are necessary to get there.