Received: 6 November 2023 | Revised: 26 March 2024 | Accepted: 17 April 2024

DOI: 10.1111/ppl.14357

ORIGINAL RESEARCH

Physiologia Plantarum

Unravelling the biostimulant activity of a protein hydrolysate in lettuce plants under optimal and low N availability: a multi-omics approach

Sonia Monterisi[1†] | Pascual Garcia-Perez[2†] | Valentina Buffagni[2†] | Monica Yorlady Alzate Zuluaga[1] | Michele Ciriello[3] | Luigi Formisano[3] | Christophe El-Nakhel[3] | Mariateresa Cardarelli[4] | Giuseppe Colla[4] | Youssef Rouphael[3] | Francesco Cristofano[3] | Stefano Cesco[1] | Luigi Lucini[2] | Youry Pii[1]

[1]Faculty of Agricultural, Environmental and Food Sciences, Free University of Bozen/Bolzano, Bolzano, Italy

[2]Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Piacenza, Italy

[3]Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

[4]Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy

Correspondence
Youry Pii,
Email: youry.pii@unibz.it

Funding information
Ministero dell'Università e della Ricerca, Grant/Award Number: 2017FYBLPP

Edited by J.-.C. Avice

Abstract

The application of protein hydrolysates (PH) biostimulants is considered a promising approach to promote crop growth and resilience against abiotic stresses. Nevertheless, PHs bioactivity depends on both the raw material used for their preparation and the molecular fraction applied. The present research aimed at investigating the molecular mechanisms triggered by applying a PH and its fractions on plants subjected to nitrogen limitations. To this objective, an integrated transcriptomic-metabolomic approach was used to assess lettuce plants grown under different nitrogen levels and treated with either the commercial PH Vegamin® or its molecular fractions PH1(>10 kDa), PH2 (1–10 kDa) and PH3 (<1 kDa). Regardless of nitrogen provision, biostimulant application enhanced lettuce biomass, likely through a hormone-like activity. This was confirmed by the modulation of genes involved in auxin and cytokinin synthesis, mirrored by an increase in the metabolic levels of these hormones. Consistently, PH and PH3 upregulated genes involved in cell wall growth and plasticity. Furthermore, the accumulation of specific metabolites suggested the activation of a multifaceted antioxidant machinery. Notwithstanding, the modulation of stress-response transcription factors and genes involved in detoxification processes was observed. The coordinated action of these molecular entities might underpin the increased resilience of lettuce plants against nitrogen-limiting conditions.

In conclusion, integrating omics techniques allowed the elucidation of mechanistic aspects underlying PH bioactivity in crops. Most importantly, the comparison of PH with its fraction PH3 showed that, except for a few peculiarities, the effects induced were equivalent, suggesting that the highest bioactivity was ascribable to the lightest molecular fraction.

[†] These authors contributed equally to the work.

1 | INTRODUCTION

Management of plant nutrition is of utmost importance to sustain plant growth and development as well as to ensure both yield and quality in horticultural crops (Marschner 2012). However, the agricultural sector is required to face an important challenge in the coming years; the rapidly increasing World population will require a parallel increase in food production, which is expected to rise by 50% by the year 2030 and double by 2050 (FAO 2009; Tilman et al. 2011). In the past century, crop yield has been enhanced by increasing the external inputs of mineral fertilizers, especially nitrogen(N)-based ones, and pesticides (Lassaletta et al. 2014). Indeed, N is an essential macronutrient for plants, pivotal in synthesising many compounds, including nucleic acids, amino acids and proteins, chlorophyll, and other secondary metabolites (Marschner 2012). Therefore, N shortage can represent one of the major limiting factors for plant growth and productivity. Nonetheless, in the last decades, crop yields have stagnated due to a decrease in the Nutrient Use Efficiency (NUE) of crops, thus suggesting that further increasing the inputs of agrochemicals will not guarantee a parallel increase in productivity (Zhang et al. 2010; Baligar and Fageria 2015). Furthermore, the intensive use of N-based fertilizers and the reduction in crops' NUE posed not only economic issues but also environmental ones, mainly related to (1) the mobility of these nutrient pools in soils (e.g. leaching, run-off) (Udvardi et al. 2021), (2) the exacerbation of greenhouse gas emissions and (3) the increase in nitrogen volatilization (Saud et al. 2022).

To favour the transition towards more sustainable agricultural approaches aimed at increasing yields and yet reducing the adverse effects of intensive fertilizer application, the adoption of innovative strategies appears mandatory. In this vision, using plant biostimulants might represent a promising solution (Di Mola et al. 2019; Siwik-Ziomek and Szczepanek 2019; Cristofano et al. 2021; Carillo et al. 2022). According to the EU regulation 2019/1009, a plant biostimulant (PB) has been defined as a product that can promote plant growth and health by enhancing either (1) nutrient use efficiency, (2) tolerance to abiotic stress, (3) crop quality traits, (4) availability of nutrients confined in the soil and rhizosphere or a combination of the above. Overall, PBs show a diversified nature, thereby including a wide range of substances like beneficial microorganisms (e.g., mycorrhiza or plant growth promoting rhizobacteria), humic substances, seaweed extracts, and protein hydrolysates (PH) (Calvo et al. 2014; Battacharyya et al. 2015; Canellas et al. 2015; Colla et al. 2015, 2017b; Halpern et al. 2015; Pii et al. 2015; Rouphael et al. 2015; Alzate Zuluaga et al. 2022). Among these, PH is composed of a mixture of peptides and free amino acids obtained through the partial hydrolysis, either chemical or enzymatic, of a protein source, deriving from either the animal or plant kingdom (Colla et al. 2015, 2017b). Previous studies have assessed that the application of PH-based biostimulants can increase the NUE and the tolerance to abiotic stresses (e.g., salinity, drought and high temperature) in horticultural crops (Calvo et al. 2014; Halpern et al. 2015; Lucini et al. 2015; Colla et al. 2017a, 2017b; Carillo et al. 2019b, 2019a; Alzate Zuluaga et al. 2022; Zuluaga et al. 2023), albeit the mode of action has not been fully elucidated yet. Several authors have ascribed the positive effects brought about by the administration of PHs to direct and indirect mechanisms, as i) the upregulation of C and N metabolism by the stimulation of key enzymes, ii) the enhancement of the antioxidant defence systems, and iii) the modulation of hormone-like activities, which could, in turn, affect the growth and the biology of the root systems, thereby influencing mineral nutrient uptake/assimilation and, ultimately, crop productivity (Colla et al. 2017b). However, several pieces of evidence have also highlighted that PHs produced from plants with a diverse botanical origin are characterized by a different composition in terms of peptides and amino acids, thus exerting distinct effects on the growth or metabolism of different crops (Ceccarelli et al. 2021; Cristofano et al. 2023a; Zuluaga et al. 2023). In this vision, understanding the different contributions of single PH components in biostimulation is still elusive, and its comprehension might represent a challenging goal to achieve. Conversely, the complete elucidation of the bioactive compounds and their effects on plants might allow choosing the best source for PHs production and designing hydrolysis processes that could enrich the final product in the effectors of interest (Lucini et al. 2020). To date, very limited research has been carried out to understand the bioactivity of different PHs fractions on horticultural crops. El-Nakhel and co-workers (2023a) demonstrated that a *Gramineae*-derived PH and its molecular fractions provided different effects (*i.e.*, accumulation of fresh biomass, modulation of Na^+, Cl- and organic acids concentration in leaves, differential accumulation of antioxidant compounds) when administrated to lettuce plants grown under salinity stress, demonstrating that each fraction might produce distinct effects on plants. Similarly, Lucini et al. (2020), through metabolomics-based analyses, demonstrated that the lightest fraction (0.5–1 kDa) of a *Fabaceae*-derived PH was the most active in inducing the rooting of tomato seedlings through an auxin-like mode of action. Indeed, the lowest fractions are rich in small metabolites and oligopeptides that could act in plant cells as signalling molecules and orchestrate cell development and response to environmental stimuli (Hirakawa and Sawa 2019). Despite these pieces of research, several aspects of the molecular regulation induced in plants by the administration of PHs and/or their fractions deserve further investigation, suggesting that integrating different omics techniques might be necessary.

In the present research paper, a greenhouse experiment was carried out on soilless-grown lettuce (*Lactuca sativa* L.), either under optimal or low N availability, to investigate the impact of the commercial biostimulant Vegamin® (Hello Nature USA Inc, Anderson, IN 46016 US) and its fractions. Lettuce is among the most demanded leafy vegetable crops, with a global market bankrolled by over 29 million tons in the 2019 harvest (Medina-Lozano et al. 2021). In recent years, the upward demand has been driven by consumers' needs, including the increasing focus on food safety issues, since lettuce is notoriously an excellent source of minerals and health-beneficial compounds (Medina-Lozano et al. 2021). Nutrient limitation, particularly N shortage, harms crops due to its negative impact on plant metabolism, which consequentially reduces the nutritional and functional quality of the products (Marschner 2012). In lettuce, N limitation reduced total polyphenols and antioxidant activity with deleterious effects on photosynthetic performance (Broadley et al. 2001; Coria-Cayupán et al. 2009). In particular, the work was focused on addressing the effects of

biostimulant application in terms of molecular reprogramming in lettuce by integrating transcriptomic and metabolomic analyses that, to our knowledge, has never been performed before. From one side, the results obtained here could assist in selecting the most suitable source for the production of PH while supporting the choice of the most bio-active fraction(s) to achieve the desired effect on crops.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Lettuce plants (*Lactuca sativa* L. cv. "Maravilla De Verano Canasta"; Pagano Domenico and Figli, Scafati, Italy), at the phenological stage of 2–3 true leaves, were transplanted into pots (14 cm diameter and 1.6 L total volume) filled with a mixture (*v/v*) of 10% perlite (Perlite Italiana s.r.l., Corsico, Italy) and 90% 3-mm quartz sand (Vaga, Sabbie e Ghiaie Silicee, Costa de' Nobili, Italy) at a density of 14 plants m^{-2}. Plants have been regularly irrigated with a nutrient solution composed as follows: 1.5 mM of P, 4 mM of K, 4 mM of Ca, 2.5 mM of S, 1.25 mM of Mg, 20 μM of Fe, 9 μM of Mn, 0.3 μM of Cu, 1.6 μM of Zn, 20 μM of B, and 0.3 μM of Mo, pH 5.8 and electrical conductivity of 1.6 dS m^{-1}. After the transplant, lettuce plants were exposed to two levels of nitrogen (Optimal N – 8 mM NO_3^- and Low N – 1 mM NO_3^-), supplemented through irrigation, and treated with a protein hydrolysate (PH) biostimulant and its molecular fractions. The PH adopted for this study was the commercial product Vegamin® (Hello Nature USA Inc.) and its molecular fractions, *i.e.*, PH1(>10 kDa), PH2 (1–10 kDa) and PH3 (<1 kDa), based on the evidence previously collected (Cristofano et al. 2023a). Vegamin® is a vegetal-based biostimulant obtained through enzymatic hydrolysis of proteins from legume crops (Cristofano et al. 2023b). The Vegamine biostimulant was applied at a rate of 3 mL biostimulant L^{-1} of solution, as recommended by the manufacturer. The rates of fractions (PH1, PH2 and PH3) application were adjusted in order to provide an equal amount of nitrogen as compared to the non-fractioned product (Cristofano et al. 2023a). Control plants were treated with distilled water instead of protein hydrolysates. A total of five applications were carried out weekly through the experimental trial, starting 13 days after the transplant. The experiment was carried out in an unheated glass greenhouse with passive ventilation at the Department of Agriculture, Federico II University of Naples, Italy (Portici, Italy; 40°48′ N, 14°20′ E, 29 m.a.s.l.) during the autumn season 2020. Plants were harvested at commercial maturity, 44 days after transplant, and samples were either dried or frozen in liquid N for subsequent analyses.

2.2 | Morphometric, Photosynthesis performance and Biochemical assay

At harvest, the shoot dry weight and the leaf area were determined in three plants per experimental unit. The latter parameter was determined by the digital image analysis as previously described (Ciriello et al. 2022b). On the day before harvest, measurements of leaf gas exchanges, *i.e.*, net CO_2 assimilation (A_{CO2}; μmol CO_2 m^{-2} s^{-1}), in addition to SPAD index, were made on young fully expanded leaves of two replicate plants using LCi T (ADC Bioscientific Ltd.) and Minolta SPAD-502 chlorophyll meter (Minolta Camera Co. Ltd.), respectively.

To determine the activity of antioxidant enzymes [catalase (CAT) and ascorbate peroxidase (APX)] and markers of oxidative stress [malondialdehyde acetate (MDA) and hydrogen peroxide (H_2O_2)], as described previously (El-Nakhel et al. 2023b), four leaves per replicate were harvested and immediately immersed in liquid nitrogen and stored at −80°C.

2.3 | UHPLC/QTOF-MS untargeted metabolomics

Fresh material (3 leaf batches per treatment) was powdered under liquid nitrogen with a pestle and mortar and then subjected to solvent extraction. Briefly, 2 g of fresh leaves were mixed with 20 mL of the solvent MeOH:H_2O:HCOOH (80:19.9:0.1, *v/v/v*), vortexed and further homogenized using a high-speed rotor (Polytron® PT 1600-E) for 1 min. The obtained extracts were later centrifuged at 8000 *g* at 4°C for 10 min (Eppendorf® 5810). Supernatants were collected and filtered through 0.22 μm pore-size syringe filters and transferred to HPLC vials for analysis.

Samples were analyzed by an untargeted ultra-high performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS). The chromatographic procedure was performed in a 1290 Infinity II-LC system (Agilent Technologies®, Santa Clara, US) equipped with a reverse-phase Poroshell 120 PFP column (Agilent®, 2.0 × 100 mm, 3 μm). The separation was achieved by applying a binary gradient of water and acetonitrile, starting from 6 to 94% organic phase in 32 min. The injection volume was 6 μL, and the flow rate was set at 0.2 mL min^{-1}. The chromatographic system was coupled to a G6550 QTOF mass spectrometer (Agilent®) *via* a JetStream electrospray ionization source. The mass spectrometer was set in positive polarity (ESI+) and SCAN modes, and an extended dynamic range of 100–1200 *m/z* was selected, with a mass resolution of 30,000 FWHM. Nitrogen was used as sheath gas (12 L min^{-1}, 315°C) and drying gas (14 L min^{-1}, 250°C). Nebulizer pressure was set at 45 psi, nozzle voltage at 350 V, and capillary voltage at 4,000 V. The analysis was performed in duplicate, making six analytical replicates per treatment ($n = 6$).

Features annotation was performed by the 'find-by-formula' algorithm, based on the monoisotopic accurate mass, isotope patterns, and isotopes ratio, by the MassHunter Profinder software (v. 10.0, Agilent®), using the database PlantCyc (v. 15.1.1, from Plant Metabolic Network, available at https://pmn.plantcyc.org/). Data filtering was carried out by considering the features present in at least 66.6% of replicates. The identification of features was achieved according to the Level 2 set by the COSMOS Metabolomics Standard Initiative: putatively annotated compounds.

2.4 | RNA-seq analysis

Total RNA was extracted from frozen lettuce leaves using Spectrum Plant Total RNA Kit (Sigma-Aldrich) following the manufacturer's instructions. RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). mRNA was purified from total RNA using poly-T magnetic beads, followed by fragmentation with divalent cations under elevated temperature. First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H^-), whereas second-strand cDNA was synthesized by using DNA Polymerase I and RNase H. Blunt ends were obtained via exonuclease/polymerase activities. The 3′ ends of DNA fragments were adenylated, and adaptors with hairpin loop structure were ligated. Library fragments were purified through the AMPure XP system (Beckman Coulter) to enrich the fraction with lengths ranging between 370 and 420 bp. PCR was then carried out using Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Indexed Primer. Finally, PCR products were purified (AMPure XP system), and library quality was assessed using the Agilent Bioanalyzer 2100 system.

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform, and 150 bp paired-end reads were generated. Raw data were cleaned by removing reads containing adapter, ploy-N and low-quality sequences. All the downstream analyses were based on clean, high-quality data.

Reference genome and gene model annotation files were downloaded directly from the genome website. Hisat2 v2.0.5 was exploited to build the reference genome and to align paired-end clean reads. The quantification of gene expression level was carried out using featureCounts v1.5.0-p3, whereas Fragments Per Kilobase of transcript sequence per Million base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. The differential expression between treated and control plants (three biological replicates per condition) was calculated using the DESeq2 R package (1.20.0) (Love et al. 2014). The resulting P-values were adjusted using Benjamini and Hochberg's approach for false discovery rate. Gene Ontology (GO) enrichment analysis of differentially expressed genes was carried out by exploiting clusterProfiler R package; GO terms with corrected P-value lower than 0.05 were considered significantly enriched by differentially expressed genes

2.5 | Statistical analysis

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 N, and Tukey's HSD test was performed to separate both the biostimulant mean effect and Nitrogen level × biostimulant interaction. All tests were deemed significant at $p = 0.05$.

Concerning metabolomics, the dataset was subjected to alignment, normalization at the 75th percentile, log2 transformation, and baselined to the median abundance of all samples before their analysis using Mass Profiler Professional (v. 15.1, Agilent®). The multivariate chemometrics analysis consisted of an unsupervised hierarchical cluster analysis (HCA) derived from a fold change-based heatmap (Euclidean distance, Ward's linkage rule) and a supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA), using the SIMCA software (v. 16, Umetrics®, Malmö, Sweden). Furthermore, OPLS-DA was combined with a variable importance in projection (VIP) analysis to detect those metabolites showing the highest influence in the discrimination between treatments, the so-called VIP markers, assuming a VIP score threshold of 1.15. The quality of OPLS-DA models was further evaluated in terms of goodness-of-fit (R^2Y) and goodness-of-prediction (Q^2Y), being statistically validated through cross-validation analysis of variance (CV-ANOVA) and excluding the overfitting by permutation test (n = 200). The metabolomics dataset was further analyzed in terms of one-way ANOVA followed by Tukey's *post hoc* test (Bonferroni multiple testing correction, $p < 0.05$) and fold change analysis (FC, cut-off = ±1.5) to identify the differential metabolites by the Mass Profiler Professional software. Differential metabolites were subjected to pathway analysis and interpreted through the Omics Viewer Pathway by PlantCyc (Plant Metabolic Network®; available at https://pmn.plantcyc.org).

2.6 | Multi-omics integration of metabolomics and transcriptomics outputs

The raw metabolomics dataset and the DEGs dataset were mutually analyzed by applying the Data Integration Analysis for Biomarker discovery using Latent variable approaches for Omics studies (DIABLO) framework core as part of the "mixOmics" R package (version 6.22). An initial sparse partial least squares model (sPLS) was used to determine the correlation between both datasets. The DIABLO model was then optimized through the tuning function by calculating the optimal number of components with the associated lowest overall balanced error rate provided by calculating centroid distances. Once optimized, the DIABLO model, based on sPLS multiblock discriminant analysis, was performed to decipher the correlation between datasets at the component level of the tested treatments. Finally, an integrative cluster was provided to get insight into treatments' combined influence on metabolomics and transcriptomics outputs. A DIABLO model for each nutritional level, optimal and low nitrogen levels, was established.

3 | RESULTS

3.1 | Morpho-physiologic and enzymatic analyses

The effects of the treatments imposed (two levels of N and PH supplementation) on lettuce plants were assessed by evaluating morpho-

TABLE 1 Effects of different levels of nitrogen (Optimal and Low) and biostimulant fractions (Control, PH, PH1, PH2, and PH3) on the activity of antioxidant enzymes [catalase (CAT) and ascorbate peroxidase (APX)] and oxidative stress markers [malondialdehyde acetate (MDA) and hydrogen peroxide (H_2O_2)].

Source of variance	MDA (mg g^{-1} fw)	H_2O_2 (mg g^{-1} fw)	CAT (mmol mg $protein^{-1}$ min^{-1})	APX (mmol mg $protein^{-1}$ min^{-1})
Nitrogen (N)				
Optimal (O)	0.84 ± 0.03	0.47 ± 0.01	23.97 ± 2.79	0.07 ± 0.01
Low (L)	1.35 ± 0.04	1.16 ± 0.03	62.54 ± 6.25	0.27 ± 0.03
t-test	***	***	***	***
Biostimulant (B)				
Control	1.13 ± 0.17	0.81 ± 0.14	24.42 ± 3.29 c	0.27 ± 0.07 a
PH	1.11 ± 0.1	0.76 ± 0.14	45.27 ± 12.62 b	0.17 ± 0.05 b
PH1	1.08 ± 0.14	0.85 ± 0.17	46.27 ± 9.23 b	0.16 ± 0.05 b
PH2	1.07 ± 0.1	0.82 ± 0.16	33.82 ± 8.24 bc	0.15 ± 0.04 b
PH3	1.06 ± 0.12	0.83 ± 0.17	66.51 ± 13.29 a	0.09 ± 0.02 b
	n.s.	n.s.	***	***
N × B	n.s.	n.s.	**	**

physiological parameters, as reported in Supplementary Figure S1 and Table 1. Supplementary Figure S1 shows the mean effects of the experimental factors (Nitrogen and Biostimulant) on leaf area (A) and shoot dry weight (B). Regarding the N effect, optimal N treatment (Optimal) increased leaf area and shoot dry weight by approximately 4.3 and 3.6 times, respectively, compared to low N treatment (Low). Relative to the biostimulant effect (B), the treatment with PH3 increased leaf area and shoot dry weight by 13.10 and 14.45%, respectively, compared to the control.

The mean effects of the experimental factors on the SPAD index and the net assimilation of CO_2 (A_{CO2}) are shown in Supplementary Figure S2. The average N effect significantly affected the SPAD index and the net assimilation of CO_2 (A_{CO2}), with the highest values recorded under Optimal N conditions. Differently, the mean biostimulant effect (B) had a significant effect only on A_{CO2}, where the PH3 fraction resulted in a 25.40% increase compared to the control.

The average effects of the factors on the activity of antioxidant enzymes and oxidative stress markers are shown in Table 1. Compared to the average N effect, a significant influence was observed for all parameters, with the highest values recorded in Optimal treatment. On the contrary, the mean biostimulant effect (B) significantly influenced only catalase (CAT) and ascorbate peroxidase (APX). Specifically, the highest CAT values were recorded in plants treated with the PH3 biostimulant fraction, in contrast to APX, where the highest values were recorded in Control plants.

3.2 | Metabolomics profiling of lettuce plants

The untargeted metabolomics analysis let us putatively annotate more than 2800 chemical entities using the PlantCyc database (Supplementary Table S1). Overall, the unsupervised hierarchical cluster analysis (HCA) pointed out N supply as the first determining factor on metabolic signatures (Figure 1A). Nevertheless, HCA identified relevant similarities/dissimilarities across treatments. PH2 and PH3 clustered together either under optimal or low nitrogen conditions. PH-treated plants were the closest to control groups under low N, whereas plants treated with PH1 modulated their metabolism more likely PH2 and PH3.

Therefore, supervised statistics was performed on two separate subsets of metabolomics data, according to the N availability regimen. The orthogonal prediction of latent structures-discriminant analysis (OPLS-DA) was first performed on the metabolic profile of lettuce plants under optimal nitrogen supply to discern predictive and orthogonal components of variance (Figure 1B). The model was validated for its robustness and predictive power ($R^2Y = 0.967$, $Q^2 = 0.740$, p (CV-ANOVA) $= 5.49\ e^{-06}$), and permutation testing excluded model overfitting. The model distinguished treatments by separating groups of data primarily along the first latent vector; one group included control samples closely related to PH- and PH1-treated plants, and then PH2 and PH3 both separated among them and the rest of the treatments, confirming the HCA output (Figure 1A). Afterwards, the variable importance in projection (VIP) analysis identified 156 metabolites with the highest discriminant potential (VIP score >1.15) (Supplementary Table S2). Secondary metabolites were the most represented compounds, essentially flavonoids, isoprenoids, and alkaloids. Primary metabolites were included to a lesser extent, such as carbohydrates, organic acids, nucleic acid components, and lipids, including glycerolipids and phospholipids. Considering hormones and their precursors, VIPs included metabolites associated with indole-3-acetate (IAA) metabolism, an N-glucoside cytokinin, brassinosteroids and gibberellins. Other VIP markers consisted of compounds related to the photosynthetic apparatus, *i.e.*, xanthophyll and organic heterocyclic compounds and molybdopterin cofactor. Interestingly, antioxidant compounds were also spotted as VIP markers, including tocopherol and tocotrienol, and five (L-cysteine glycine)-*S*-conjugates involved in glutathione-mediated detoxification.

In parallel, supervised statistics were performed on from the metabolic profile of low-N stressed plants to obtain an OPLS-DA model of

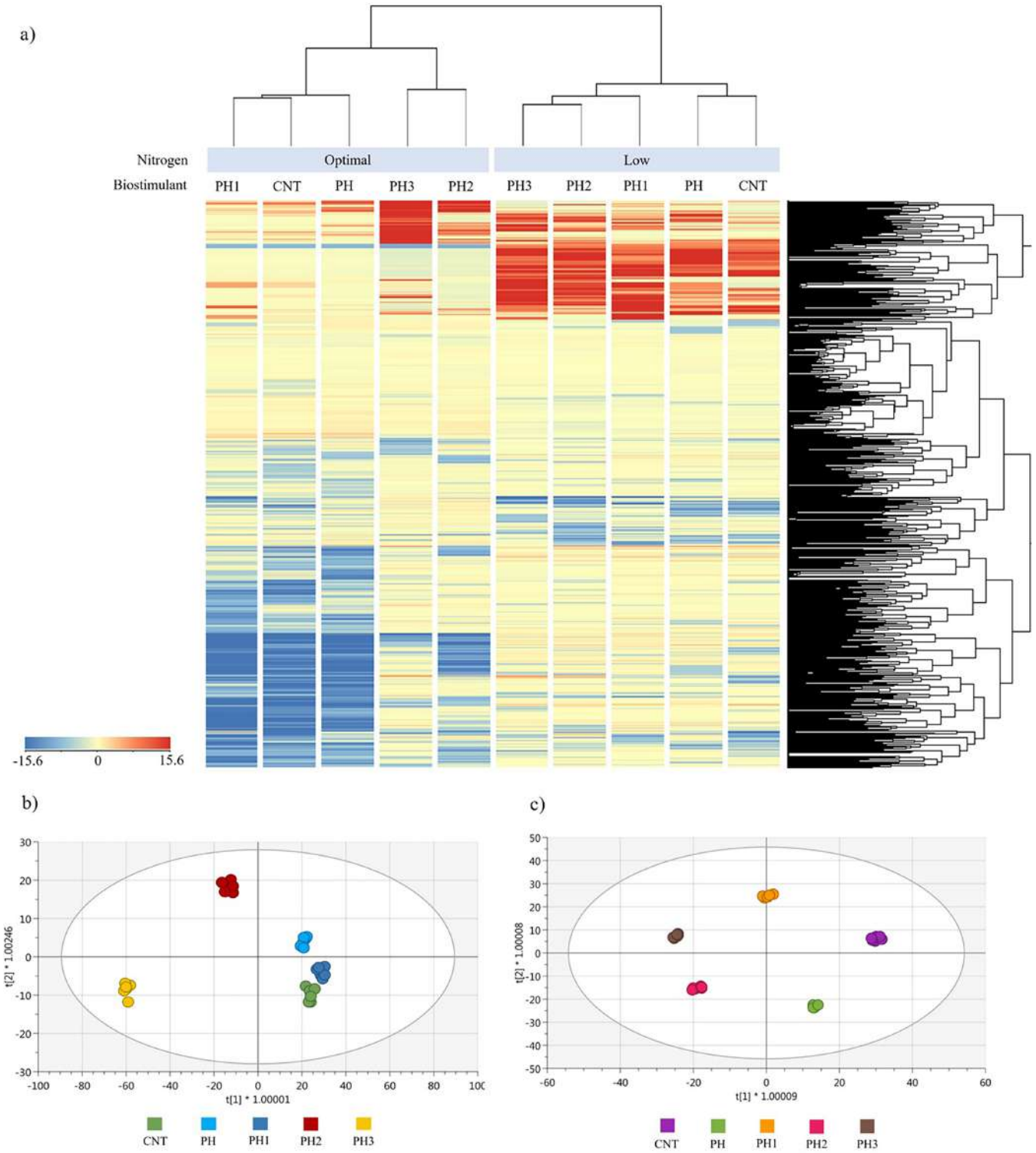

FIGURE 1 a) Hierarchical cluster analysis; b) OPLS-DA Optimal N; c) OPLS-DA Low N.

high quality, as indicated by its parameters: $R^2Y = 0.996$, $Q^2Y = 0.890$, and p (CV-ANOVA) = 1.83777e-12 (Figure 1C). Under low N conditions, all treatments were independently separated into the score plot because of PH-dependent response. Finally, 224 metabolites were identified throughout VIP analysis (VIP score >1.15) as the most responsible for the separation between treatments (Supplementary Table S3), suggesting a broader metabolic modulation due to biostimulant treatment than that reported under optimal conditions. Among discriminant compounds, secondary metabolites were featured, being mainly represented by flavonoids, isoprenoids, and nitrogen-containing compounds (NCCs), such as alkaloids, aliphatic glucosinolates and related N-hydroxylated amino acids involved in their biosynthesis. As well, phytohormones were spotted as discriminant compounds, including IAA-conjugates (IAA-Ala, IAA-Leu, IAA-Ile, IAA-CoA), cytokinins

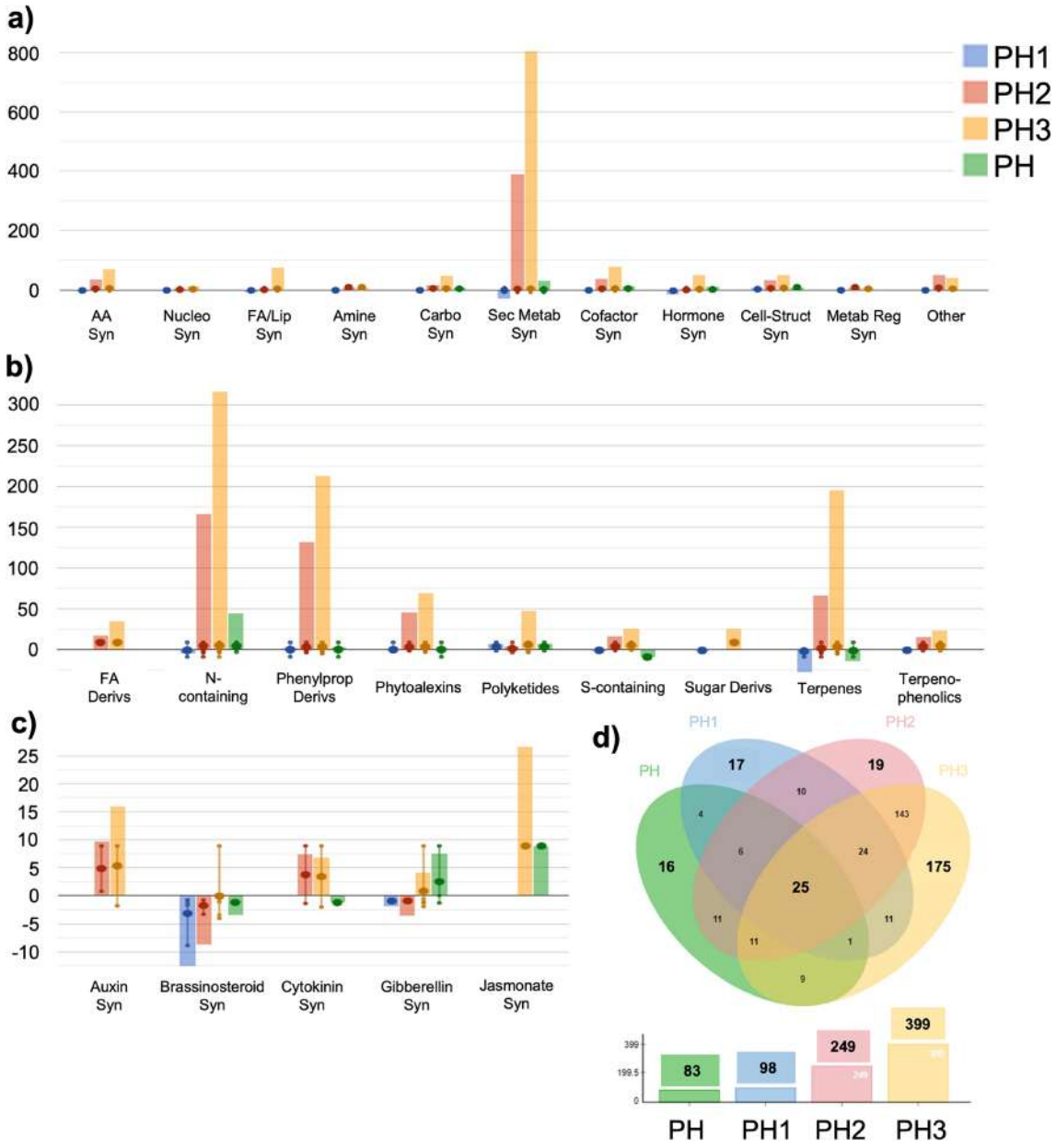

FIGURE 2 Biosynthetic pathways modulated by biostimulants on lettuce plants grown under Optimal N condition (a). Secondary metabolism (b). Phytohormone (c). Venn diagram indicating peculiar and commonly modulated metabolites following different treatments (d). Significative compounds modulated by PH and its fractions PH1, PH2 and PH3 (ANOVA Tukey HSD $p = 0.05$, FC cut-off 1.5).

(dihydrozeatin, zeatin and zeatin riboside), gibberellins, jasmonates and their CoA-derived precursors, and 1-aminocyclopropane. Other discriminant compounds included primary metabolites, like nucleic acid components, lipids, and organic acids, as well as vitamin B and the cofactor FMN, phytoalexins and geranylgeranyl chlorophyll a.

3.2.1 | Metabolic modulation by PH fractions under optimal nitrogen supply

The application of statistical and fold change analyses in PH-treated lettuce, compared to control under optimal N conditions, allowed the selection of 466 significant compounds (one-way ANOVA, $p < 0.05$, FC >1.5, <−1.5; Supplementary Table S4). To describe the metabolic outcome attributed to biostimulant treatments with respect to control conditions, the significant metabolites were subjected to pathway analysis through the Omics Viewer of PlantCyc in terms of general biosynthetic metabolism (Figure 2A), secondary metabolism (Figure 2B), and phytohormone biosynthesis (Figure 2C). Concerning the significant compounds involved in this analysis due to different biostimulant treatments, the corresponding Venn diagram (Figure 2D) indicated that PH3 treatment significantly influenced the presence of almost 400 metabolites, whereas PH2 impacted more than 200, and PH1 and PH modulated less than 100 compounds themselves. This observation suggests that PH3 and PH2 greatly modulated the biosynthesis of lettuce plants compared with PH1 and PH. Such a result

is reinforced by the results on the biosynthetic metabolism of lettuce plants since PH3 and PH2 had the most incisive impact on plant metabolism with respect to the control, playing a positive role in secondary metabolism (Figure 2B). In particular, NCCs, phenylpropanoids, terpenes, and phytoalexins accumulated as a common response, mainly to PH3 and PH2 fractions. On the other hand, plants treated with PH showed a mild accumulation of NCCs while it drove a slight down-accumulation of terpenoids, as also observed for PH1. NCCs were essentially represented by glucosinolate precursors, such as 2-[(3′-methylsulfanyl)propyl]malate, (*E*)-phenylacetaldehyde oxime, and (*Z*)-2-phenyl-1-thioacetohydroximate, and indole-terpenoid alkaloids, such as vindoline and catharanthine (logFC = 8.89 for all compounds in PH3 and PH2-treated plants). The effect of PH2 and PH3 on phenylpropanoids involved the accumulation of polyphenols, essentially flavonoids (*i.e.*, (−)-epiafzelechin, baicalin, and isorhamnetin-3-*O*-glucoside; logFC = 8.89 for all compounds), and lignans (*i.e.*, (−)-yatein and 6-methoxypodophyllotoxin; logFC = 8.89 for all compounds), coupled with a decrease in the accumulation of methoxylated flavonoids, such as myricetin and quercetin trimethoxy derivatives (logFC = −4.86 – -2.68). In the case of terpenoids, besides indole-terpenoid alkaloids, an evident accumulation of carotenoids was observed in both PH2- and PH3-treated plants, as observed for 4′,4′-dihydroxyadonixanthin, (3S,3'S)-astaxanthin and norbixin (logFC = 8.89 for all compounds in both treatments).

Considering the phytohormone profile (Figure 2C), PH2 and PH3 increased levels of auxins, as shown for the indole-3-acetic acid (IAA) derivative (indole-3-yl)acetonitrile (logFC = 8.89 for both treatments), and PH3 also promoted the accumulation of IAA-CoA while decreasing methyl-IAA levels (logFC = 8.89 and − 1.77, respectively). Besides auxins, all treatments negatively affected brassinosteroid biosynthesis by reducing levels of brassinolide precursors and campestanol (logFC = −8.89 – −1.18), whereas PH, followed by PH3, promoted a net accumulation of gibberellins, motivated by the high rates reported for GA_9 and GA_5, respectively (logFC = 8.89 in both cases). Additionally, PH3 promoted the accumulation of jasmonate precursors, such as 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-(3R-hydroxybutanoyl)-CoA, 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-butanoyl-CoA (logFC = 8.89 for both compounds), while all treatments drove a clear decrease of the abscisic acid precursor (3*S*,5*R*,6*R*)-3,5-dihydroxy-6,7-didehydro-5,6-dihydro-12′-apo-β-caroten-12′-al (logFC = −8.89 – -1.42). Finally, low-molecular-weight PH fractions, PH3 and PH2, promoted the accumulation of the cytokinin kinetin-7-N-glucoside (logFC = 8.89 for both treatments).

Besides secondary metabolism, treatments modulated primary metabolism to a lesser extent. A common increment of amino acid precursors was reported in PH2- and PH3-treated plants, especially those involved in Met, Phe, Pro, Ser, and Tyr biosynthesis (logFC = 8.89 in all cases for both treatments). Lipid metabolism was also affected by the same treatments, reporting an impairment of lipid cutin components and membrane-associated glycolipids (logFC = −4.76 – −0.88 for galactosyl-diacylglycerol derivatives). Among vitamins and cofactors, positive modulations regarding the Vitamin E pathway, particularly tocopherols and vitamin B6, were observed for all treatments. Finally, intermediates involved in glutathione biosynthesis and its recycling pathway were found to accumulate specifically by PH2 and PH3, as mainly observed by the accumulation of γ-L-glutamyl-L-cysteine and L-cysteinylglycine (logFC = 8.89 in all cases).

3.2.2 | Metabolic modulation by PH fractions under low nitrogen supply

Under low N conditions, 391 compounds were found to be significantly modulated following the biostimulant application compared to control grown under low N conditions (one-way ANOVA, $p < 0.05$, FC >1.5, <−1.5; Supplementary Table S5), and further imported into the Omics Viewer of Plantcyc to determine the impact of PH treatments on the biosynthetic metabolism of lettuce plants with respect to the untreated control (Figure 3). Regarding the individual impact of biostimulants, the Venn diagram on the significant compounds modulated by treatments revealed that PH2 affected the accumulation of 277 compounds, followed by PH3, which affected 200 compounds (Figure 3D), thus suggesting that both low-molecular-weight fractions promoted the strongest metabolic reprogramming under stress. With respect to the PH-mediated modulation, all treatments induced a marked accumulation of secondary metabolites, especially in the cases of PH3 and PH2 fractions. In contrast, the biosynthetic metabolism of amino acids, nucleotides, lipids, cofactors and phytohormones was affected to a lesser extent (Figure 3A).

As stated before, secondary metabolism showed the highest impact, exhibiting a general accumulation of NCCs, terpenes, and phenylpropanoids by the biostimulant treatments, which followed a fraction-dependent effect: NCCs and terpenoids were mostly accumulated by low-molecular-weight fractions (PH3 and PH2, respectively). Meanwhile, phenylpropanoids were mostly accumulated by PH and PH1 (Figure 3B). Among NCCs, glucosinolates were found accumulated following PH2 and PH3 treatments (*i.e.*, 3-carboxy-9-(methylsulfanyl)-2-oxononanoate, 7-(methylsulfanyl)-2-oxoheptanoate, 3-(methylsulfanyl)propyl-desulfoglucosinolate; logFC = 8.55 in all cases), together with a decrease in their major precursor *N*-hydroxyhomomethionine (logFC = −8.55 for both treatments). Regarding terpenoids, all PH fractions promoted the accumulation of the sesquiterpenoid precursor methyl (2*E*, 6*E*)-farnesoate and PH2 and PH3 promoted the accumulation of the phytoalexin gypsogenin-28-β-D-glucoside (logFC = 8.55 in all cases). Finally, a biostimulant-dependent accumulation of phenylpropanoids was detected, reaching the highest rates for PH, as shown for the flavonoid glycosides quercetin-3-*O*-rhamnoside and delphinidin-3-*O*-(6''-*O*-malonyl)-β-glucoside-3'-*O*-)-β-glucoside (logFC = 8.55 for both compounds), and PH1, inducing the accumulation of apigenidin 5-*O*-glucoside and 6,7,4′-trihydroxyisoflavone (logFC = 8.55 for both compounds).

Concerning the phytohormone profile, all biostimulant treatments caused a significant impact under stress (Figure 3C). Jasmonates accumulated under all treatments with PH fractions, following the trend PH3 > PH2 > PH1, whereas PH-induced a decrease in their accumulation. In contrast, all treatments promoted a decrease in gibberellin

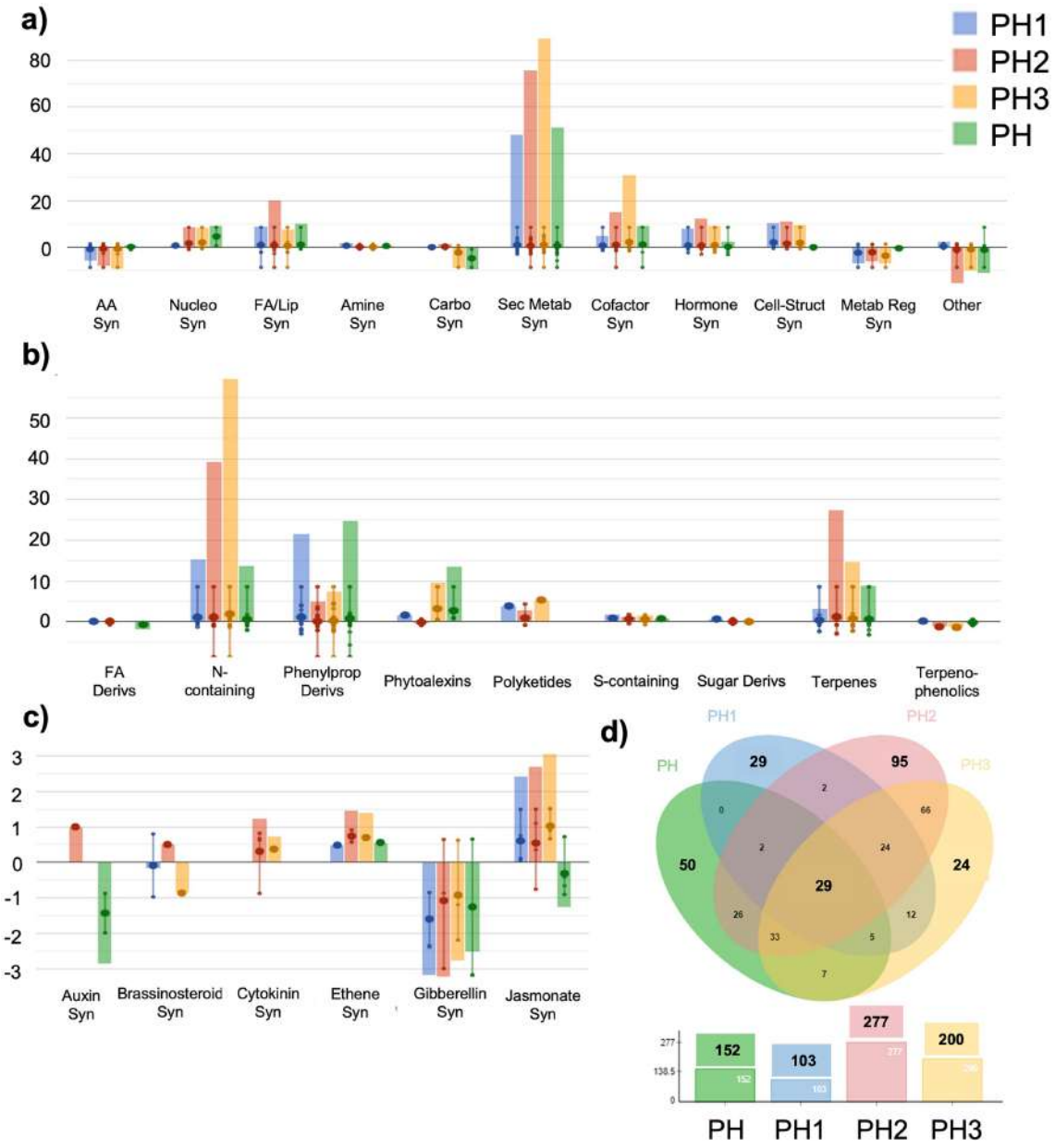

FIGURE 3 Biosynthetic pathways modulated by biostimulants on lettuce plants grown under low N condition (a). Secondary metabolism (b). Phytohormone (c). Venn diagram indicating peculiar and commonly modulated pathways following different treatments (d).Significative compounds modulated by PH and its fractions PH1, PH2 and PH3 (ANOVA Tukey HSD $p = 0.05$, FC cut-off 1.5).

levels, mediated by the precursor *ent*-kaurenol (logFC = −1.20 – −0.85) and gibberellin A_{25} (logFC = −3.17 – -2.20). Concerning the accumulation of cytokinins, positive effects were exclusively attributed to low-molecular-weight fractions for *trans*-zeatin, dihydrozeatin, and isopentenyladenine-7-*N*-glucoside (logFC values ranging 0.35–0.82 for PH2 and PH3). Interestingly, the same treatments promoted the accumulation of ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC, logFC = 0.57–0.66).

Considering primary metabolism, amino acid accumulation was slightly modulated by biostimulant treatments. For instance, the treatments involving PH fractions induced the accumulation of Gln (logFC = 0.75–1.05), suggesting an enhanced mobilization of N under low supply conditions, as well as a marked accumulation of Leu (logFC = ∼ 1.50 for all treatments), as well as the accumulation of the Pro catabolite *(S)*-1-pyrroline-5-carboxylate by PH2 and PH3 (logFC = 1.53 and 1.39, respectively). In the same way, different components of cellular membranes were significantly affected by biostimulants. Indeed, glycolipids (mono/di-galactosyldiacilglycerols) and a phosphatidylglycerol (18:2–16:0 PG) accumulated in treated plants, with PH2 promoting the strongest effect. Indeed, the same treatment elicited the accumulation of long-chain unsaturated fatty acids, such as (11Z,14Z,17Z)-icosatrienoyl-CoA, (2E,7Z,10Z,13Z,16Z,19Z)-docosahexaenoyl-CoA, and (3R,5Z,8Z,11Z,14Z,17Z)-3-hydroxydocosapentaenoyl-CoA (logFC = 0.49, 0.62, and 1.02, respectively for PH2-treated plants).

Other compounds elicited by biostimulants included vitamins, which were mostly accumulated following PH3 treatment, *i.e.*, riboflavin and 7,8-dihydroneopterin 3′-triphosphate, a folate precursor (logFC = 8.55 for both compounds).

3.3 | RNA-seq analysis on lettuce plants

Based on the results presented above, the effects of protein hydrolysates PH and its fraction PH3 on lettuce plants' transcriptome, grown under different N availability, were investigated through an RNA-seq analysis. The gene expression quantification pointed out the presence of genes uniquely expressed in each treatment. In particular, in the Optimal N provision, PH and PH3 have respectively 239 and 394 genes differentially expressed between them and compared to the control, which has 215 genes uniquely expressed. On the other hand, all the samples share 18737 genes that do not show any modification in their expression level (Figure 4A).

In low N conditions, the number of genes co-expressed in control lettuce plants and biostimulated ones remains almost the same as before (18649). However, control plants in low N conditions showed an increased number of differentially expressed genes equal to 439. Plants treated with PH and PH3 had an opposite trend compared to plants grown in optimal N conditions since they had 178 and 273 genes showing changes in the expression level, respectively (Figure 4B).

In optimal N conditions, the PH treatment significantly modulated gene expression, whereby 793 and 525 were up-regulated and down-regulated, respectively, as compared to control plants (Figure 5A).

Differently, plants treated with the fraction PH3 and subjected to optimal N supply showed 2891 genes significantly up-regulated, whilst 2171 were down-regulated (Figure 5B). In low N conditions, instead, the PH biostimulant and its fraction PH3 showed a comparable impact on the modulation of lettuce transcriptome, inducing the up-regulated of 3213 and 3049, respectively, and the down-regulation of 2260 and 2166, respectively (Figure 5C and D).

3.3.1 | Functional analysis of differentially expressed genes in optimal nitrogen condition

The gene ontology (GO) enrichment approach was applied as a classification system to determine which biological functions are significantly associated with DEGs. The genes reported in the following sections were selected among those showing a |log2FC| ≥ 2 and belonging to the molecular function (MF) and biological process (BP) terms of GO. By representing the 30 most significant GO terms (padj <0.05) in plants treated with PH under optimal N supply conditions, a significant impact on the BP category, encompassing *cell recognition*, *pollination*, *pollen-pistil interaction*, *multi-multicellular organisms process*, *recognition of pollen*, *multi-organism process*, *reproduction*, *reproductive process* and *multicellular organismal process* terms, was observed (Figure 6A and Supplementary Table S6). For the terms highlighted, 16 annotated genes showed statistical significance in the differential expression, except for the *multicellular organismal process* term, which featured 17 annotated genes. Among the DEGs, 14 genes were down-regulated, highlighting how all these biological processes were decreased with the PH treatment (Supplementary Table S6). In particular, the genes *LSAT_3X42080* and *LSAT_2X133640* encoding for G-type lectin S-receptor-like serine/threonine-protein kinases showed a log2FC higher than 2 and lower than −2, respectively (Supplementary Table S7). The *response to oxidative stress* term was also significantly affected. In fact, nine out of eleven annotated genes were up-regulated, suggesting that PH could enhance the antioxidant activity of treated plants with respect to controls (Supplementary Table S6). Indeed, three genes belonging to this GO term encoding for peroxidases (*LSAT_2X92781*, *LSAT_9X84700* and *LSAT_5X85721*) had a log2FC ranging from 1.3 to 2.7, whilst one gene encoding for a basic peroxidase (*LSAT_8X111620*) had the log2FC ≤ −2 (Supplementary Table S7).

The same analysis performed on PH3-treated plants and grown in optimal N supply (Figure 6B) highlighted that the most significant

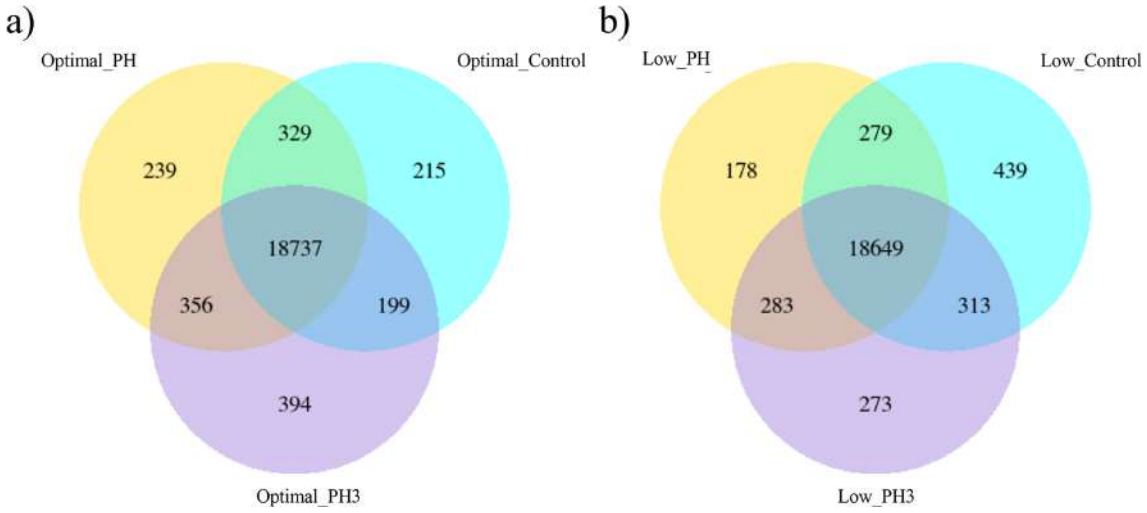

FIGURE 4 Venn diagrams reporting the number of DEGs in lettuce plants grown in either Optimal (a) or Low (b) N conditions and either not treated (Control) or treated with the biostimulant (PH) or its fraction (PH3).

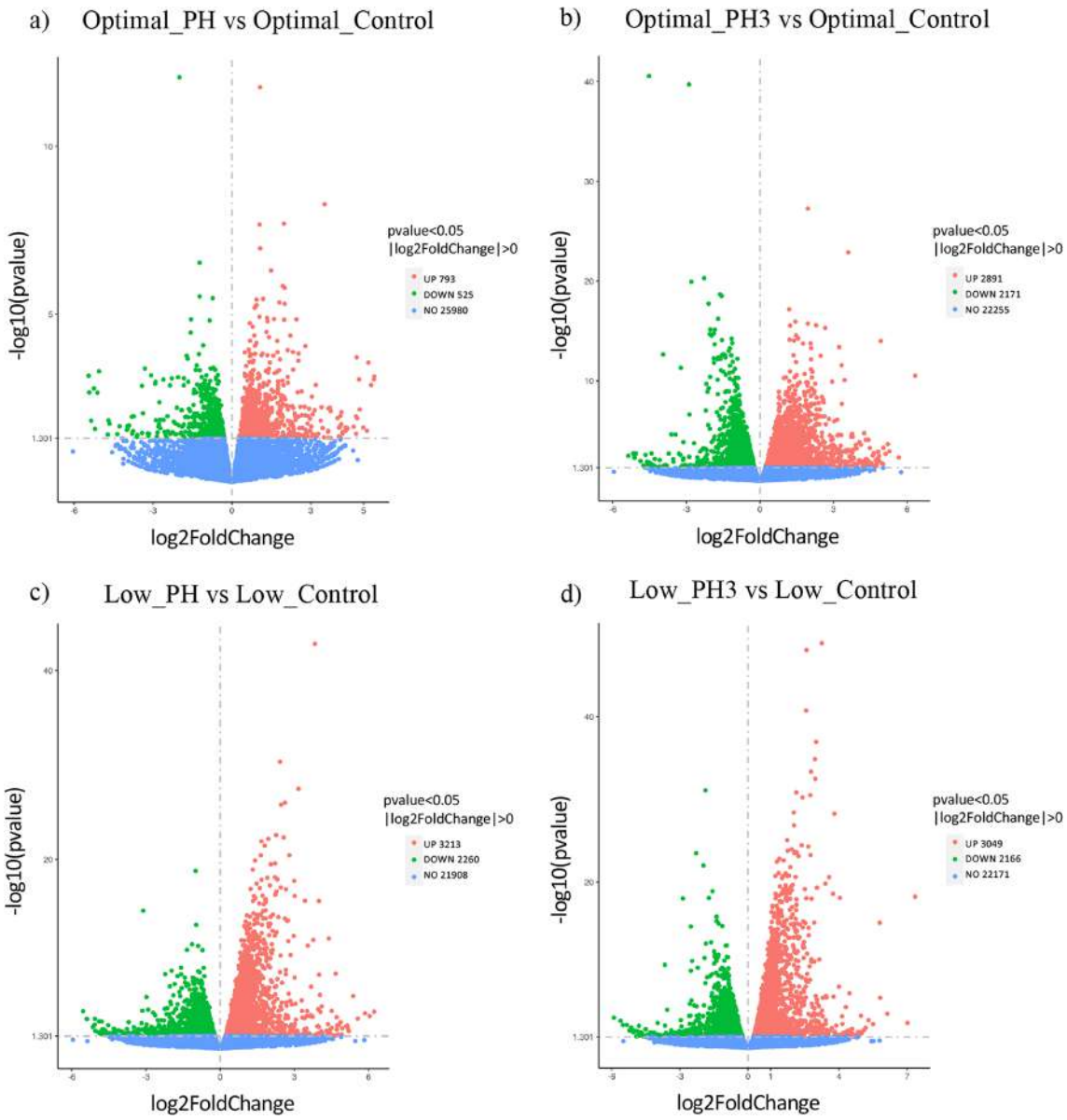

FIGURE 5 Volcano Plot of DEGs recorded in lettuce plants exposed to different N conditions and biostimulant treatments. (a) Optimal N, PH vs. Control, (b) Optimal N, PH3 vs. Control, (c) Low N, PH vs. Control, (d) Low N, PH3 vs. Control. The DEGs were selected using a threshold of |Log2FC| > 0 and P < 0.05. UP, upregulated; DOWN, downregulated; NO, not changed.

differences in gene expression were found in the BP term *response to auxin*, where 18 out of 23 annotated genes were down-regulated (Supplementary Table S8). Among these, six genes, namely *LSAT_8X126641*, *LSAT_5X60281*, *LSAT_8X127520*, *LSAT_9X20900*, *LSAT_8X127640*, encoded for small auxin up-regulated RNA (SAUR) proteins and one, *LSAT_8X127621*, encoded for auxin-induced protein 15A (Supplementary Table S7).

On the other hand, when the MF category was considered, the GO terms *transferase activity*, *transferring glycosyl groups* and *UDP-glycosyltransferase activity* featured the up-regulation of genes encoding for zeatin O-xylosyltransferases (*LSAT_5X147980* and *LSAT_5X160181*), zeatin O-glucosyltransferase (*LSAT_5X160161*), xyloglucan endotransglucosylase proteins (*LSAT_7X83841*, *LSAT_7X83821*, *LSAT_8X67301*, *LSAT_8X67360*, *LSAT_8X67401* and *LSAT_8X67381*), UDP-glycosyltransferases (*LSAT_4X130401*, *LSAT_4X130441*, *LSAT_1X89721*, *LSAT_5X24900*, *LSAT_5X24781*, *LSAT_4X130341*, *LSAT_9X16980* and *LSAT_4X152421*) and cellulose synthase-like proteins (*LSAT_9X47581* and *LSAT_5X2221*) (Supplementary Table S7).

3.3.2 | Functional analysis of differentially expressed genes under low nitrogen conditions

In low N conditions, the GO enrichment analyses (Figure 7) highlighted common features in lettuce plants treated with PH and PH3. Indeed, it was highlighted that the BP terms *translation*, *peptide biosynthetic process*, *amide biosynthetic process*, *peptide metabolic process* and *cellular amide metabolic process* were significantly affected by

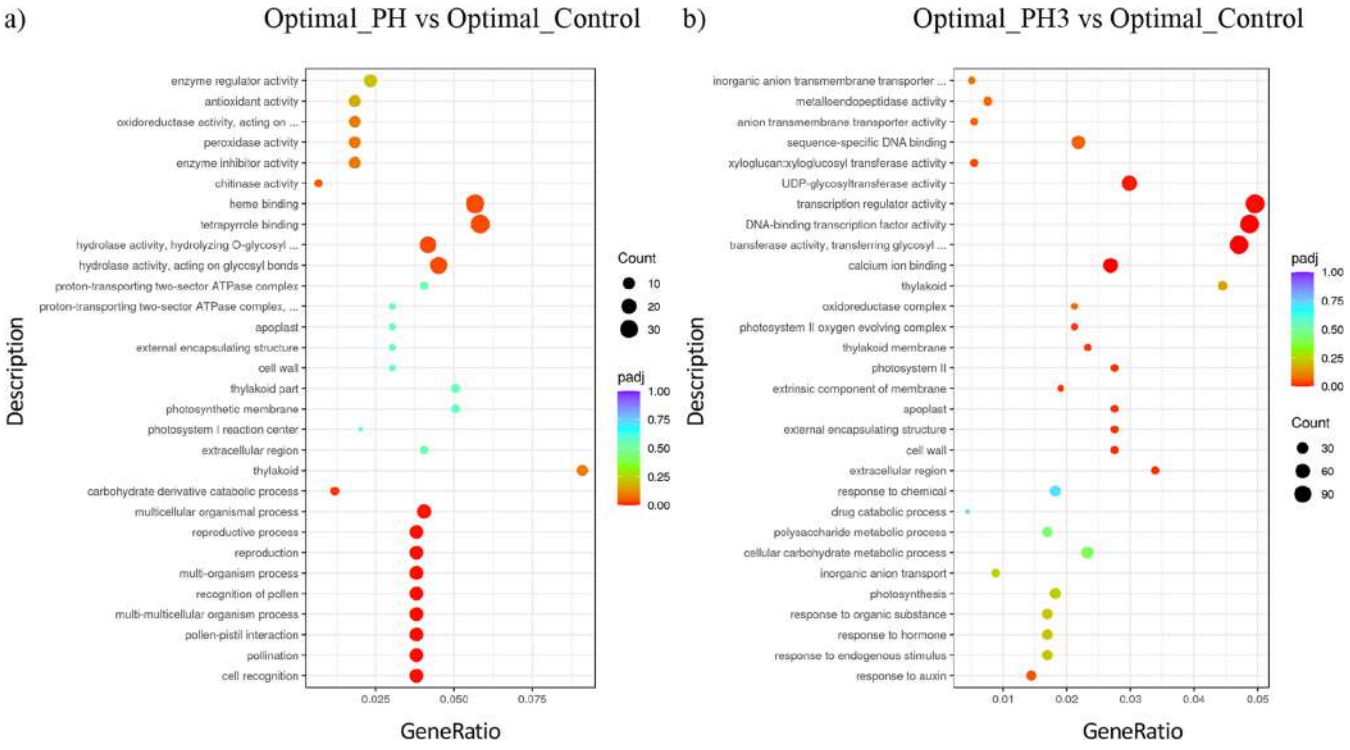

FIGURE 6 Gene Ontology Enrichment analyses for plants grown in Optimal N condition. (a) GO Enrichment analyses in plants treated with PH with respect to Control plants. (b). GO Enrichment analyses in plants treated with PH3 with respect to Control plants. The top 30 ranked GO terms according to gene count, adjusted p-value and gene ratio. 'Gene count' is the number of genes enriched in a GO term. 'Gene ratio' is the percentage of total DEGs in the given GO term.

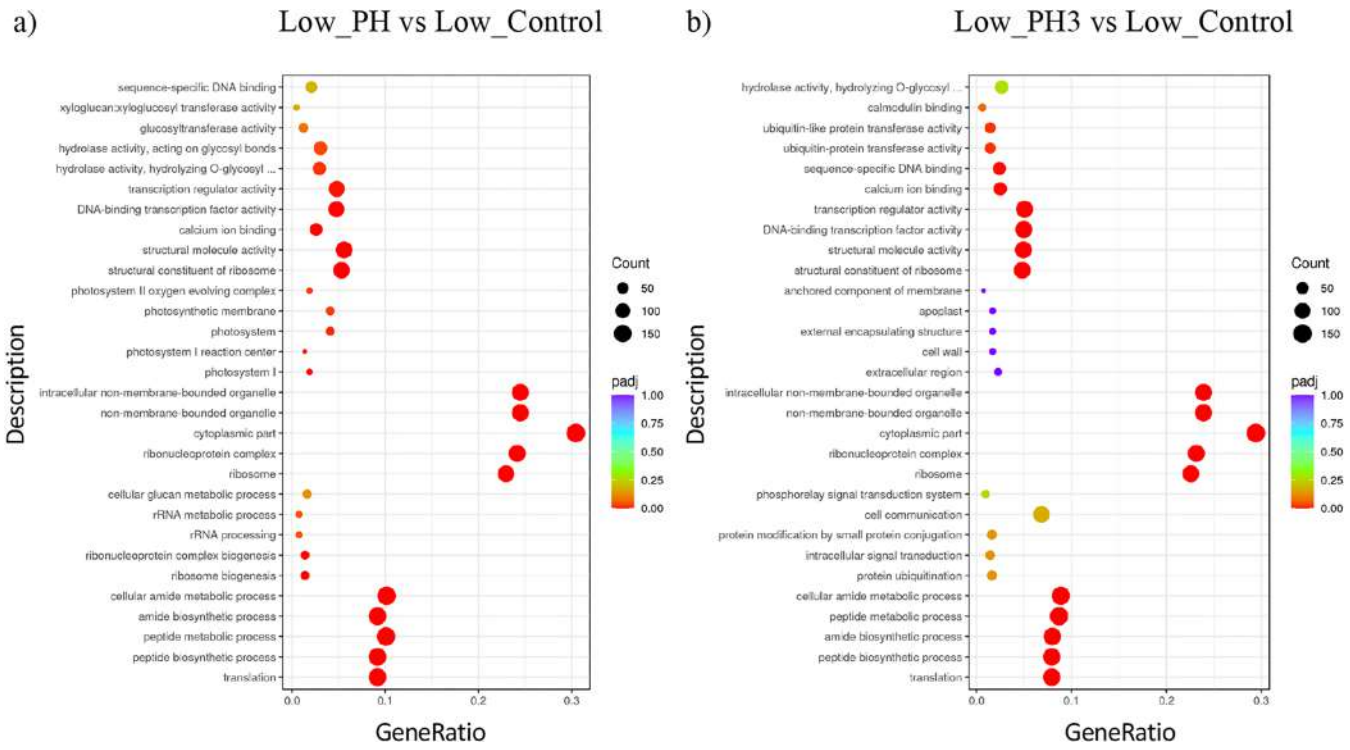

FIGURE 7 Gene Ontology Enrichment analyses for plants grown in Low N condition. (a) GO Enrichment analyses in plants treated with PH with respect to Control plants. (b). GO Enrichment analyses in plants treated with PH3 with respect to Control plants. The top 30 ranked GO terms according to gene count, adjusted p-value and gene ratio. 'Gene count' is the number of genes enriched in a GO term. 'Gene ratio' is the percentage of total DEGs in the given GO term.

PH and PH3, which induced a general up-regulation of 80 and 95% of the genes detected for each group, respectively (Table S9 and S10). In particular, within these GO terms, genes encoding for probable glutathione S-transferase (*LSAT_7X84721* and *LSAT_8X81900*) and alkaline ceramidase (*LSAT_1X72081*) were up-regulated, whereas a peptide chain release factor (*LSAT_8X83140*), a glutathione S-transferase T1 (*LSAT_6X5961*) and a ribosomal protein S11 (*LSAT_6X69061*) were down-regulated (Supplementary Table S7). In addition, also the GO terms *structural constituent of ribosome* and *structural molecule activity* resulted in 83 and 84%, and 98 and 97% of up-regulated genes after PH and PH3-treated plants, respectively (Table S9 and S10). On the contrary, the up-regulation of genes belonging to BP terms *ribosome biogenesis*, *ribonucleoprotein complex biogenesis*, *rRNA processing* and *rRNA metabolic process* terms was detected in PH-treated plants, suggesting that the biostimulant enhanced the ribosomal activity and possibly the protein content (Table S9).

The MF terms *sequence-specific DNA binding*, *ubiquitin-protein transferase activity*, *ubiquitin-like protein transferase activity*, instead, are significantly affected only in samples treated with PH3 fraction with the up-regulation of 65% of genes belonging to the first group, and of 64% of genes of the others two (Supplementary Table S10). Within the *sequence-specific DNA binding* term, genes encoding for a protein responsive to ABA and salt (*LSAT_9X35220*) and for a protein ZW2 (*LSAT_6X107880*) had a log2FC ≥2, whilst a gene coding for DOG1-like protein (*LSAT_9X99800*) was down-regulated (Supplementary Table S7). Regarding the *ubiquitin-protein transferase activity* and *ubiquitin-like protein transferase activity*, instead, only one gene encoding for a ubiquitin-protein ligase PUB23 (*LSAT_6X33780*) was up-regulated (Supplementary Table S7).

3.3.3 | Common responses to biostimulants irrespective of N nutrition

Despite the differences highlighted previously, the treatment with PH and PH3, independently from the N levels in the growth medium, could also induce similar responses in the modulation of gene expression. In this context, the treatment with PH affected the regulation of genes belonging to the GO terms *hydrolase activity*, *acting on glycosyl bonds*, *hydrolase activity*, *hydrolysing O-glycosyl compounds*, *tetrapyrrole binding* and *heme binding* of the MF category. For all these groups, most of the differentially regulated genes were up-regulated (Supplementary Table S6 and S9). Within the *hydrolase activity* and *tetrapyrrole and heme binding* term, four genes encoding for xyloglucan endotransglucosylase proteins (*LSAT_7X83841*, *LSAT_7X83821*, *LSAT_8X67360* and *LSAT_8X67301*) and six genes encoding for three cytochrome P450 (*LSAT_5X97860*, *LSAT_4X120220*, and *LSAT_6X40941*), two peroxidases (*LSAT_5X95980* and *LSAT_5X85721*) and one geraniol 8-hydroxylase (*LSAT_7X47061*), respectively, were up-regulated. Conversely, within the same terms, a germacrene A hydroxylase (*LSAT_3X61420*), a basic peroxidase (*LSAT_8X111620*) and a polygalacturonase encoding genes were down-regulated (Tables S6, S7 and S9).

Similarly, the treatment with PH3 significantly affected the MF GO terms *DNA-binding transcription factor activity* and *transcription regulator activity* by upregulating approximately 80% of the annotated genes, independently of N levels (Table S8 and S10). Among these, ethylene-responsive transcription factors (*LSAT_6X81041*, *LSAT_5X108800*, *LSAT_6X92940*, *LSAT_2X130801*, *LSAT_2X130761*, *LSAT_2X107800*, *LSAT_6X13121*, *LSAT_6X84541*, *LSAT_6X92840*, *LSAT_7X39941*, *LSAT_9X41960*, *LSAT_4X138540*, *LSAT_3X5680*, *LSAT_1X5340*, *LSAT_2X130781*, *LSAT_2X130880*, *LSAT_2X109761*, *LSAT_2X133380*, *LSAT_4X46601*, *LSAT_4X166640*, *LSAT_4X166720* and *LSAT_3X121961*), heat stress transcription factor (*LSAT_8X6800*, *LSAT_5X185701*), dehydratation-responsive element-binding proteins (*LSAT_9X54101*, *LSAT_7X3988*, *LSAT_9X53921* *LSAT_6X13081*, *LSAT_6X41680*, *LSAT_2X130641* and *LSAT_6X13081*), a heat shock factor protein (*LSAT_5X48320*) and probable WRKY transcription factors (*LSAT_7X15520*, *LSAT_6X220* and *LSAT_8X147781* and *LSAT_8X147781*) can be listed. Within these terms only one gene encoding for an ethylene-responsive transcription factor WIN1 (*LSAT_1X44740*) was down-regulated (Table S7, S8 and S10).

3.4 | Multi-omics integration of metabolomic and transcriptomic datasets

The outputs for both metabolomic and transcriptomic approaches were integrated through the DIABLO framework. The results of such integrative analysis are displayed in Figure 8. In the case of optimal N conditions, the model indicated that both datasets showed a high correlation for the two optimized components of 0.96 and 0.97 (Supplementary Figure S3A). For each block contribution (Figure 8A), the first major component allowed discriminating PH3 from the rest of the treatments (control and PH), whereas the second component provided a clear separation between all groups. In general terms, the integrative cluster showed a clear segregation for PH3 treatment into a specific subcluster, being unable to discriminate between PH and the control (Figure 8B), suggesting that PH3 played the highest impact on both metabolome and transcriptome of lettuce plants under optimal N conditions. Due to the supervised nature of DIABLO modelling, the variables showing the highest discrimination power between treatments for both approaches were obtained (Supplementary Table S11). As expected, the markers showing the highest influence due to the first component are greatly associated with PH3, whereas the markers derived from the second component allowed digging into the differences between all groups.

Thus, the metabolite markers mainly associated with PH3 support the previous results on the accumulation of secondary metabolites, being represented by NCCs, essentially glucosinolates like 3-(methylsulfanyl)propanoate and *p*-hydroxymandelonitrile, and alkaloids, such as berbamurine and harmol. Moreover, a wide range of polyphenols were also attributed to PH3 treatment, including flavonoids, *i.e.*, baicalin, kaempferide, and apigenin 7-*O*-neohesperidoside, and lignans, for instance, (−)-pluviatolide and (−)-medicarpin-3-*O*-glucoside. PH3-associated phytohormones covered different

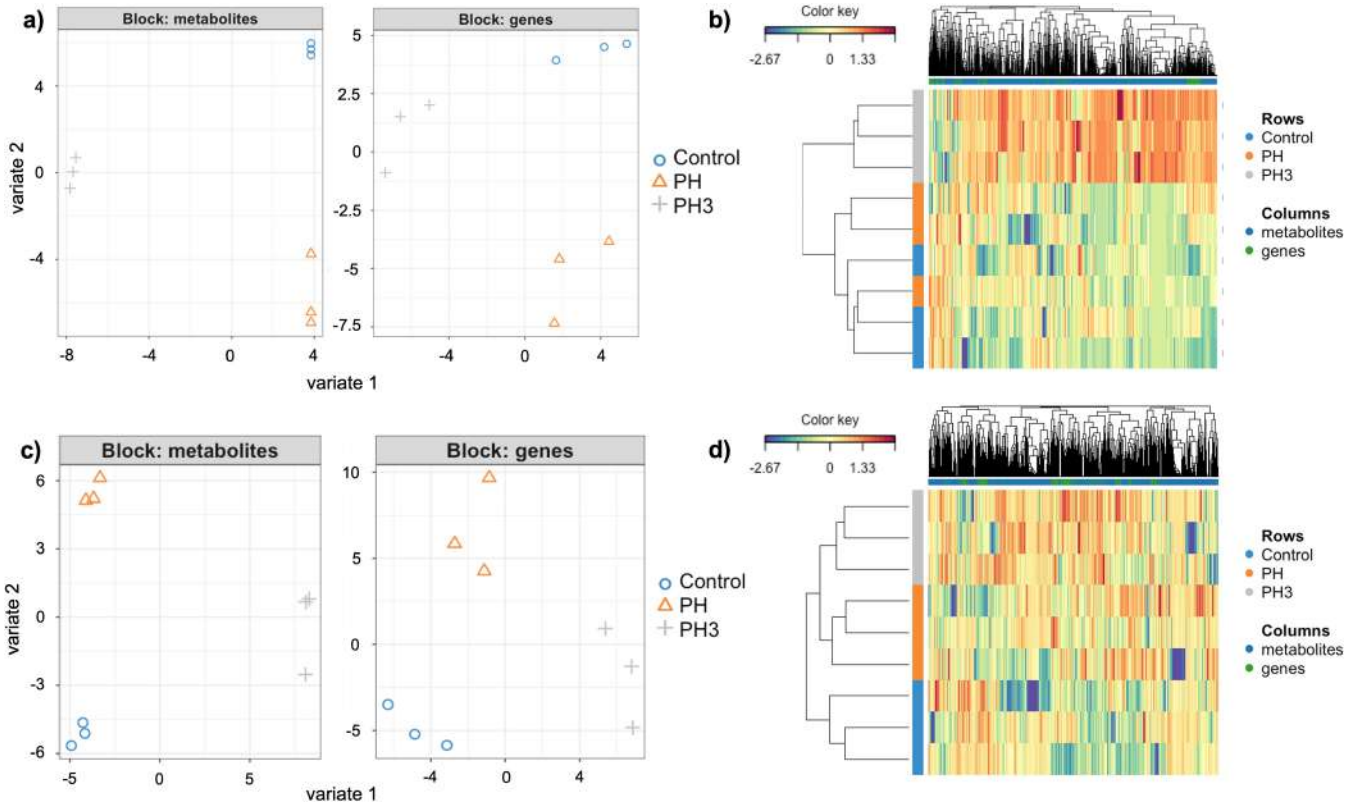

FIGURE 8 (a) Block contributions for metabolomics and transcriptomics outputs discriminating among control, PH, and PH3 treatments in lettuce plants grown under Optimal N conditions. (b) Heatmap-based cluster co-ordinately involving metabolomics and transcriptomics outputs in lettuce plants grown under Optimal N conditions. (c) Block contributions for metabolomics and metagenomics outputs discriminating among control, PH, and PH3 treatments in lettuce plants grown under Low N conditions. (d) Heatmap-based cluster co-ordinately involving metabolomics and transcriptomics outputs in lettuce plants grown under Low N conditions.

subfamilies, ranging from IAA-derivatives (for example, (indol-3-yl)acetyl-L-Trp and (indol-3-yl)acetyl-L-Pro), and gibberellins, which were found previously accumulated in PH3-treated lettuce plants, whereas brassinosteroids, as homobrassinolide and homocastasterone derivatives were negatively associated to PH3 treatment. In contrast, the markers represented by primary metabolites included a wide range of carbohydrates, such as phytate, galactopinitol, and dithioerythritol, and amino acids and derivatives, *i.e.*, L-pipecolate, histidinal, and *O*-phospho-L-homoserine, among others. According to the discrimination with respect to PH and the control, PH showed a low number of associated biomarkers, suggesting its intermediate effect between PH3 and the control. The latter is mostly associated with carotenoids, which were previously found to show decreased levels in the control treatment.

These responses found at a metabolic level were combined with those at the transcriptomic level, where a heterogeneous group of genes resulted in differentially expressed due to PH3 treatment, as mostly shown for genes encoding for UDP-glycosyl transferases (*LSAT_5X24781*, *LSAT_9X16980*, *LSAT_1X25561*, *LSAT_6X28801*, *LSAT_6X28780*, *LSAT_1X52580*, *LSAT_2X126380*, *LSAT_6X28821*, *LSAT_9X12380*) and zeatin-O-glycosyltransferases (*LSAT_5X147980*, *LSAT_5X160161*, *LSAT_5X162041*). Moreover, PH3 was positively associated with the expression of ethylene-responsive transcription factors (*LSAT_6X92840*, *LSAT_9X50660*, *LSAT_2X126980*, *LSAT_5X182281*, *LSAT_6X92900*, *LSAT_6X92940*, *LSAT_2X130781*, *LSAT_3X10461*, etc.) and heat stress transcription factors (*LSAT_4X48361*, *LSAT_1X50141*, *LSAT_7X25320*), as previously described. In parallel, other gene ontologies were found to be associated with PH, showing higher expression rates for genes encoding for different peroxidases (*LSAT_5X175321*, *LSAT_2X66161*, *LSAT_2X92781*), as well as cytochromes (*LSAT_9X121260*, *LSAT_5X63400*, *LSAT_4X120220*, *LSAT_8X12301*). Finally, control plants were found to reflect specific gene markers involved in G-type lectin S-receptor-like serine/threonine-protein kinase (*LSAT_3X41281*, *LSAT_1X68181*, *LSAT_5X5521*).

In parallel, another model was developed for plants grown in low N conditions, in which performance was assessed through high-quality correlation parameters for the two previously optimized components, 0.96 and 0.95 (Supplementary Figure S3B). As it occurred under optimal N conditions, the independent block contributions (Figure 8C) showed a coordinated outcome between metabolomic and transcriptomic approaches, showing a PH3 discrimination through the first component, whereas all treatments were discriminated accordingly to the second component. In this case, under low N conditions, the integrative cluster (Figure 8D) indicated that PH3 was independently clustered, whereas control and PH were efficiently separated within the same subcluster, suggesting an exclusive

PH3-guided response at both metabolomics and transcriptomics levels. The analysis of the markers associated with the two principal components (Supplementary Table S12) highlighted that the first component mostly featured PH3-associated markers, whereas those attributed to PH and control were mostly derived from the second component. Concerning PH3, the majority of metabolic markers were represented by: 1) NCCs, which were previously found accumulated in this treatment, essentially glucosinolates (3-(methylsulfanyl)propyl-desulfoglucosinolate, and 3-carboxy-9-(methylsulfanyl)-2-oxononanoate) and alkaloids like (S)-nandinine; 2) terpenoids, such as the carotenoids β-D-gentiobiosyl crocetin, and 8′,10-diapocarotene-8′,10-dial; 3) vitamins and cofactors, such as FMN and 7,8-dihydroneopterin 3′-triphosphate. With respect to polyphenols, they were differentially associated with both PH3 and PH treatments, as described earlier. Thus, flavonoids like kaempferol 7-*O*-glucoside and quercetin 3-*O*-rhamnoside were associated with PH3 treatment, and quercetin 7-*O*-glucoside was associated with PH together with the anthocyanin delphinidin-3-*O*-(6''-*O*-malonyl)-β-glucoside-3'-*O*-β-glucoside and the isoflavone ononin, suggesting a diversified response on phenylpropanoid biosynthesis. In contrast, control samples were associated with gibberellins and precursors, as found for gibberellin A_{25} and *ent*-kaurenol, respectively, being in accordance with the highest presence of this group of phytohormones with respect to treated plants.

The response at the transcriptomic level was coordinated with that of metabolomics, supporting the previous evidence on the effect of PH treatments on lettuce plants under optimal N conditions. Thus, PH3 was associated with the up-regulation of the expression of genes mostly involved in glutathione S-transferases (*LSAT_9X46740*, *LSAT_5X120361*, *LSAT_6X29540*), ethylene-responsive transcription factors (*LSAT_6X13161*, *LSAT_1X125981*, *LSAT_4X166540*, *LSAT_5X136680*), heat shock factor proteins (*LSAT_5X48320*, *LSAT_4X165921*), and heat stress transcription factors (*LSAT_8X6800*, *LSAT_5X185701*, *LSAT_2X133880*, *LSAT_3X8400*). Interestingly, the model identified several markers involved in the expression of calcium-calmodulin system associated with PH3 (*LSAT_5X159920*, *LSAT_3X53980*, *LSAT_8X148521*, *LSAT_2X106341*, *LSAT_6X94740*, *LSAT_9X95381*, *LSAT_2X87480*, *LSAT_3X113400*, *LSAT_9X7881*, *LSAT_5X91960*, among others). PH and control showed an influence to a much lesser extent at a transcriptomic level, with PH-associated markers encompassing the expression of WRKY transcription factor (*LSAT_4X48480*) and dehydration-responsive element-binding protein (*LSAT_7X39881*), whereas the control was associated with a wide range of genes encoding for chloroplast ribosomal proteins (for instance, *LSAT_6X113160*, *LSAT_2X91740*, *LSAT_6X108161*).

4 | DISCUSSION

4.1 | PH fractions enhanced lettuce growth and antioxidant activities

Nitrogen is an essential macro-element for plants (Djidonou and Leskovar 2019), and its limited availability has a negative impact on the growth and yield of sensitive plants, for instance, lettuce (*Lactuca sativa* L.) (Broadley et al. 2000; Geary et al. 2015). However, it is important to emphasize that N deficiency in lettuce is more detrimental in the later growth stage of the crop cycle than in the early ones. According to previous data (Fallovo et al. 2009; Djidonou and Leskovar 2019), our results confirm that by reducing N levels (Low; 1 mM NO_3^-), leaf area and shoot dry weight decreased as compared with plants grown in optimal N conditions (8 mM NO_3^-). This could be related to the beneficial effects of N on the absorption and utilisation of photosynthetically active radiation (Djidonou and Leskovar 2019), as also suggested by the increased net CO_2 assimilation (A_{CO2}) and SPAD index in optimal N conditions (Miras-Moreno et al. 2020; Yue et al. 2020).

The improvement in biometric indices (leaf area and shoot dry weight) following the application of plant-derived protein hydrolysates is in line with the results shown in recent pieces of research (Carillo et al. 2022; Ciriello et al. 2022a; Giordano et al. 2022; Zuluaga et al. 2023). Specifically, except for PH2 treatment, all biostimulants evaluated in this work improved leaf area compared to the control, regardless of N level. As already reported (Rouphael et al. 2018), biostimulant products trigger a range of physiological and molecular mechanisms under suboptimal growth conditions, including i) the production of phytochemical substances, ii) the translocation and absorption of nutrients, and iii) the enhancement of photosynthetic performance. This latter is also partially confirmed by our results, particularly when the improvement of the net CO_2 assimilation (A_{CO2}) by the PH3 fraction is considered.

The increase in H_2O_2 and MDA (a general indicator of lipid peroxidation in the cell membrane) observed under N-limiting conditions is consistent with the results obtained in tomato and lettuce, respectively (Francesca et al. 2022; El-Nakhel et al. 2023b). As recently highlighted by Formisano et al. (2020), plants possess an efficient antioxidant system composed of enzymes such as CAT (catalase) and APX (ascorbate peroxidase), among others. As expected, in N-limiting conditions, the levels of CAT and APX were also more pronounced. However, although Candido et al. (2023) described an increase in the biosynthesis of antioxidant enzymes after applying biostimulants, in the present work, this pattern was only observed in the case of CAT, being more activated by the treatment with PH3 fraction.

4.2 | PH fractions affected both primary and secondary metabolism in lettuce

The results for the metabolomics-based profile of lettuce plants indicate that low-molecular-weight (LMW) fractions of PH biostimulants, PH2 and PH3, promoted significant metabolic reprogramming under optimal or low N conditions. The enhanced response attributed to these fractions may respond to a higher assimilation rate of LMW fractions during fertilization, as recently hypothesized in lettuce plants, which developed an increase in N use and uptake efficiency when treated with vegetal protein hydrolysates (Choi et al. 2022). Indeed, applying untargeted metabolomics suggested that PH-derived fractions promoted differential reprogramming at a metabolic level under both optimal and low N conditions. In this sense, N metabolism

was significantly affected by the application of these biostimulants, involving N-containing primary metabolites, such as amino acids, nucleotides, and phytohormones, as well as secondary metabolites, for instance, glucosinolates and alkaloids, mainly. Interestingly, the biological activity associated with LMW hydrolysates was previously assessed to stimulate both carbon and nitrogen metabolism of lettuce under abiotic stress (Colla et al. 2017b), as demonstrated by our results on the accumulation of amino acid precursors. Due to the differential responses by PH biostimulants reported under optimal and low N conditions, multivariate and chemometric analyses were performed separately for each condition.

Thus, under optimal conditions, a combined resilient response integrating phytohormones and secondary metabolites accumulation was reported for LMW fraction-treated lettuce plants. With respect to phytohormone response, PH2 and PH3 promoted the accumulation of auxins, gibberellins and cytokinins, which are well-known to coordinately improve plant growth. Particularly, PH3 treatment also promoted the combined accumulation of jasmonates and brassinosteroids and led to a decrease in ABA precursors, which are considered stress-related phytohormones that work together to develop abiotic stress resistance (Peng et al. 2011). This specialized response related to stress mitigation has been recently reported in greenhouse-grown lettuce plants treated with legume-based protein hydrolysates under salinity stress (Rouphael et al. 2022). The modulation of secondary metabolism attributed to both fractions acts similarly since a diversified antioxidant response was observed in PH2- and PH3-treated plants. The accumulation of polyphenols, such as flavonoids and lignans, coupled with the decrease in methoxylated flavonoids, indicates an efficient mobilization of this family of phenolic compounds with associated antioxidant activity (Laoué et al. 2022). Moreover, the accumulation of intermediates of the ascorbate-glutathione system and vitamins with antioxidant properties (like vitamin E and B6) by PH3 reinforces the induction of such a multifaceted antioxidant response, which constitutes an effective mechanism to prevent the development of plant stress phenomena since they are generally guided by an initial onset of oxidative stress at a cellular level. In detail, according to previous studies (Zha et al. 2020), the induction of the ascorbate-glutathione cycle is an efficient mechanism to cope with light-induced oxidative stress by lettuce plants. Besides this antioxidant response, the resilient response mainly observed in PH3-treated lettuce plants also involved the accumulation of stress-related specialized metabolites, *i.e.*, glucosinolates, terpenoid-indole alkaloids, and carotenoids, which mainly play defensive roles in plants thanks to their antimicrobial properties. Because of the associated biological activities of these metabolites, the application of biostimulants has been recognized as a solid strategy to improve the nutritional value of lettuce (Ćavar Zeljković et al. 2023).

As stated earlier, the application of PH-based biostimulants on lettuce plants under low N stress drove a different response at a metabolic level. In this case, all treatments, including PH and its fractions, conferred significant metabolic signatures on lettuce plants, affecting both primary and secondary metabolism in a fraction-dependent manner. Nitrogen metabolism was significantly affected under these conditions, as all PH fractions promoted an accumulation of glutamine (Gln), an amino acid closely related to N assimilation in plants. Consequently, such a response represents an effective mechanism to mitigate the N deficiency-driven stress by lettuce, driven by a decrease in glutamine synthase activity (Zhou et al. 2021). In parallel, the application of biostimulants exhibited a marked impact on the phytohormone profile of lettuce plants under low N supply. Thus, all PH-treated plants were found to show a marked decrease in the levels of gibberellins and their precursors, regardless of the molecular weight characterizing the specific PH fraction. This observation could be motivated by the fact that gibberellins play a critical role in the regulation of nitrogen uptake (Wang et al. 2020) and, therefore, their biosynthesis could be eventually repressed under low N availability as a mechanism to optimize the limited resources of lettuce plants in these conditions. In contrast, PH2 and PH3 fractions drove the accumulation of key phytohormones, *i.e.*, cytokinins and jasmonates, thus performing highly efficient mitigation of plant stress coupled with promoting plant growth. In this sense, the combination of jasmonates and cytokinins was established as a mitigation mechanism in wheat seedlings under oxidative stress (Avalbaev et al. 2021), suggesting that this coordinated response could also be observed in this case following PH treatment. At the same time, the accumulation of glucosinolates and proline (Pro) catabolites mediated by these LMW fractions reinforces the hypothesis of the efficient mitigation of stress in lettuce plants. Indeed, the catabolism of proline could be regarded as a dual solution by lettuce plants under N deficiency since it may lead to the dissipation of stress-related events and serve as an endogenous source for the biosynthesis of other amino acids, such as glutamate (Maswada et al. 2021). Interestingly, 1-Aminocyclopropane 1-Carboxylic Acid (ACC), considered the direct precursor of ethylene, was also found accumulated in plants treated with LMW fractions. Although ethylene has been classically recognized as a stress-promoting phytohormone in leaf tissues, some authors have claimed the role of ACC as an ethylene-independent growth regulator and stress-releasing mediator in a series of plant species (Polko and Kieber 2019), which is supposed to play that role in the present study under PH treatment.

The PH fraction-dependent biostimulant effect reported in lettuce under low N conditions also affected the antioxidant systems. In this sense, while PH and PH1 fractions efficiently improved the accumulation of polyphenols, PH2 and PH3 fractions promoted a diversified and multifaceted antioxidant response. In the case of PH2 fraction, an enhanced lipid profile mediated by the accumulation of glycolipids and polyunsaturated fatty acids was reported. Recent reports highlight the implication of membrane-associated lipids remodelling under nutrient stress in different lettuce cultivars to contribute to enhancing photosynthetic performance and plant growth (Körber et al. 2023). Finally, in the case of PH3 fraction-treated lettuce plants under stress, an accumulation of vitamin B2 and folate pathway intermediaries was observed. Interestingly, the accumulation of B vitamins is also recognized as a stress-releasing mechanism in plants, motivated by their role as cofactors in important physiological features, such as C and N metabolism or photosynthesis, as well as playing a secondary

role as antioxidants thanks to their protective properties against oxidative stress (Hanson et al. 2016).

4.3 | RNAseq highlighted common and peculiar responses in lettuce plants treated with protein hydrolysates

4.3.1 | Optimal Nitrogen conditions

Lettuce plants treated with PH and grown under optimal N regime showed a significant up-regulation of 793 genes over the total number of genes differentially expressed. The PH impacted the transcription of genes involved in the *cell recognition* pathway. In particular, the modulation of two different G-type lectin S-receptor-like serine/threonine protein kinases was induced, most likely involved in different responses in dicots plants (Dombrowski and Martin 2012). Protein kinases are known for their role in plant development and response to abiotic stress (Dombrowski and Martin 2012), and, in particular, receptor-like protein kinase (RLK) family have been demonstrated to play an active role in several physiological processes, including plant growth (Hématy and Höfte 2008; Nibau and Cheung 2011).

In addition, different peroxidases and cytochrome P450 (CYP) genes, belonging to the *response to oxidative stress*, were upregulated in plants treated with PH. These genes are included in t*etrapyrrole* and *heme binding* terms since heme, a well-known member of the tetrapyrrole group (Layer et al. 2010), acts as a prosthetic group of many enzymes, including peroxidases and CYPs (Matsunaga and Shiro 2004). Class III peroxidases, widely present in the plant kingdom (Hiraga et al. 2001; Tognolli et al. 2002), have a pivotal role in many plants physiological processes, such as cell wall metabolism, lignification, suberization, fruit growth and ripening, seed germination, ROS metabolism and stress tolerance (Pandey et al. 2017). In this specific case, a significant over-expression of two peroxidases and a simultaneous down-regulation of a basic peroxidase indicate how the biostimulant application in adequate N supplementation could potentially have beneficial effects on plants due to the increase of antioxidant enzyme activity, thus stimulating/priming plant defence system (Alzate Zuluaga et al. 2022).

Four genes encoding for CYPs showed a significant up-regulation in lettuce treated with PH. CYPs are involved in several physiological processes in plants, including biosynthesis of structural polymers, defence against pathogens, herbicide resistance, stress tolerance and hormonal signalling (Minerdi et al. 2023). Focusing on this last feature, two of the three up-regulated CYPs are involved in hormonal pathways. In particular, cytochrome P450 734A1 belongs to a ubiquitarian subfamily in the plant kingdom and can inactivate brassinosteroid hormones (Neff et al. 1999; Turk et al. 2003; Ohnishi et al. 2006; Sakamoto et al. 2011; Thornton et al. 2011). On the other hand, cytochrome P450 94C1 is known to be involved in the jasmonic acid (JA)-isoleucine (Ile) conjugate oxidation, which has been demonstrated to be the major catabolic pathway for the JA-Ile hormone turnover (Heitz et al. 2012). Within the same gene super-family, the over-expression of geraniol 8-hydroxylase, membrane-bound cytochrome-P450 monooxygenase belonging to the CYP76 family (Sintupachee et al. 2015) was also detected. This enzyme has been suggested as a potential regulation site of secologanin production, which is involved in secondary metabolites biosynthesis, such as the monoterpenoid indole alkaloids and iridoid monoterpenoids pathways (Höfer et al. 2013). Within the same GO term, a significant simultaneous down-regulation of a germacrene A hydroxylase gene was detected. This is a key enzyme in costunolide biosynthesis, which belongs to the sesquiterpene lactone (STL) compounds and is used as an active principle of different drugs (Thakur et al. 2020).

The PH3 fraction, instead, induced the significant up-regulation of 2891 and the down-regulation of 2171 genes in lettuces in optimal N condition. A general down-regulation of auxin-responsive genes was recorded. In particular, six genes encoding for different small auxin up-regulated RNA (SAUR) proteins, identified as SAUR12, SAUR19 and SAUR51, were significantly repressed. Among these, three genes encoding SAUR19 proteins are known to play a pivotal role in cell expansion in *Arabidopsis* when over-expressed in elongating tissues (Spartz et al. 2012). Despite the majority of SAUR genes (approximately 70% in the *Arabidopsis* genome) induced by the phytohormone auxin, there is also a group of SAUR that is auxin-insensitive and rather regulated by other signals (van Mourik et al. 2017), as shown in different plant species. The characterization of SAUR in different plants (e.g. tomato, poplar, citrus, maize) highlighted that the different SAUR genes can show a peculiar expression pattern depending on the plant developmental stage (Stortenbeker and Bemer 2019). In addition, these studies demonstrated that SAUR genes can be either positively or negatively regulated by hormones, with auxin among them (van Mourik et al. 2017), as well as by environmental stressors (Stortenbeker and Bemer 2019). Therefore, based on these pieces of information, it cannot be ruled out that PH3 could have exerted the auxin-like activity as previously demonstrated (Colla et al. 2014).

Differently, the PH3 treatment promotes a significant up-regulation of several genes involved in *DNA-binding transcription factor activity* and *transcription regulator activity*. As previously demonstrated, the activation of transcription factors could mediate the plant response to biostimulant application (Wilson et al. 2015). In our case, we observed the up-regulation of genes belonging to the APETALA2/ethylene response factor (ERF) transcription factor superfamily, and in particular, genes encoding for ethylene-responsive transcription factors (ERFs) and dehydration-responsive element binding factors (DREB). Noteworthy, ERFs and DREB are accounted as members of the APETALA2/ERF superfamily (Sakuma et al. 2002). These regulatory proteins are generally involved in the control of primary and secondary metabolism, as well as in response to biotic and abiotic stress (Licausi et al. 2013).

Moreover, among the up-regulated genes, a probable WRKY and heat stress transcription factors that are generally involved in response to different abiotic stresses (Guo et al. 2016; Jiang et al. 2017), together with an *abscisic acid insensitive 5* (*ABI5*) protein which belongs to the basic leucine zipper (bZIP) family (Collin

et al. 2021) have been observed. Interestingly, only one gene belonging to the *transcription activity* term, *i.e.*, ERF WIN1 transcription factor, showed a significant down-regulation. Interestingly, ERF WIN1 has been demonstrated to promote epidermal wax deposition when over-expressed in *Arabidopsis* plants (Broun et al. 2004). Indeed, epidermal wax is important in plants' water retention and repelling atmospheric water (Riederer and Schreiber 2001).

Within the term *transferase activity*, PH3 induced the significant over-expression of genes involved in different biological functions. Among these, three genes were found to encode enzymes involved in zeatin metabolism, in particular, two for zeatin *O*-xylosyltransferase and one for zeatin *O*-glucosyltransferase. Considering that zeatin is a plant hormone belonging to the cytokinin family, the up-regulation of these enzymes is committed to regulating the level of active cytokinins in plants (Mok et al. 2000), further demonstrating that biostimulant applications could affect the plant hormone metabolisms as shown previously (Colla et al. 2014). In this case, it is worth mentioning that plant hormone-like activity is retained by the PH3 fraction, which is characterized by the compounds with the lowest molecular weight.

The application of PH3 also induced the up-regulation of different members of the UDP-glucosyltransferase (UGT) gene family. The UGT are key enzymes in the flavonoid pathway for anthocyanins synthesis (Peng et al. 2016). In detail, UGT 89B2 was annotated as a potential contributor to the accumulation of glycosylated linalool, hotrienol, α-terpineol, geraniol, and cis-rose oxide in grape (Wang et al. 2021). In contrast, UGT 85C2 and 74G1 might be possibly involved in the steviol glycosides biosynthesis pathway (Ghaheri et al. 2019). The up-regulation of UGTs further confirms that the biostimulant application, particularly the fraction PH3, could positively affect the plant's secondary metabolism and flavonoid accumulation (Lucini et al. 2015).

Furthermore, both PH and PH3 induced the up-regulation of genes encoding for xyloglucan endotransglucosylase/hydrolases proteins (XTHs), exerting a xyloglucan endotransglucosylase activity (XET) (Fry et al. 1992) and for cellulose synthase-like proteins (CSL). The primary role of XTHs is to cleave and reassemble xyloglucan chains in the plant cell wall, thus regulating cell wall assembly and growth (Sharples et al. 2017), whereas CSL subfamily D is involved in plant cell wall carbohydrate-based polymer biosynthesis (Hu et al. 2019). These observations confirmed that both PH and PH3 in optimal N conditions could affect lettuce plant growth, thereby promoting cell elongation and remodelling the cell wall.

4.3.2 | Low nitrogen

PH biostimulant induced the significant up-regulation of 3213 genes and the down-regulation of 2260 genes in lettuce plants grown in low N conditions. Similarly, the PH3 fraction significantly up-regulated 3049 and down-regulated 2166 genes.

Both Ph and PH3 significantly impacted the GO term *translation*. In fact, they induced the significant up-regulation of genes encoding for probable glutathione S-transferase (GST) and alkaline ceramidase. The first gene belongs to a vast protein family, including several classes that have different roles in plant development, endogenous secondary metabolisms, stress tolerance, and xenobiotic detoxification (Nianiou-Obeidat et al. 2017). Alkaline ceramidase, instead, is involved in sphingolipids metabolism and catabolism, affecting their homeostasis and playing essential roles in plant development and stress responses in *Arabidopsis* (Wu et al. 2015).

The two treatments also induced the significant down-regulation of a peptide chain release factor (PrfB3) and of a GST belonging to the T1, whereas only PH-induced the up-regulation of one PrfB3. In *Arabidopsis*, PrfB3 was shown to be a protein involved in the stability of *petB* transcript. *petB* is a chloroplastic gene encoding cytochrome b_6 that plays a pivotal role in photosynthetic electron transport (Eckardt 2011). Moreover, it has been demonstrated that the PrfB3 expression is highly responsive to light and stress conditions (Eckardt 2011). The down-regulated GST belong to the Theta (T) class that possesses both significant glutathione-dependent hyperoxidase (GPOX) activity on fatty acid hydroperoxides and glutathione conjugation activity towards cytotoxic lipid peroxidation products (Nianiou-Obeidat et al. 2017). These functions permit this class of GST to be directly involved in abiotic stress tolerance by decreasing the generation of reactive electrophile species (Dixon et al. 2010). It is interesting to point out that these observations could highlight the molecular mechanisms activated by these specific biostimulants' administration in alleviating plant stress conditions at the photosynthetic and reactive compounds production level.

Considering the GO term encompassing the transcription factors, both PH and PH3 induced the up-regulation of several genes encoding for ERF, DREB, probable WRKY and ABI transcription factors. In contrast, only PH3 up-regulated heat stress and heat shock transcription factors. All these genes are involved in the stress response, as previously mentioned, for plants treated with PH3 in optimal N conditions.

Moreover, both PH and PH3 significantly positively affected the expression of the long hypocotyl 5 (HY5) transcription factor, which is a high hierarchical regulator of transcriptional cascades for photomorphogenesis, resulting in its promotion (Lee et al. 2007).

On the other hand, only the PH3 fraction induced the up-regulation of a ribosomal protein 40S S14-3, designated as a stress-responsive translational protein (Panda et al. 2020), whereas only PH caused the down-regulation of a 30S ribosomal protein S11, a second class protein in ribosomal assembly (Culver and Narayanaswamy 2008).

Biostimulant PH significantly affected gene regulation in the *hydrolase activity* GO term. In particular, it up-regulated three XTHs genes, as observed in plants treated with both PH and PH3 in optimal N conditions. Moreover, PH-induced the up-regulation of a glucan endo-1,3-beta-glucosidase and a lichenase enzyme. The first enzyme is recognised as a pathogenesis-related protein abundant in many plant species after infection by different types of pathogens and it was shown to be involved in cell division, pollen development, pollen tube growth, regulation of plasmodesmata signalling, cold response, seed germination, and maturation (Doxey et al. 2007). Lichenase,

instead, encodes for a hydrolytic enzyme able to solubilise β-glucans from cereal grains (Saulnier and Quemener 2009). On the other hand, PH down-regulated a polygalacturonase, an enzyme involved in the hydrolysis of α-1,4 glycosidic bonds of pectic acid (Yahia et al. 2019), especially at the cell wall level.

Finally, PH3 significantly up-regulated a ubiquitin-protein ligase PUB23, belonging to the *ubiquitin-protein transferase* GO term. This enzyme is involved in pathogen-associated molecular patterns (PAMPs), down-regulating the PAMP responses (Stegmann et al. 2012).

4.4 | Biological and functional insight through multi-omics data integration

The generalized increase in the growth and biomass accumulation observed in lettuce plants, independently from the N nutritional status and the biostimulants treatment, can be generally ascribed to a hormone-like activity of PH. In this context, a transcriptional modulation of genes involved in auxin biology and the synthesis of cytokinins has been observed. This effect is mirrored by an increase in the metabolic levels of these hormones, which are responsible for different aspects of plant growth. However, the impact of protein hydrolysates on hormone regulation revealed quite a complex picture; several members of the CYP gene family, which are generally involved in detoxification processes as well as in the turnover of JA and ABA, were significantly modulated and putatively related to the increase of the same hormones at the metabolomic level. Interestingly, as mentioned above, these effectors could possibly be involved in increasing the resilience of lettuce plants against abiotic stress. This function could also be coordinated with i) the increase in the concentration of ACC, that, despite being the precursor of the stress hormone ethylene, it is also believed to act as an ethylene-independent growth regulator and stress-releasing mediator in different plant species, and ii) the induction of several transcription factors (e.g. ERF, DREB, WRKY) that are usually involved in the activation of the machinery responding to abiotic stresses.

Interestingly, plant growth at the cellular level is also sustained by other functions, stimulated by PH and its fraction PH3. In particular, lettuce plants treated with biostimulants showed the up-regulation not only of xyloglucan endotransglucosylase activity (XET), involved in the cell wall assembly and plasticity but also of cellulose synthase-like proteins (CLS), suggesting a promoting effect on cell growth and elongation. This effect could also be achieved by the intervention of RLK, namely G-type lectin S-receptor-like serine/threonine protein kinases, which are involved in different responses in dicot plants, including plant growth.

The metabolomic analysis also pointed out the activation of a multifaceted antioxidant response following the application of biostimulants, independently of N nutrition, with the accumulation of specific metabolites involved in the mitigation of the oxidative stress, including glucosinolates, catabolites of Proline and vitamin B2. These observations are indeed in line with the increase in CAT activity. On the contrary, at the transcriptomic level, we observed the modulation of transcription factors involved in the stress response and in the regulation of primary and secondary metabolism, and the up-regulation of GST genes related to detoxification processes, and of UGT gene, which plays a role in the synthesis of flavonoids and anthocyanins. In this case, it is worth mentioning that, by integrating transcriptomic and metabolomic datasets, a direct correspondence is not obvious since these two techniques provide complementary information (Culver and Narayanaswamy 2008).

In conclusion, the study hereby reported showed that the integration of physiological, biochemical, transcriptomic and metabolomic approaches can elucidate different aspects, sometimes overlapping and sometimes complementary, of the same biological process, namely the plant biostimulation through protein hydrolysates. In addition, the comparison of the whole PH and its fraction PH1-3 showed that, apart from a few peculiarities, the effects induced on plants by PH were equivalent to those produced by PH3, thus suggesting that the most bioactive fraction might be the lightest.

AUTHOR CONTRIBUTIONS

Experimental Design, GC, YR, LL, YP; Experiments execution, SM, VB, MYAZ, MC, LF, CEN, MC, FC; Data collection, SM, VB, MYAZ, PGP, LL, YP; Data analyses and visualization, SM, VB, MYAZ, PGP, LL, YP; Data interpretation, SM, VB, MYAZ, PGP, LL, YP; Manuscript writing and critical revision, SM, VB, PGP, LL, SC, YP; Financial support, LL, YP.

ACKNOWLEDGEMENTS

The authors thank the "Romeo ed Enrica Invernizzi" foundation (Milan, Italy) for its kind support of the metabolomics facility at Università Cattolica del Sacro Cuore. The work by P.G.-P. was financed by the Spanish Ministry of Universities under the application 33.50.460A.752 and by the European Union NextGenerationEU/PRTR through a Margarita Salas contract by the Universidade de Vigo.

FUNDING INFORMATION

This work was financially supported by the Italian Ministry of Education, University and Research (MIUR) through the project PHOBOS coded 2017FYBLPP. The funding bodies played no role in the design of the study and collection, analysis, interpretation of data, and in writing the manuscript.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. The RNA sequencing data have been deposited in the NCBI SRA database (accession no. PRJNA1009415).

ORCID

Luigi Lucini https://orcid.org/0000-0002-5133-9464
Youry Pii https://orcid.org/0000-0002-1613-851X

REFERENCES

Alzate Zuluaga MY, Miras-Moreno B, Monterisi S, Rouphael Y, Colla G, Lucini L, Cesco S, Pii Y (2022) Integrated metabolomics and

morpho-biochemical analyses reveal a better performance of *Azospirillum brasilense* over plant-derived biostimulants in counteracting salt stress in tomato. *International Journal of Molecular Sciences* 23: 14216.

Avalbaev A, Allagulova C, Maslennikova D, Fedorova K, Shakirova F (2021) Methyl jasmonate and cytokinin mitigate the salinity-induced oxidative injury in wheat seedlings. *Journal of Plant Growth Regulation* 40: 1741–1752.

Baligar VC, Fageria NK (2015) Nutrient use efficiency in plant: an overview. In: Rakshit A, Singh HB, Sen A (eds) *Nutrient Use Efficiency: from Basics to Advances*. Springer, New Delhi.

Battacharyya D, Babgohari MZ, Rathor P, Prithiviraj B (2015) Seaweed extracts as biostimulants in horticulture. *Scientia Horticulturae* 196: 39–48.

Broadley MR, Escobar-Gutiérrez AJ, Burns A, Burns IG (2000) What are the effects of Nitrogen deficiency on growth components of lettuce? *New Phytologist* 147: 519–526.

Broadley MR, Escobar-Gutiérrez AJ, Burns A, Burns IG (2001) Nitrogen-limited growth of lettuce is associated with lower stomatal conductance. *New Phytologist* 152: 97–106.

Broun P, Poindexter P, Osborne E, Jiang C-Z, Riechmann JL (2004) WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. *Proceedings of the National Academy of Sciences* 101: 4706–4711.

Calvo P, Nelson L, Kloepper JW (2014) Agricultural uses of plant biostimulants. *Plant and Soil* 383: 3–41.

Candido V, Boari F, Cantore V, Castronuovo D, Denora M, Sergio L, Todorovic M, Schiattone MI (2023) Interactive effect of water regime, Nitrogen rate and biostimulant application on physiological and biochemical traits of wild rocket. *Agricultural Water Management* 277: 108075.

Canellas LP, Olivares FL, Aguiar NO, Jones DL, Nebbioso A, Mazzei P, Piccolo A (2015) Humic and fulvic acids as biostimulants in horticulture. *Scientia Horticulturae* 196: 15–27.

Carillo P, Colla G, El-Nakhel C, Bonini P, D'Amelia L, Dell'Aversana E, Pannico A, Giordano M, Sifola MI, Kyriacou MC, De Pascale S, Rouphael Y (2019a) Biostimulant application with a tropical plant extract enhances *Corchorus olitorius* adaptation to sub-optimal nutrient regimens by improving physiological parameters. *Agronomy* 9: 249.

Carillo P, Colla G, Fusco GM, Dell'Aversana E, El-Nakhel C, Giordano M, Pannico A, Cozzolino E, Mori M, Reynaud H, Kyriacou MC, Cardarelli M, Rouphael Y (2019b) Morphological and physiological responses induced by protein hydrolysate-based biostimulant and Nitrogen rates in greenhouse spinach. *Agronomy* 9: 450.

Carillo P, De Micco V, Ciriello M, Formisano L, El-Nakhel C, Giordano M, Colla G, Rouphael Y (2022) Morpho-anatomical, physiological, and mineral composition responses induced by a vegetal-based biostimulant at three rates of foliar application in greenhouse lettuce. *Plants* 11: 2030.

Ćavar Zeljković S, Štefelová N, Hron K, Doležalová I, Tarkowski P (2023) Preharvest abiotic stress affects the nutritional value of lettuce. *Agronomy* 13: 398.

Ceccarelli AV, Miras-Moreno B, Buffagni V, Senizza B, Pii Y, Cardarelli M, Rouphael Y, Colla G, Lucini L (2021) Foliar application of different vegetal-derived protein hydrolysates distinctively modulates tomato root development and metabolism. *Plants* 10: 326.

Choi S, Colla G, Cardarelli M, Kim H-J (2022) Effects of plant-derived protein hydrolysates on yield, quality, and Nitrogen Use Efficiency of greenhouse grown lettuce and tomato. *Agronomy* 12: 1018.

Ciriello M, Formisano L, El-Nakhel C, Corrado G, Rouphael Y (2022a) Biostimulatory action of a plant-derived protein hydrolysate on morphological traits, photosynthetic parameters, and mineral composition of two basil cultivars grown hydroponically under variable electrical conductivity. *Horticulturae* 8: 409.

Ciriello M, Formisano L, Kyriacou MC, Carillo P, Scognamiglio L, De Pascale S, Rouphael Y (2022b) Morpho-physiological and biochemical responses of hydroponically grown basil cultivars to salt stress. *Antioxidants* 11: 2207.

Colla G, Cardarelli M, Bonini P, Rouphael Y (2017a) Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience horts* 52: 1214–1220.

Colla G, Hoagland L, Ruzzi M, Cardarelli M, Bonini P, Canaguier R, Rouphael Y (2017b) Biostimulant action of protein hydrolysates: Unraveling their effects on plant physiology and microbiome. *Frontiers in Plant Science* 8: 2202.

Colla G, Nardi S, Cardarelli M, Ertani A, Lucini L, Canaguier R, Rouphael Y (2015) Protein hydrolysates as biostimulants in horticulture. *Scientia Horticulturae* 196: 28–38.

Colla G, Rouphael Y, Canaguier R, Svecova E, Cardarelli M (2014) Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Frontiers in Plant Science* 5: 448.

Collin A, Daszkowska-Golec A, Szarejko I (2021) Role of ABSCISIC ACID INSENSITIVE 5 (ABI5) and ABSCISIC ACID-RESPONSIVE ELEMENT BINDING FACTORs (ABFs) in ABA signaling in different developmental stages in plants. *Cells* 10: 1996.

Coria-Cayupán YS, Sánchez de Pinto MI, Nazareno MA (2009) Variations in bioactive substance contents and crop yields of lettuce (*Lactuca sativa* L.) cultivated in soils with different fertilization treatments. *Journal of Agricultural and Food Chemistry* 57: 10122–10129.

Cristofano F, El-Nakhel C, Colla G, Cardarelli M, Pii Y, Lucini L, Rouphael Y (2023a) Tracking the biostimulatory effect of fractions from a commercial plant protein hydrolysate in greenhouse-grown lettuce. *Antioxidants* 12: 107.

Cristofano F, El-Nakhel C, Colla G, Cardarelli M, Pii Y, Lucini L, Rouphael Y (2023b) Modulation of morpho-physiological and metabolic profiles of lettuce subjected to salt stress and treated with two vegetal-derived biostimulants. *Plants* 12: 709.

Cristofano F, El-Nakhel C, Rouphael Y (2021) Biostimulant substances for sustainable agriculture: origin, operating mechanisms and effects on cucurbits, leafy greens, and nightshade vegetables species. *Biomolecules* 11: 1103.

Culver GM, Narayanaswamy K (2008) Assembly of the 30S Ribosomal Subunit. *EcoSal Plus* 3: https://doi.org/10.1128/ecosalplus.2.5.3

Di Mola I, Cozzolino E, Ottaiano L, Giordano M, Rouphael Y, Colla G, Mori M (2019) Effect of vegetal- and seaweed extract-based biostimulants on agronomical and leaf quality traits of plastic tunnel-grown baby lettuce under four regimes of Nitrogen fertilization. *Agronomy* 9: 571.

Dixon DP, Skipsey M, Edwards R (2010) Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry* 71: 338–350.

Djidonou D, Leskovar DI (2019) Seasonal changes in growth, Nitrogen nutrition, and yield of hydroponic lettuce. *HortScience horts* 54: 76–85.

Dombrowski JE, Martin RC (2012) Abiotic stresses activate a MAPkinase in the model grass species *Lolium temulentum*. *Journal of Plant Physiology* 169: 915–919.

Doxey AC, Yaish MWF, Moffatt BA, Griffith M, McConkey BJ (2007) Functional divergence in the Arabidopsis *β-1,3-Glucanase* gene family inferred by phylogenetic reconstruction of expression states. *Molecular Biology and Evolution* 24: 1045–1055.

Eckardt NA (2011) PrfB3: a nuclear-encoded protein recruited for regulation of transcript stability in arabidopsis chloroplasts. *The Plant Cell* 23: 2474.

El-Nakhel C, Cristofano F, Colla G, Pii Y, Lucini L, Rouphael Y (2023a) A *Graminaceae*-derived protein hydrolysate and its fractions provide differential growth and modulate qualitative traits of lettuce grown under non-saline and mild salinity conditions. *Scientia Horticulturae* 319: 112130.

El-Nakhel C, Cristofano F, Colla G, Pii Y, Secomandi E, De Gregorio M, Buffagni V, Garcia-Perez P, Lucini L, Rouphael Y (2023b) Vegetal-derived biostimulants distinctively command the physiological and metabolomic signatures of lettuce grown in depleted nitrogen conditions. *Scientia Horticulturae* 317: 112057.

Fallovo C, Rouphael Y, Rea E, Battistelli A, Colla G (2009) Nutrient solution concentration and growing season affect yield and quality of *Lactuca sativa* L. var. *acephala* in floating raft culture. *Journal of the Science of Food and Agriculture* 89: 1682–1689.

FAO (2009) Global Agriculture towards 2050, High-Level Expert Forum, How to Feed the World 2050, Rome 12–13 October 2009.

Formisano L, El-Nakhel C, Corrado G, De Pascale S, Rouphael Y (2020) Biochemical, physiological, and productive response of greenhouse vegetables to suboptimal growth environment induced by insect nets. *Biology* 9: 432.

Francesca S, Najai S, Zhou R, Decros G, Cassan C, Delmas F, Ottosen C-O, Barone A, Rigano MM (2022) Phenotyping to dissect the biostimulant action of a protein hydrolysate in tomato plants under combined abiotic stress. *Plant Physiology and Biochemistry* 179: 32–43.

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemical Journal* 282: 821–828.

Geary B, Clark J, Hopkins BG, Jolley VD (2015) Deficient, adequate and excess Nitrogen levels established in hydroponics for biotic and abiotic stress-interaction studies in potato. *Journal of Plant Nutrition* 38: 41–50.

Ghaheri M, Kahrizi D, Bahrami G, Mohammadi-Motlagh H-R (2019) Study of gene expression and steviol glycosides accumulation in *Stevia rebaudiana* Bertoni under various mannitol concentrations. *Molecular Biology Reports* 46: 7–16.

Giordano M, El-Nakhel C, Carillo P, Colla G, Graziani G, Di Mola I, Mori M, Kyriacou MC, Rouphael Y, Soteriou GA, Sabatino L (2022) Plant-derived biostimulants differentially modulate primary and secondary metabolites and improve the yield potential of red and green lettuce cultivars. *Agronomy* 12: 1361.

Guo M, Liu J-H, Ma X, Luo D-X, Gong Z-H, Lu M-H (2016) The plant Heat Stress Transcription Factors (HSFs): structure, regulation, and function in response to abiotic stresses. *Frontiers in Plant Science* 7: 114.

Halpern M, Bar-Tal A, Ofek M, Minz D, Muller T, Yermiyahu U (2015) The use of biostimulants for enhancing nutrient uptake. In: Sparks D (ed) *Advances in Agronomy*. Academic Press, pp. 141–174.

Hanson AD, Beaudoin GA, McCarty DR, Gregory III JF (2016) Does abiotic stress cause functional B vitamin deficiency in plants? *Plant Physiology* 172: 2082–2097.

Heitz T, Widemann E, Lugan R, Miesch L, Ullmann P, Désaubry L, Holder E, Grausem B, Kandel S, Miesch M, Werck-Reichhart D, Pinot F (2012) Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone jasmonoyl-isoleucine for catabolic turnover. *Journal of Biological Chemistry* 287: 6296–6306.

Hématy K, Höfte H (2008) Novel receptor kinases involved in growth regulation. *Current Opinion in Plant Biology* 11: 321–328.

Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. *Plant and Cell Physiology* 42: 462–468.

Hirakawa Y, Sawa S (2019) Diverse function of plant peptide hormones in local signaling and development. *Current Opinion in Plant Biology* 51: 81–87.

Höfer R, Dong L, André F, Ginglinger J-F, Lugan R, Gavira C, Grec S, Lang G, Memelink J, Van Der Krol S, Bouwmeester H, Werck-Reichhart D (2013) Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family of cytochrome P450 enzymes and potential for engineering the early steps of the (seco)iridoid pathway. *Metabolic Engineering* 20: 221–232.

Hu H, Zhang R, Tang Y, Peng C, Wu L, Feng S, Chen P, Wang Y, Du X, Peng L (2019) Cotton CSLD3 restores cell elongation and cell wall integrity mainly by enhancing primary cellulose production in the Arabidopsis *cesa6* mutant. *Plant Molecular Biology* 101: 389–401.

Jiang J, Ma S, Ye N, Jiang M, Cao J, Zhang J (2017) WRKY transcription factors in plant responses to stresses. *Journal of Integrative Plant Biology* 59: 86–101.

Körber TT, Sitz T, Abdalla MA, Mühling KH, Rohn S (2023) LC-ESI-MS/MS analysis of Sulfolipids and Galactolipids in green and red lettuce (*Lactuca sativa* L.) as influenced by Sulfur nutrition. *International Journal of Molecular Sciences* 24: 3728.

Laoué J, Fernandez C, Ormeño E (2022) Plant flavonoids in Mediterranean species: A focus on flavonols as protective metabolites under climate stress. *Plants* 11: 172.

Lassaletta L, Billen G, Grizzetti B, Anglade J, Garnier J (2014) 50 year trends in nitrogen use efficiency of world cropping systems: The relationship between yield and nitrogen input to cropland. *Environmental Research Letters* 9: 105011.

Layer G, Jahn D, Deery E, Lawrence AD, Warren MJ (2010) Biosynthesis of Heme and Vitamin B12. In: *Liu H-W* (Ben), Mander LBT-CNPII (eds). Elsevier, Oxford, pp. 445–499.

Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, Tongprasit W, Zhao H, Lee I, Deng XW (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. *The Plant Cell* 19: 731–749.

Licausi F, Ohme-Takagi M, Perata P (2013) APETALA2/Ethylene Responsive Factor (AP2/ERF) transcription factors: mediators of stress responses and developmental programs. *New Phytologist* 199: 639–649.

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15: 550.

Lucini L, Miras-Moreno B, Rouphael Y, Cardarelli M, Colla G (2020) Combining molecular weight fractionation and metabolomics to elucidate the bioactivity of vegetal protein hydrolysates in tomato plants. *Frontiers in Plant Science* 11: 976.

Lucini L, Rouphael Y, Cardarelli M, Canaguier R, Kumar P, Colla G (2015) The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Scientia Horticulturae* 182: 124–133.

Marschner P (2012) *Marschner's mineral nutrition of higher plants*. London.

Maswada HF, Abd El-Razek UA, El-Sheshtawy A-NA, Mazrou YSA (2021) Effect of *Azolla filiculoides* on growth, physiological and yield attributes of maize grown under water and Nitrogen deficiencies. *Journal of Plant Growth Regulation* 40: 558–573.

Matsunaga I, Shiro Y (2004) Peroxide-utilizing biocatalysts: structural and functional diversity of heme-containing enzymes. *Current Opinion in Chemical Biology* 8: 127–132.

Medina-Lozano I, Bertolín JR, Díaz A (2021) Nutritional value of commercial and traditional lettuce (*Lactuca sativa* L.) and wild relatives: Vitamin C and anthocyanin content. *Food Chemistry* 359: 129864.

Minerdi D, Savoi S, Sabbatini P (2023) Role of cytochrome P450 enzyme in plant microorganisms' communication: a focus on grapevine. *International Journal of Molecular Sciences* 24: 4695.

Miras-Moreno B, Corrado G, Zhang L, Senizza B, Righetti L, Bruni R, El-Nakhel C, Sifola MI, Pannico A, De Pascale S, Rouphael Y, Lucini L (2020) The metabolic reprogramming induced by sub-optimal nutritional and light inputs in soilless cultivated green and red butterhead lettuce. *International Journal of Molecular Sciences* 21: 1–16.

Mok DWS, Martin RC, Shan X, Mok MC (2000) Genes encoding Zeatin O-Glycosyltransferases. *Plant Growth Regulation* 32: 285–287.

van Mourik H, van Dijk ADJ, Stortenbeker N, Angenent GC, Bemer M (2017) Divergent regulation of Arabidopsis *SAUR* genes: a focus on the *SAUR10*-clade. *BMC Plant Biology* 17: 245.

Neff MM, Nguyen SM, Malancharuvil EJ, Fujioka S, Noguchi T, Seto H, Tsubuki M, Honda T, Takatsuto S, Yoshida S, Chory J (1999) BAS1: A gene regulating brassinosteroid levels and light responsiveness in Arabidopsis. *Proceedings of the National Academy of Sciences* 96: 15316–15323.

Nianiou-Obeidat I, Madesis P, Kissoudis C, Voulgari G, Chronopoulou E, Tsaftaris A, Labrou NE (2017) Plant glutathione transferase-mediated stress tolerance: functions and biotechnological applications. *Plant Cell Reports* 36: 791–805.

Nibau C, Cheung A (2011) New insights into the functional roles of CrRLKs in the control of plant cell growth and development. *Plant Signaling & Behavior* 6: 655–659.

Ohnishi T, Nomura T, Watanabe B, Ohta D, Yokota T, Miyagawa H, Sakata K, Mizutani M (2006) Tomato cytochrome P450 CYP734A7 functions in brassinosteroid catabolism. *Phytochemistry* 67: 1895–1906.

Panda A, Rangani J, Parida AK (2020) Comprehensive proteomic analysis revealing multifaceted regulatory network of the xero-halophyte *Haloxylon salicornicum* involved in salt tolerance. *Journal of Biotechnology* 324: 143–161.

Pandey VP, Awasthi M, Singh S, Tiwari S, Dwivedi UN (2017) A comprehensive review on function and application of plant peroxidases. *Biochemistry & Analytical Biochemistry* 06: 1–16.

Peng H, Yang T, Whitaker BD, Shangguan L, Fang J (2016) Calcium/calmodulin alleviates substrate inhibition in a strawberry UDP-glucosyltransferase involved in fruit anthocyanin biosynthesis. *BMC Plant Biology* 16: 197.

Peng Z, Han C, Yuan L, Zhang K, Huang H, Ren C (2011) Brassinosteroid enhances Jasmonate-induced anthocyanin accumulation in Arabidopsis seedlings. *Journal of Integrative Plant Biology* 53: 632–640.

Pii Y, Mimmo T, Tomasi N, Terzano R, Cesco S, Crecchio C (2015) Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biology and Fertility of Soils* 51: 403–415.

Polko JK, Kieber JJ (2019) 1-Aminocyclopropane 1-Carboxylic Acid and its emerging role as an ethylene-independent growth regulator. *Frontiers in Plant Science* 10: 1602.

Riederer M, Schreiber L (2001) Protecting against water loss: analysis of the barrier properties of plant cuticles. *Journal of Experimental Botany* 52: 2023–2032.

Rouphael Y, Carillo P, Garcia-Perez P, Cardarelli M, Senizza B, Miras-Moreno B, Colla G, Lucini L (2022) Plant biostimulants from seaweeds or vegetal proteins enhance the salinity tolerance in greenhouse lettuce by modulating plant metabolism in a distinctive manner. *Scientia Horticulturae* 305: 111368.

Rouphael Y, Franken P, Schneider C, Schwarz D, Giovannetti M, Agnolucci M, Pascale SD, Bonini P, Colla G (2015) Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Scientia Horticulturae* 196: 91–108.

Rouphael Y, Kyriacou MC, Petropoulos SA, De Pascale S, Colla G (2018) Improving vegetable quality in controlled environments. *Scientia Horticulturae* 234: 275–289.

Sakamoto T, Kawabe A, Tokida-Segawa A, Shimizu B, Takatsuto S, Shimada Y, Fujioka S, Mizutani M (2011) Rice CYP734As function as multisubstrate and multifunctional enzymes in brassinosteroid catabolism. *The Plant Journal* 67: 1–12.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K (2002) DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochemical and Biophysical Research Communications* 290: 998–1009.

Saud S, Wang D, Fahad S (2022) Improved Nitrogen Use Efficiency and Greenhouse Gas Emissions in Agricultural Soils as Producers of Biological Nitrification Inhibitors. *Frontiers in Plant Science* 13: 854195.

Saulnier L, Quemener B (2009) Enzymatic mapping of arabinoxylan structure. In: *Shewry PR*, Ward JLBT-HM (eds) American Associate of Cereal Chemists International. AACC International Press, pp. 191–201.

Sharples SC, Nguyen-Phan TC, Fry SC (2017) Xyloglucan endotransglucosylase/hydrolases (XTHs) are inactivated by binding to glass and cellulosic surfaces, and released in active form by a heat-stable polymer from cauliflower florets. *Journal of Plant Physiology* 218: 135–143.

Sintupachee S, Promden W, Ngamrojanavanich N, Sitthithaworn W, De-Eknamkul W (2015) Functional expression of a putative geraniol 8-hydroxylase by reconstitution of bacterially expressed plant CYP76F45 and NADPH-cytochrome P450 reductase CPR I from *Croton stellatopilosus* Ohba. *Phytochemistry* 118: 204–215.

Siwik-Ziomek A, Szczepanek M (2019) Soil extracellular enzyme activities and uptake of N by oilseed rape depending on fertilization and seaweed biostimulant application. *Agronomy* 9: 480.

Spartz AK, Lee SH, Wenger JP, Gonzalez N, Itoh H, Inzé D, Peer WA, Murphy AS, Overvoorde PJ, Gray WM (2012) The SAUR19 subfamily of SMALL AUXIN UP RNA genes promote cell expansion. *The Plant Journal* 70: 978–990.

Stegmann M, Anderson RG, Ichimura K, Pecenkova T, Reuter P, Žárský V, McDowell JM, Shirasu K, Trujillo M (2012) The Ubiquitin Ligase PUB22 targets a subunit of the exocyst complex required for PAMP-triggered responses in Arabidopsis. *The Plant Cell* 24: 4703–4716.

Stortenbeker N, Bemer M (2019) The SAUR gene family: the plant's toolbox for adaptation of growth and development. *Journal of Experimental Botany* 70: 17–27.

Thakur V, Bains S, Pathania S, Sharma S, Kaur R, Singh K (2020) Comparative transcriptomics reveals candidate transcription factors involved in costunolide biosynthesis in medicinal plant-*Saussurea lappa*. *International Journal of Biological Macromolecules* 150: 52–67.

Thornton LE, Peng H, Neff MM (2011) Rice CYP734A cytochrome P450s inactivate brassinosteroids in Arabidopsis. *Planta* 234: 1151–1162.

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America* 108: 20260–20264.

Tognolli M, Penel C, Greppin H, Simon P (2002) Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. *Gene* 288: 129–138.

Turk EM, Fujioka S, Seto H, Shimada Y, Takatsuto S, Yoshida S, Denzel MA, Torres QI, Neff MM (2003) CYP72B1 inactivates brassinosteroid hormones: an intersection between photomorphogenesis and plant steroid signal transduction. *Plant Physiology* 133: 1643–1653.

Udvardi M, Below FE, Castellano MJ, Eagle AJ, Giller KE, Ladha JK, Liu X, Maaz TM, Nova-Franco B, Raghuram N, Robertson GP, Roy S, Saha M, Schmidt S, Tegeder M, York LM, Peters JW (2021) A research road map for responsible use of agricultural Nitrogen.

Wang W, Feng J, Wei L, Khalil-Ur-Rehman M, Nieuwenhuizen NJ, Yang L, Zheng H, Tao J (2021) Transcriptomics integrated with free and bound terpenoid aroma profiling during "Shine Muscat" (*Vitis labrusca* × *V. vinifera*) grape berry development reveals coordinate regulation of MEP pathway and terpene synthase gene expression. *Journal of Agricultural and Food Chemistry* 69: 1413–1429.

Wang Y, Yao Q, Zhang Y, Zhang Y, Xing J, Yang B, Mi G, Li Z, Zhang M (2020) The role of gibberellins in regulation of Nitrogen uptake and physiological traits in maize responding to Nitrogen availability. *International Journal of Molecular Sciences* 21: 1824.

Wilson HT, Xu K, Taylor AG (2015) Transcriptome analysis of gelatin seed treatment as a biostimulant of cucumber plant growth. *The Scientific World Journal* 2015: 391234.

Wu J-X, Li J, Liu Z, Yin J, Chang Z-Y, Rong C, Wu J-L, Bi F-C, Yao N (2015) The Arabidopsis ceramidase *AtACER* functions in disease resistance and salt tolerance. *The Plant Journal* 81: 767–780.

Yahia EM, Carrillo-López A, Bello-Perez LA (2019) *Carbohydrates*. In: Yahia EMBT-PP and B of F and V (ed). Woodhead Publishing, pp. 175–205.

Yue X, Hu Y, Zhang H, Schmidhalter U (2020) Evaluation of Both SPAD Reading and SPAD Index on Estimating the Plant Nitrogen Status of Winter Wheat. *International Journal of Plant Production* 14: 67–75.

Zha L, Liu W, Yang Q, Zhang Y, Zhou C, Shao M (2020) Regulation of ascorbate accumulation and metabolism in lettuce by the red:blue

ratio of continuous light using LEDs. *Frontiers in Plant Science* 11: 704.

Zhang F, Shen J, Zhang J, Zuo Y, Li L, Chen X (2010) Rhizosphere processes and management for improving nutrient use efficiency and crop productivity: implications for China. Advances in Agronomy. Elsevier Inc, pp. 1–32.

Zhou W, Liang X, Li K, Dai P, Li J, Liang B, Sun C, Lin X (2021) Metabolomics analysis reveals potential mechanisms of phenolic accumulation in lettuce (*Lactuca sativa* L.) induced by low Nitrogen supply. *Plant Physiology and Biochemistry* 158: 446–453.

Zuluaga MYA, Monterisi S, Rouphael Y, Colla G, Lucini L, Cesco S, Pii Y (2023) Different vegetal protein hydrolysates distinctively alleviate salinity stress in vegetable crops: A case study on tomato and lettuce. *Frontiers in Plant Science* 14: 1077140.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Monterisi, S., Garcia-Perez, P., Buffagni, V., Zuluaga, M.Y.A., Ciriello, M., Formisano, L. et al. (2024) Unravelling the biostimulant activity of a protein hydrolysate in lettuce plants under optimal and low N availability: a multi-omics approach. *Physiologia Plantarum*, 176(3), e14357. Available from: https://doi.org/10.1111/ppl.14357