Research Article

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Foliar applications of a Malvaceae-derived protein hydrolysate and its fractions differentially modulate yield and functional traits of tomato under optimal and suboptimal nitrogen application

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Abstract

BACKGROUND: Protein hydrolysates (PHs) can enhance plant nitrogen nutrition and improve the quality of vegetables, depending on their bioactive compounds. A tomato greenhouse experiment was conducted under both optimal (14 mM) and suboptimal (2 mM) nitrogen ($N\text{-}NO_3$) conditions. Tomatoes were treated with a new Malvaceae-derived PH (MDPH) and its molecular fractions (MDPH1, >10 kDa; MDPH2, 1–10 kDa and MDPH3, <1 kDa).

RESULTS: Under optimal N conditions, the plants increased biomass and fruit yield, and showed a higher photosynthetic pigment content in leaves in comparison with suboptimal N, whereas under N-limiting conditions, an increase in dry matter, soluble solid content (SSC) and lycopene, a reduction in firmness, and changes in organic acid and phenolic compounds were observed. With 14 mM $N\text{-}NO_3$, MDPH3 stimulated an increase in dry weight and increased yield components and lycopene in the fruit. The MDPH2 fraction also resulted in increased lycopene accumulation in fruit under 14 mM $N\text{-}NO_3$. At a low N level, the PH fractions showed distinct effects compared with the whole MDPH and the control, with an increase in biomass for MDPH1 and MDPH2 and a higher pigment content for MDPH3. Regardless of N availability, all the fractions affected fruit quality by increasing SSC, whereas MDPH2 and MDPH3 modified organic acid content and showed a higher concentration of flavonols, lignans, and stilbenes.

CONCLUSION: The molecular weight of the peptides modifies the effect of PHs on plant performance, with different behavior depending on the level of N fertilization, confirming the effectiveness of fractioning processes.

Supporting information may be found in the online version of this article.

Keywords: biostimulants; nutritional stress; fruit quality; lycopene; UHPLC; untargeted metabolomics; phenolic profiling

INTRODUCTION

Greenhouse horticulture is an intensive agriculture system that focuses on the production of high-value vegetables and requires very efficient resource utilization (water, fertilizers, etc.) to maximize the yield, guarantee early development and yield stability, and produce quality in a sustainable way. Its global market was valued at 32.3 billion US dollars in 2021, and it was projected to grow at a compound annual growth rate (CAGR) of 7.8% from 2022 to 2030 (https://www.alliedmarketresearch.com/greenhouse-horticulture-market). Greenhouse horticulture relies on a substantial use of fertilizers, especially nitrogen per unit area, in comparison with other cropping systems.[1,2] Nitrogen plays a key role in various essential processes, including growth, leaf-area

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expansion, biomass production, crop yield, produce quality, and response to environmental stresses.[3,4] Nitrogen fertilizers have been often overused because of the high crop N requirements under greenhouse conditions, with little attention paid to the negative impact on the environment and human health;[5] nonetheless it has been verified that there is no linear correlation between high N fertilizer supply and uptake, transport, and storage of this element.[1] Conversely, it has been shown that increasing N fertilization can reduce the N use efficiency and can negatively affect both yield and product quality negatively.[6-8] Nitrogen application that exceeds crop uptake and utilization limits results in nitrogen losses in the agroecosystem through surface run off, leaching, denitrification, and volatilization; these losses contribute to increase environmental issues linked to nitrogen pollution such as the emission of greenhouse gases, contamination of water bodies, eutrophication, soil acidification, and a decline in biodiversity.[9-12]

To address this issue, the European Commission has developed the European Green Deal strategy to reduce fertilizer consumption by 20% by 2030 in comparison with 1990 levels (EU, 2020). To meet this challenge, the use of protein hydrolysate- (PH-)based biostimulants is an agronomic tool that can enable a shift from a resource-intensive to sustainable greenhouse production systems, safeguarding yield and quality production. Protein hydrolysates can reduce the fertilizer application rate by improving the efficiency of the nutrient acquisition processes and by increasing quality traits (Regulation (EU) 2019/1009 of the European Parliament). In particular, PHs have been shown to influence plant nutritional status by modulating several physiological and molecular mechanisms such as the stimulation of the genes involved in the N assimilation processes;[7,13] they can boost endogenous hormones by their phytohormones-like activity, and they can support mineral nutrition due to modulation of the root system.[14-17] An extensive body of scientific literature is available regarding the PH effects mentioned above at different N rates, especially on leafy vegetables. For instance, recent studies demonstrated that the application of a legume-derived PH increased the marketable yield of spinach[1] and lamb's lettuce,[7] especially under low nitrogen rates. Di Mola *et al.*[18] reported that the foliar application of a vegetal-based PH stimulated plant growth and boosted the marketable yield and functional quality of baby rocket grown under a suboptimal nitrogen fertilization regime. Applications of a PH at suboptimal N regime enhanced tomato growth at early development stage, upregulating gene expression for amino acid transporter and glutamine synthetase.[13]

Nevertheless, the mode of action of PHs is still not understood completely. Peptides are the most abundant bioactive molecules in enzimatically-produced PHs; peptides exhibit their main biological regulatory activity by acting as signaling molecules.[19-21] On the other hand, the variability in the molecular characteristics (e.g., amino acid sequences) of peptides impedes generalization of their mode of action. In view of this, molecular fractionation could be a useful process to shed light on the effects of different molecular weight peptides.

Indeed, Lucini *et al.*[22] showed that the smallest fraction (PH1; <1 kDa) of the PH Trainer was the most active in promoting the growth of adventitious roots of tomato cuttings in a laboratory assay; the same authors observed similar metabolic signature between PH1 and IBA-treated cuttings thereby inferring the auxin-like activity of this fraction. Furthermore, in recently published papers the fractions of two different PHs differentially modulated lettuce nutritional quality, thereby highlighting their different mode of action.[23,24]

As far as the authors of the current study are aware, no research has been conducted concerning the effect of peptides at different molecular weights in interaction with different N levels on agronomical, physiological, and fruit quality response of an important vegetable crop such as fresh market tomato grown under greenhouse conditions. Tomato is indeed an important source of nutrients and antioxidant-rich phytochemicals including carotenoids (mainly lycopene) and bioactive phenols, which helps to counter many chronic diseases, as argued by Ali *et al.*[25] Thus, the aim of this work was to evaluate a new Malvaceae-derived PH (MDPH) and its molecular fractions (MDPH1, <1 kDa; MDPH2, 1–10 kDa and MDPH3, >10 kDa) on yield and phytochemical profile of tomato fruit under both optimal (14 mM) and suboptimal (2 mM) nitrogen regimes. Finally, this approach can provide a 'knowledge package' that includes innovative solutions and multidisciplinary know how regarding the contribution of each specific fraction to the greenhouse vegetable production process which in turn can lead to the development of a new generation of functional biostimulants.

MATERIALS AND METHODS

Growth conditions, experimental design, and plant material

The experiment was carried out in the autumn and winter of 2020/2021, in a polyethylene greenhouse at the Experimental Farm 'Nello Lupori' of Tuscia University, Viterbo, central Italy (42° 25' N; 12° 08' E; 310 m a.s.l.). The daily air temperature inside the greenhouse was maintained between 18 and 28 °C by forced ventilation and day/night air relative humidity was 55/85%. On October 4, 2020, seedlings of *Solanum lycopersicum* L. cv. Pralyna (SAIS Sementi, Cesena, Italy) were transplanted at the three-true leaves stage into pots (Ø = 24 cm, 9.5 L) containing 1.5 L of quarziferous sand with a particle size between 0.4 and 0.8 mm. Plant rows were 0.9 m apart, and the space between plants within a row was 0.3 m. The distance between the centers of double rows was 1.2 m, resulting in a plant density of 3.5 plants m^{-2}. Crops were pruned at the seventh truss stage. The experimental design consisted of a factorial combination of two N levels of the nutrient solution (14 mM N-NO_3: optimal and 2 mM N-NO_3: suboptimal) with five biostimulant (B) treatments – an untreated-control and four biostimulants (MDPH, MDPH1, MDPH2, MDPH3) – arranged in a randomized complete block design with four replicates. In total, the design employed 40 experimental units (2 N × 5B × 4 replicates), each consisting of 12 tomato plants (n = 480 plants).

Biostimulant characteristics and application

A PH derived from Malvaceae biomass, referred to here as MDPH, and its fractions (MDPH1, MDPH2, MDPH3) with different molecular weights, were chosen for this trial. The MDPH was obtained by enzymatic hydrolysis of vegetal-derived proteins, as described previously.[26] Total N and carbon were quantified through the Dumas method using an elemental analyzer (Elemental vario MAX CN, Langenselbold, Germany). Quantitative analysis of this PH showed carbon and N content of 178 and 53.7 g kg^{-1}, respectively. The fractionation process was carried out using centrifuge filtering tubes (Amicon Ultra 15, Merck KGaA, Darmstadt, Germany) to obtain the fractions >10 kDa (MDPH1) and 1–10 kDa (MDPH2) and using 1 molecular cut-off cellulose acetate membranes (VWR, Milan, Italy) for the lowest molecular weight fraction

(MDPH3). Due to the use of water for partition, N concentration was diluted in each fraction obtained; the content of N was therefore again determined as follows: 1.5 g kg^{-1} for MDPH1 (>10 kDa); 1.1 g kg^{-1} for MDPH2 (1–10 kDa); 0.4 g $kg^{-1}$for MDPH3 (<1 kDa). The treated plants were sprayed uniformly 11 times during the growing cycle at 10-day intervals with a solution containing 2.48 g L^{-1} (MDPH), 88.8 g L^{-1} (MDPH1), 121.2 g L^{-1} (MDPH2), and 333.2 g L^{-1} (MDPH3) using a 2.5 L stainless-steel sprayer. Control plants were sprayed with tap water. Fraction dosage rates were defined in order to apply the same N level across treatments (0.133 g N L^{-1}). Foliar applications were initiated 7 days after transplanting (October 11).

Nutrient solution management

The nutrient solution was distributed through fertigation with a nutrient concentration of: 1.5 mM P, 2.5 mM S, 5.0 mM K, 1.25 mM Mg, 20.0 μM Fe, 9.0 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B, and 0.3 μM Mo. Two differential amounts of N were added to this base nutrient solution so that the N concentration of the solution was 14 mM N-NO_3 and 2 mM N-NO_3 for optimal and suboptimal nutritional treatment (N), respectively. In the suboptimal N treatment, calcium was adjusted to the same concentration in the optimal N concentration solution (7 mM Ca) through the addition of $CaCl_2$. All plants were drip irrigated using one emitter per plant with a flow rate of 2 L h^{-1}. Low-tension tensiometers (LT-Irrometer, Riverside, CA, USA) were used to schedule and manage fertirrigation, depending on substrate matric potential.[27] The beginning (−5 kPa) and end (−1 kPa) of fertirrigation, which correspond to the high- and low-tension set points for the majority of the growth media, were regulated by tensiometers that were coupled to an electronic programmer.[28] Each fertirrigated cycle was timed to be long enough to allow at least 35% of the nutrient solution draining from the pots.

Plant growth measurement, yield, and fruit quality assessment

At 48 days after transplanting (DAT) (i.e., on December 22), leaf tissues from three plants per experimental unit were collected, transferred into liquid nitrogen, and stored at −80 °C to determine pigments. The harvest of fully ripe fruits (mature red stage) started on December 16 (42 DAT) and continued until January 26 (112 DAT), on each plant of each experimental unit, recording total and marketable yield, number, and mean weight of marketable fruits.

A subsample of marketable fruits was selected, from each experimental unit, to evaluate fruit quality parameters. Dry matter (DM) content was determined by drying 20 g of homogenized tomato sample in a ventilated oven at 65 °C until constant weight. Firmness was measured at the equator of the fruit by using a portable penetrometer (FT 40 Wagner Instruments, Greenwich CT, USA) fitted with a 6 mm diameter stainless-steel tip. The soluble solid content (SSC) was assessed at 20 °C using an Atago N1 digital refractometer (Atago Co. Ltd., Tokyo, Japan) and expressed as °Brix. Other fruits were immediately frozen in liquid nitrogen and stored at −80 °C for lycopene and phenolic profiling, and ferric reducing antioxidant activity (FRAP) and2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

At the end of the experiment, stems, leaves, and roots were dried in ventilated oven at 65 °C until a constant weight was obtained to determine the total dry biomass.

Dried fruit samples (250 mg) were used to analyze organic acids content as described by El-Nakhel *et al.*[29] Briefly, the dry material was diluted in ultrapure water (50 mL; Milli-Q, Merck Millipore, Darmstadt, Germany) and then placed in a water bath (80 °C; ShakeTemp SW22, Julabo, Seelbach, Germany) and shaken for 10 min. The filtered samples were then identified for their organic acid content using ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) and quantified with ion chromatography coupled to a conductivity detector. Then, citric, malic, and oxalic acids were expressed in mg g^{-1} dry weight (DW).

Leaf chlorophylls and carotenoids

Total chlorophyll, chlorophyll a, b, and carotenoids were assessed as described by Lichtentaheler.[30] Briefly, 0.5 g of frozen leaf samples were extracted in 10 mL of acetone (80% v/v) using a mortar and pestle for 15 min in darkness and the extracts were then centrifuged at 3000 × *g* for 5 min. Chlorophyll a, b and carotenoids concentration were determined using ultraviolet (UV-) visible spectrophotometry (Beckman DU-50 UV–visible; Beckman Instruments, Inc., Fullerton, CA, USA) measuring the absorbance solution at 647, 664 and 470 nm, respectively. Total chlorophyll was calculated as the sum of chlorophyll a and chlorophyll b. All pigments were expressed as mg g^{-1} fresh weight (FW).

Fruit lycopene content and phenolic profiling using untargeted metabolomics

Lycopene determination was performed as described by Sadler *et al.*[31] Two grams of fresh tomato fruits were ground with mortar and pestle and mixed with 50 mL of a mixture of *n*-hexane:acetone:ethanol (2:1:1) at 0.5% of 2,6-di-tert-butyl-4metyl-phenol. After 30 min the absorbance was read at 472 nm with a spectrophotometer (Beckman DU-50 UV-visible; Beckman Instruments, Inc., Fullerton, CA, USA). Pure lycopene (Sigma, St. Louis, MO, USA) was used to build the calibration curves. Lycopene content was then expressed as mg g^{-1} FW.

Tomato fruit sampled from both optimal and suboptimal conditions, together with biostimulant treatments, were monitored to explore phenolic profiles by untargeted metabolomics as previously reported.[32] A solution (10 mL) containing of 80% methanol (v/v) and 0.1% of formic acid was used to extract 1 g of each frozen sample followed by homogenizing (Polytron PT1200 E, Kinematica AG, Switzerland). A combination of ultra-high-pressure liquid chromatography (UHPLC, 1290 series) and high-resolution mass spectrometry (QTOF analyzer, G6550 iFunnel) (Agilent Technology, Santa Clara, CA, USA) was applied for profiling. The separation was provided by a Knauer Blue Orchid C18 column (100 × 2 mm i.d., 1.8 μm), a binary mixture of water, and acetonitrile acidified with 0.1% (v/v) formic acid as a mobile phase (LC–MS grade, VWR, Milan, Italy). In each injection, 6 μL of sample solution is introduced into the column using a gradient of acetonitrile from 6 to 94% at 33 min intervals (with a flow rate of 0.200 mL min^{-1}). Acquisition was carried out in SCAN mode (100–1000 m/z, at 35 000 FWHM resolution). Data alignment and feature extraction were completed postacquisition using Agilent Profinder software B.10.0 (Agilent Technologies, Santa Clara, CA, USA). Identity confirmation of compounds was based on monoisotopic mass, isotopic ratio, and isotopic spacing, considering quasi-molecular ions and the possible adducts. Compound annotation was achieved recursively based on a 'find-by-formula' algorithm used with the Phenol-Explorer 3.6 database (http://phenol-explorer.eu). As a post annotation filter, only features measured in 75% or more of internal replicates within at least one treatment were retained. Afterwards, phenolic compounds were first ascribed to classes and then their semi-quantitative

values were estimated using calibration curves as described previously.[33]

Fruit DPPH and FRAP

The antioxidant activity was evaluated after extraction of 1 g of fresh tomato material with 10 mL of methanol and using the Hach DR 2000 UV–visible spectrophotometer (Hach Co., Loveland, CO, USA). Extraction was carried out by homogenizing the frozen samples with pestle and mortar. The DPPH method involved incubation of 200 µL of extract from each microgreen sample, at room temperature for 10 min with 1 mL of DPPH solution (4 mg of DPPH in 10 mL of methanol) and reading the extracts at 517 nm.[34]

The FRAP method involved incubation for 4 min of 150 µL of tomato material extract at room temperature with 2.850 mL of FRAP working solution (1.25 mL), consisting of 10 mM 2,4,6- tripyridylstriazine (TPTZ) in HCl (40 mM), 1.25 mL of $FeCl_3$ (20 mmol) in water and 12.5 mL of 0.3 mol L^{-1} sodium acetate buffer 0.3 mol L^{-1} (pH 3.6). The absorbance of the samples was read at a length of 593 nm, thanks to the reduction of ferric tripyridyltriazine, Fe (III) – TPTZ, to colored ferrous tripyridyltriazine (Fe (II) – TPTZ).[35]

For both methods the antioxidant activity was expressed as mg Trolox equivalents (TE) g^{-1} FW.

Statistical analysis

All experimental data were subjected to two-way analysis of variance (ANOVA) using the software package SPSS 20 for Windows 10 (IBM, Armonk, NY, USA). The N level mean effect was compared using *t*-tests, while Tukey's HSD test, at $P = 0.05$, was used to separate the main effects of biostimulants and their interactions with the measurement parameters.

Agilent Mass Profiler Professional B.12.06 software was applied to normalize the metabolomics-based data. Similarities/dissimilarity across treatments was measured with unsupervised hierarchical cluster analysis (HCA) (Euclidean distance and the Ward linkge method). A supervised statistics method based on orthogonal projections to latent structures discriminant analysis (OPLS-DA) was also used to assess the discriminant metabolites. Afterwards, variable importance in projection (VIP) ranking was used to find the discriminant power of each discriminant marker. Here, variables with a VIP score greater than 1 were considered important in our models. Compounds listed as VIP markers were given a VIP score. The VIP ranking allows to evaluate importance of individual variables from predictors block and their influence on the PLS model. The logarithm of fold change (logFC) was quantified to assess the accumulation trend of each discriminant compound between treatments. The quality and validity of the models were also evaluated by the goodness-of-fit (R2X and R2Y) and prediction ability (Q2Y) parameters.

Finally, the differences in phenolics content between treatments were assessed using a one-way ANOVA followed by Tukey's *post hoc* test was performed with SPSS software (v.n25, IBM Corp.), adjusting the significance level at $\alpha = 0.05$.

RESULTS

Plant growth and yield assessment

Total plant dry weight was affected significantly by N level ($P \leq 0.001$) and N level × biostimulant interaction ($P \leq 0.01$, reported in Fig. 1). Plants treated with the lower molecular weight of PH (MDPH3) and fertilized with the optimal N dose provided the highest dry biomass when in comparison with all other

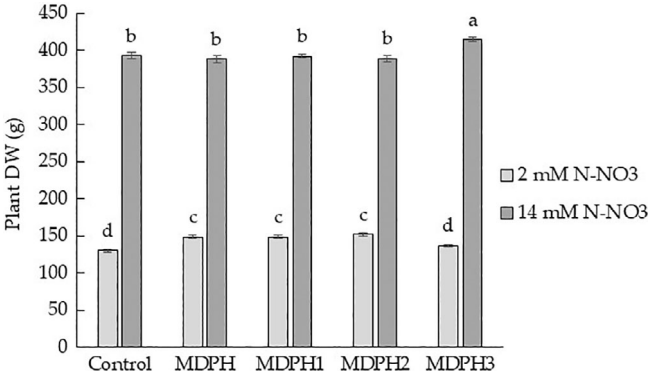

Figure 1. Total dry biomass (leaves, stems, fruits and roots) of tomato plants grown under two nitrogen rates (optimal N-14 = = 14 mM N-NO_3, and suboptimal N-2 = 2 mM N-NO_3) and treated with Malvaceae-derived protein hydrolysate (MDPH) and its molecular fractions (MDPH1, MDPH2, and MDPH3 corresponding to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively). All data are expressed as means ± standard errors ($n = 4$). Different letters above the bars indicate significant differences according to Tukey's HSD test, performed at $P = 0.05$. DW: dry weight.

treatments, whereas treatments MDPH, MDPH1, and MDPH2 sustained biomass accumulation under N deficiency.

Table 1 reports the effect of N level and biostimulant treatment on total and marketable yield and marketable yield components of tomato plants. All yield variables were significantly affected by N input with a strong decrease in all mean values by reducing the N level from 14 to 2 mM. There was a significant interaction between N level and biostimulant for total and marketable yield, and for number of marketable fruits under 14 mM of N. MDPH3 treatment provided the highest increase of total and marketable yield and yield components in comparison with untreated control under optimum N dose whereas there were not significant benefits from the application of biostimulant treatments in increasing marketable yield and its components. However, under suboptimal N level, MDPH2 enhanced total yield by 23% in comparison with untreated control (Table 1).

Total chlorophyll, chlorophyll a and b and carotenoids

Table 2 reports the effect of the nitrogen level and biostimulant treatment on chlorophyll a and b, and total chlorophyll (a + b) level, as well as carotenoid content in tomato leaves. Except for chlorophyll b, foliar pigment concentrations were significantly enhanced by increasing N level. All foliar pigment concentrations were significantly affected by a N level × biostimulant interaction. At the optimal N dose, chlorophyll a, total chlorophyll, and carotenoids were highest in leaves of plants treated with the low molecular fractions of PHs (MDPH2 and MDPH3). At 2 mM of N-NO_3, MDPH3 treatment provided the highest values of chlorophyll a, total chlorophyll, and carotenoids in tomato leaves. The increase in chlorophyll a, total chlorophyll, and carotenoids caused by MDPH3 application was much more pronounced under a suboptimal N level than an optimal N level.

Quality traits of tomato fruits

The effect of N fertilization rate and PH treatments on physical, chemical, and nutritional traits of tomato fruit is shown in Table 3. Fruit dry matter was only affected by the N level with the highest values under suboptimal N supply. Significant interaction was found for fruit firmness with a slight and generalized

Table 1. Effect of N level and biostimulant treatment on total and marketable yield, and number and mean weight of marketable fruits of tomato plants

Source of variance	Total yield (kg plant^{-1})	Marketable yield (kg plant^{-1})	Marketable fruit	
			Number (n. plant^{-1})	Mean weight (g fruit^{-1})
N level (mM N-NO_3)				
14	2.39 ± 0.04a	2.08 ± 0.05a	116.52 ± 2.88a	18.04 ± 0.51 a
2	0.90 ± 0.02b	0.84 ± 0.02b	74.10 ± 2.19b	11.53 ± 0.25b
Biostimulant (B)				
Control	1.54 ± 0.23	1.41 ± 0.21	90.93 ± 7.12	14.70 ± 1.28
MDPH	1.66 ± 0.19	1.40 ± 0.13	93.79 ± 5.65	14.57 ± 0.82
MDPH1	1.63 ± 0.24	1.52 ± 0.21	97.70 ± 7.99	14.92 ± 1.03
MDPH2	1.70 ± 0.22	1.47 ± 0.17	96.58 ± 6.51	14.99 ± 1.39
MDPH3	1.74 ± 0.34	1.48 ± 0.28	95.89 ± 11.80	14.49 ± 1.30
N level × B				
N-14 × Control	2.43 ± 0.09b	2.18 ± 0.09b	119.00 ± 4.47b	19.41 ± 1.13
N-14 × MDPH	2.23 ± 0.08c	1.80 ± 0.05d	108.50 ± 2.97c	16.79 ± 0.63
N-14 × MDPH1	2.33 ± 0.03bc	2.14 ± 0.05bc	120.20 ± 2.53b	17.87 ± 0.49
N-14 × MDPH2	2.40 ± 0.10b	2.01 ± 0.11c	112.33 ± 3.77c	18.35 ± 1.93
N-14 × MDPH3	2.64 ± 0.04a	2.37 ± 0.11a	130.50 ± 2.25a	18.25 ± 0.90
N-2 × Control	0.82 ± 0.02e	0.76 ± 0.02e	72.22 ± 3.56d	11.17 ± 0.67
N-2 × MDPH	0.91 ± 0.04de	0.86 ± 0.04e	74.17 ± 3.57d	11.61 ± 0.50
N-2 × MDPH1	0.92 ± 0.05de	0.90 ± 0.05e	75.20 ± 3.65d	11.98 ± 0.46
N-2 × MDPH2	1.01 ± 0.05d	0.93 ± 0.06e	80.83 ± 2.46d	11.62 ± 0.49
N-2 × MDPH3	0.84 ± 0.03de	0.77 ± 0.05e	68.20 ± 3.39d	11.48 ± 0.69
Significance				
N level	***	***	***	***
B	ns	ns	ns	ns
N level × B	**	***	*	ns

Note: All data are expressed as means ± standard errors; $n = 4$. ns, *, **, ***: non-significant or significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means (N-14 = 14 mM N-NO_3, N-2 = 2 mM N-NO_3) were compared by *t*-tests. Different letters within each column indicate significant differences as determined by Tukey's honestly significant difference (HSD) test ($P = 0.05$). MDPH: Malvaceae-derived protein hydrolysate; MDPH1, MDPH2 and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively.

decrease of mean values in PH-treated plants when in comparison with untreated control at optimal N level whereas no significant effects of biostimulant treatments were recorded on fruit firmness under a suboptimal N level. The SSC was significantly affected by N treatment and PH application. The decrease in the N concentration increased the SSC content by 10% in fruits. When averaged across N level, MDPH1, MDPH2, and MDPH3 increased the SSC content of fruits in comparison with control by 8.7, 6.6 and 7.5%, respectively. Citric acid was the most commonly represented organic acid in tomato fruits, and it was generally reduced by lowering the N supply (Table 3). At optimal N supply, citric acid was increased in fruits by all molecular fractions in comparison with untreated control (avg. +11%), whereas oxalic acid decreased with the lowest fraction of PH treatment (MDPH3) (−17.5%). Under a low N level, citric acid was reduced only by MDPH1 treatment whereas oxalic acid was lower in all biostimulant treatments than in the control. Malic acid was affected significantly only by N treatment with a lower value (−36%) at 2 mM N-NO_3 in comparison with 14 mM N-NO_3. Nitrogen availability also had an impact on the fruit nutrient concentration, as the concentration of P decreased significantly from 3.6 mg g^{-1} DW under optimal N to 2.1 mg g^{-1} DW with N deficiency; similarly, K concentration changed from 34.3 to 27.2 mg g^{-1} DW and magnesium from 1.6 to 1.0 mg g^{-1} DW. On the other hand, reducing the N level from 2 to 14 mM N-NO_3 increased the Ca concentration by 18% (0.9 to 1.0 mg g^{-1} DW), the S concentration by 8% (0.54 to 0.58 mg g^{-1} DW), and the Cl concentration by 421% (1.13 to 5.87 mg g^{-1} DW). No significant differences were recorded in fruit mineral concentration as a result of changing the biostimulant treatment (Table S1).

The fruit lycopene content was significantly affected by N level × biostimulant treatment interaction (Fig. 2). Lycopene content was generally reduced by increasing the N supply. At the optimal N level, MDPH2 and MDPH3 increased lycopene content in fruit in comparison with an untreated control (by 30% and 40%, respectively) whereas biostimulant applications did not enhance lycopene content of fruits under suboptimal N levels.

No interaction was noted between both factors (nitrogen and biostimulant) regarding FRAP and DPPH antioxidant activities in tomato fruits (Table 4). In comparison with N-2, FRAP, and DPPH antioxidant activity in fruit was higher under N-14 by 28.7 and 89.4%, respectively (Table 4). Biostimulant treatment affected only FRAP with a significant increase in comparison with the untreated control in MDPH, MDPH1, and MDPH3 treatments by 31.0, 42.6 and 36.1%, respectively.

Phenolic profile of tomato fruits

Untargeted metabolomics using Ultra high performance liquid chromatography coupled to a quadropole time of flight mass spectrometer (UHPLC-ESI/QTOF) was performed to screen

Table 2. Effect of N level and biostimulant treatment on chlorophyll a and b, total chlorophyll, and carotenoids in tomato leaves

Source of variance	Chlorophyll a mg g^{-1} FW	Chlorophyll b mg g^{-1} FW	Total Chlorophylls mg g^{-1} FW	Carotenoids mg g^{-1} FW
N level (mM N-NO_3)				
14	0.90 ± 0.02a	0.21 ± 0.01	1.12 ± 0.03a	0.26 ± 0.01a
2	0.44 ± 0.02b	0.22 ± 0.01	0.67 ± 0.02b	0.10 ± 0.01b
Biostimulant (B)				
Control	0.62 ± 0.12b	0.24 ± 0.04	0.86 ± 0.08b	0.15 ± 0.05c
MDPH	0.62 ± 0.10b	0.18 ± 0.00	0.81 ± 0.11b	0.17 ± 0.03b
MDPH1	0.63 ± 0.08b	0.20 ± 0.00	0.84 ± 0.09b	0.16 ± 0.02bc
MDPH2	0.72 ± 0.13a	0.24 ± 0.01	0.96 ± 0.15a	0.21 ± 0.05a
MDPH3	0.75 ± 0.06a	0.23 ± 0.01	0.98 ± 0.07a	0.21 ± 0.02a
N level × B				
N-14 × Control	0.88 ± 0.10b	0.15 ± 0.05c	1.04 ± 0.06b	0.27 ± 0.01b
N-14 × MDPH	0.85 ± 0.03b	0.21 ± 0.01bc	1.06 ± 0.03b	0.24 ± 0.01bc
N-14 × MDPH1	0.83 ± 0.03b	0.20 ± 0.01bc	1.04 ± 0.03b	0.22 ± 0.01c
N-14 × MDPH2	1.03 ± 0.03a	0.27 ± 0.01ab	1.31 ± 0.04a	0.32 ± 0.01a
N-14 × MDPH3	0.99 ± 0.05a	0.24 ± 0.01b	1.24 ± 0.05a	0.29 ± 0.01a
N-2 × Control	0.35 ± 0.00d	0.34 ± 0.02a	0.69 ± 0.02d	0.03 ± 0.01f
N-2 × MDPH	0.40 ± 0.01d	0.16 ± 0.01c	0.56 ± 0.01e	0.11 ± 0.01e
N-2 × MDPH1	0.44 ± 0.01d	0.12 ± 0.01bc	0.64 ± 0.02de	0.12 ± 0.01e
N-2 × MDPH2	0.41 ± 0.01d	0.21 ± 0.02bc	0.62 ± 0.02de	0.10 ± 0.01e
N-2 × MDPH3	0.62 ± 0.01c	0.21 ± 0.03bc	0.83 ± 0.03c	0.17 ± 0.01d
Significance				
N level	***	ns	***	***
B	**	ns	***	***
N level × B	**	***	***	***

Note: All data are expressed as means ± standard errors; $n = 4$. ns, **, ***: non-significant or significant at $P \leq 0.01$ and 0.001, respectively. Nitrogen level means (N-14 = 14 mM N-NO_3, N-2 = 2 mM N-NO_3) were compared by *t*-tests. Different letters within each column indicate significant differences according to Tukey's honestly significant difference (HSD) test ($P = 0.05$). MDPH: Malvaceae-derived protein hydrolysate; MDPH1, MDPH2 and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively.

comprehensive profiles of phenolic compounds in tomato fruits treated with biostimulants with different molecular weights under optimal and suboptimal N conditions. A total of 252 phenolic compounds were identified, representing an extensive diversity that included anthocyanins, flavonoid-related subclasses (isoflavonoids, flavanols, flavonols), lignans, phenolic acids, stilbenes, and tyrosols. The complete list of annotated compounds and isomeric structures, along with other characteristics, including individual abundances and composite mass spectra, is given in Supporting Information, Table S2. Further, to give a visual sight of comparison in each class, a semi-quantification was also carried out (Table 5), which summarizes the identified compounds into various classes according to their structure. Isoflavonoids, flavonols, tyrosols, and other polyphenol-related compounds were among the classes that were significantly affected by the N availability level. The concentration of isoflavonoids increased by 25% and flavonols increased by 33%, whereas tyrosols decreased by 35%, and other polyphenols decreased by −21% in the optimal N conditions in comparison with the suboptimal N conditions. In the case of biostimulant application, in general, compounds related to anthocyanins, flavanols, flavonols, lignans, and stilbenes accumulated in fruits treated with different molecular fractions of PH in comparison with the control. Among different molecular weight biostimulants, generally, MDPH2 and MDPH3 showed higher concentrations of flavonols (up to 0.244 mg eq. g^{-1} DW), lignans (up to 0.633 mg eq. g^{-1} DW), and stilbenes (up to 0.082 mg eq. g^{-1} DW). However, phenolic acids were negatively affected by biostimulants, except for MDPH2, which did not show a significant difference in comparison with the untreated control under suboptimal N conditions. Table 5 shows that the concentration of lignans, stilbenes, flavonols, and phenolic acids was significantly influenced by the interaction of N level and biostimulant treatment. Regarding lignans, and stilbenes, the higher content belonged to MDPH3 treatment under low nitrogen conditions recorded by 0.69, and 0.09 mg eq. g^{-1} DW, respectively.

Figure 3 portrays the unsupervised HCA used to decipher the differences among the phenolic profiles. The HCA is based on three factors including N availability level, biostimulant treatment, and the interaction between N level and biostimulant treatment, which highlights the effect of each factor and their potential interplays. In fact, this clustering involves creating clusters that have predominant ordering from top to bottom and pictures both similarities and differences between the metabolomics fingerprints. Considering the HCA, the level of N has clearly resulted in two distinct clusters (i.e., optimal and suboptimal N levels), indicating different phenolic profiles. The behavior of biostimulants with different molecular weights was influenced by the N level. Under optimal N conditions there was no clear separation between control and biostimulants except for MDPH3, which was clearly separated from others. However, different behavior was identified under the suboptimal N condition as highlighted by well separated clusters of individual biostimulants and also the control, indicating the effectiveness of fractioning processes. In

Table 3. Effect of N level and biostimulant treatment on dry matter, firmness, soluble solids content, and organic acids of tomato fruits

Source of variance	Dry matter (%)	Firmness (N mm^{-1})	Soluble solids content (°Brix)	Citric acid (mg g^{-1} DW)	Malic acid (mg g^{-1} DW)	Oxalic acid (mg g^{-1} DW)
N level (mM N-NO_3)						
14	8.94 ± 0.09b	1.42 ± 0.02 a	7.20 ± 0.08b	45.90 ± 0.92 a	5.49 ± 0.10 a	0.54 ± 0.01b
2	9.63 ± 0.06 a	1.21 ± 0.02b	7.94 ± 0.07 a	29.15 ± 0.82b	3.53 ± 0.10b	0.74 ± 0.02 a
Biostimulant (B)						
Control	9.14 ± 0.12	1.42 ± 0.04 a	7.22 ± 0.23b	36.43 ± 2.39b	4.49 ± 0.40	0.73 ± 0.06 a
MDPH	9.29 ± 0.08	1.36 ± 0.03 ab	7.42 ± 0.16 ab	36.85 ± 3.55b	4.52 ± 0.39	0.61 ± 0.02b
MDPH1	9.24 ± 0.15	1.22 ± 0.03c	7.85 ± 0.15 a	36.01 ± 4.50b	4.25 ± 0.46	0.63 ± 0.04b
MDPH2	9.28 ± 0.09	1.31 ± 0.03bc	7.70 ± 0.14 a	40.87 ± 3.16 a	4.87 ± 0.34	0.65 ± 0.03b
MDPH3	9.41 ± 0.02	1.27 ± 0.03bc	7.76 ± 0.16 a	39.47 ± 3.09 a	4.44 ± 0.38	0.61 ± 0.05b
N level × B						
N-14 × Control	8.67 ± 0.22	1.61 ± 0.03 a	6.72 ± 0.21	42.12 ± 1.25b	5.46 ± 0.30	0.57 ± 0.01de
N-14 × MDPH	8.89 ± 0.13	1.45 ± 0.03b	7.07 ± 0.08	45.42 ± 2.64 ab	5.53 ± 0.19	0.58 ± 0.03de
N-14 × MDPH1	8.91 ± 0.27	1.31 ± 0.05c	7.50 ± 0.13	47.49 ± 2.46 a	5.41 ± 0.24	0.53 ± 0.02 ef
N-14 × MDPH2	8.95 ± 0.21	1.45 ± 0.03b	7.32 ± 0.20	48.92 ± 1.82 a	5.66 ± 0.29	0.56 ± 0.03de
N-14 × MDPH3	9.26 ± 0.24	1.28 ± 0.04cd	7.37 ± 0.07	47.57 ± 0.49 a	5.41 ± 0.21	0.47 ± 0.03 f
N-2 × Control	9.61 ± 0.19	1.23 ± 0.03cde	7.72 ± 0.15	30.74 ± 1.90c	3.53 ± 0.22	0.89 ± 0.02 a
N-2 × MDPH	9.70 ± 0.12	1.26 ± 0.06cde	7.77 ± 0.20	28.29 ± 1.76c	3.51 ± 0.14	0.64 ± 0.02cd
N-2 × MDPH1	9.67 ± 0.27	1.14 ± 0.03 e	8.20 ± 0.09	24.53 ± 0.95d	3.08 ± 0.18	0.72 ± 0.04bc
N-2 × MDPH2	9.62 ± 0.15	1.17 ± 0.04de	7.87 ± 0.13	32.83 ± 0.41c	4.08 ± 0.19	0.73 ± 0.02b
N-2 × MDPH3	9.55 ± 0.03	1.25 ± 0.05cde	8.15 ± 0.12	29.37 ± 0.88c	3.49 ± 0.19	0.75 ± 0.02b
Significance						
N level	***	***	***	***	***	***
B	ns	***	**	*	ns	***
N level × B	ns	**	ns	*	ns	***

Note: All data are expressed as means ± standard errors; $n = 4$. ns, *, **, ***: non-significant or significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means (N-14 = 14 mM N-NO_3, N-2 = 2 mM N-NO_3) were compared by *t*-tests. Different letters within each column indicate significant differences according to Tukey's honestly significant differences (HSD) test ($P = 0.05$). MDPH: Malvaceae-derived protein hydrolysate; MDPH1, MDPH2 and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively. DW: dry weight.

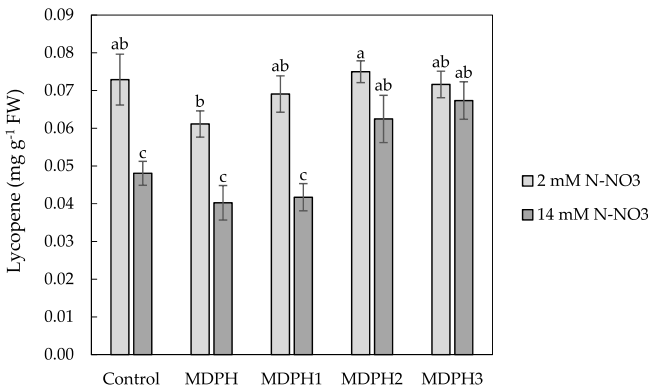

Figure 2. Effect of N level and biostimulant treatment on lycopene content in tomato fruits. MDPH: Malvaceae-derived protein hydrolysate; MDPH1, MDPH2, and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, and <1 kDa, respectively. All data are expressed as means ± standard errors, $n = 4$. Different letters above the bars indicate significant differences according to Tukey's honestly significant difference (HSD) test, performed at $P = 0.05$. FW: fresh weight.

more detail, MDPH2 and MDPH3 showed more similarities where they shared one main cluster, while control, MDPH, and MDPH1 were grouped in another main cluster. In the latter, of course, control has its own subcluster while MDPH and MDPH1 shared another subcluster. This also indicates the effect of biostimulants themselves on phenolic profiles when plants are exposed to low nitrogen in comparison with optimal nitrogen.

To gain more information regarding the phenolic differences among PH treatments under different N fertilization rate, a supervised orthogonal partial least squares discriminant analysis (OPLS-DA) was also performed (Fig. 4). This analysis was followed by VIP ranking for estimating the most discriminating compounds in the projection. The OPLS-DA model showed a differential phenolic profile between fruits treated with different molecular weight biostimulants under the two rates of N fertilization (Fig. 4 (A),(B)). Looking deeper to discover the effect of fractioning processes, DA models were also considered for the behavior of biostimulants under optimal and suboptimal N conditions. As these models show, under optimal N conditions, all fractions of biostimulants were discriminated from the control, indicating the effect of the biostimulant itself. Besides, the model also highlights differences between different fractions as it is more evident for MDPH3. Similarly, under suboptimal N condition, the same behavior was also observed, indicating the effectiveness of biostimulants when plants are under N stress. It is worth noting that the application of MDPH and its first fraction exhibited a close behavior to the control group under optimal N condition. In agreement, both supervised models were characterized by goodness of prediction of Q2 >0.7. Following by VIP selection method, 59 and 76 compounds (excluding the potential isomeric forms of phenolics) were found to be highly discriminant, respectively, under optimal

Table 4. Effect of N level and biostimulant treatment on antioxidant activity (FRAP: Ferric reducing antioxidant activity and DPPH: 2,2-diphenyl-1-picrylhydrazyl) in tomato fruits

Source of variance	FRAP mg TE g^{-1} FW	DPPH mg TE g^{-1} FW
N level (mM N-NO_3)		
14	22.03 ± 0.74a	2.50 ± 0.05a
2	17.11 ± 0.76b	1.32 ± 0.02b
Biostimulant (B)		
Control	15.47 ± 0.82b	1.99 ± 0.18
MDPH	20.28 ± 1.64a	1.90 ± 0.15
MDPH1	22.14 ± 1.27a	1.90 ± 0.16
MDPH2	18.56 ± 0.84ab	1.91 ± 0.16
MDPH3	21.07 ± 1.22a	1.97 ± 0.18
N level × B		
N-14 × Control	18.35 ± 0.38	2.66 ± 0.07
N-14 × MDPH	22.78 ± 2.51	2.46 ± 0.08
N-14 × MDPH1	23.10 ± 1.81	2.39 ± 0.14
N-14 × MDPH2	20.90 ± 0.65	2.49 ± 0.11
N-14 × MDPH3	24.57 ± 1.12	2.51 ± 0.11
N-2 × Control	12.59 ± 0.21	1.31 ± 0.06
N-2 × MDPH	17.78 ± 1.83	1.34 ± 0.06
N-2 × MDPH1	21.18 ± 1.84	1.34 ± 0.03
N-2 × MDPH2	15.45 ± 0.28	1.34 ± 0.04
N-2 × MDPH3	17.57 ± 1.29	1.26 ± 0.03
Significance		
N level	***	***
B	***	ns
N level × B	ns	ns

Note: All data are expressed as means ± standard errors; $n = 4$. ns and ***: non-significant or significant at $P \leq 0.001$, respectively. Nitrogen level means (N-14 = 14 mM N-NO_3, N-2 = 2 mM N-NO_3) were compared by *t*-tests. Different letters within each column indicate significant differences according to Tukey's honestly significant differences (HSD) test ($P = 0.05$). MDPH: Malvaceae-derived protein hydrolysate; molecular fractions (MDPH1, MDPH2 and MDPH3) correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively. TE: Trolox equivalents. FW: fresh weight.

and suboptimal N conditions through ANOVA and OPLS-DA analyses. Venn analysis also indicates only 22 compounds are shared between these two conditions (Fig. 4(C)). The list of VIP discriminant markers with VIP scores greater than 1.1 along with cross-validated standard error and regulation is provided in Supporting Information, Table S3 (14 mM N-NO_3,) and Table S4 (2 mM N-NO_3). The most abundant VIP markers under optimal N conditions were flavonoids (37.2%) mainly anthocyanins and flavanols, phenolic acids (30.5%) mainly hydroxycinnamics, followed by other polyphenols (10.9%) such as alkylphenols, and tyrosols. A trend with a slight change of percentage in each class was observed under suboptimal N condition followed by 48.7%, 19.7%, and 23.7% for flavonoids, phenolic acids, and other polyphenols, respectively. Apart from the main classes, other compounds were also spotted with a lower percentage, including stilbenes and lignans. The highest VIP scores were recorded for 4-ethylphenol (1.7), and cyanidin 3-*O*-glucosyl-rutinoside (1.6) under optimal N level while for naringin 6′-malonate (1.5), and pterostilbene (1.4) under suboptimal N level conditions. Discriminant accumulated flavonoids under biostimulant treatments in the optimal conditions were mainly represented by peonidin 3-*O*-rutinoside, theaflavin, (+)-catechin 3-*O*-gallate, and sesaminol (a lignan), hydroxycinnamic acids derivatives, and so forth. However, some flavonoid-based compounds such as narirutin 4'-*O*-glucoside, quercetin 3-*O*-rhamnoside, daidzein 4'-*O*-glucuronide, 7,4′-dihydroxyflavone, and cyanidin 3-*O*-sambubioside 5-*O*-glucoside were down-accumulated in MDPH treatments in comparison with the control. Ellagic acid arabinoside, rosmarinic acid, *p*-coumaroyl tartaric acid, and dihydrocaffeic acid were also among down-accumulated phenolic acids (Supporting Information, Table S3). On the other hand, logFC analysis showed that some compounds belong to flavonoids (such as cyanidin 3-*O*-arabinoside, daidzein 4′-*O*-glucuronide, sativanone), phenolic acids (dihydrocaffeic acid 3-sulfate, gallic acid 4-*O*-glucoside, isoferulic acid 3-*O*-glucuronide), stilbenes (resveratrol 3-*O*-glucoside), other polyphenols (isopimpinellin) were accumulated (logFC = 4) in fruits when plants treated with biostimulants under low nitrogen levels, as suggested also by the semi-quantitative analysis of classes in Table 5. However, in the same profile, compounds like cyanidin 3-*O*-(6″-dioxalyl-glucoside) (an anthocyanin), 1,2,2′-trisinapoylgentiobiose (a hydroxycinnamic acid), gallic acid 3-*O*-gallate (a hydroxybenzoic acid), and coumestrol (other polyphenols) were reduced.

DISCUSSION

Addressing food demand and fostering sustainability in agricultural systems represent key modern challenges that require innovative solutions and strategic approaches from scientists.[1] Research in this field is investigating ways to maximize yield and product quality under low-input systems based on reduced N inputs.[36] Several scientific achievements have provided evidence that the use of protein hydrolysates (PHs) in agriculture can contribute to the achievement of these objectives, while regulating and improving N use efficiency.[1,15,37-39] Beyond these achievements, which were focused on the agronomic evaluation of whole products, the current research aimed to identify the most active fractions of a Malvaceae-derived PH under optimal and suboptimal N levels. The results showed the ability of MDPH treatments to boost tomato growth (plant dry weight; Fig. 1) in comparison with untreated plants under optimal and especially suboptimal N fertilization. In fact, the best dry-weight accumulation was in plants treated with PHs (MDPH, MDPH1, and MDPH2) when grown under suboptimal N (+14.8% as average of MDPH, MDPH1, and MDPH2 treatments in comparison with untreated plants) whereas treatment MDPH3 favored dry biomass accumulation under optimal N levels. The smallest fractions of MDPH (MDPH3) containing low-molecular-weight peptides are easily adsorbed by leaves and translocated in plant tissues,[40,41] promoting the activation of specific transductional signal pathways involved in endogenous phytohormone synthesis.[18,23] Previous research conducted by Lucini *et al.*[22] showed that a low molecular fraction (molecular weight <1 kDa) of the commercial biostimulant Trainer modulated the phytohormone profile (i.e., auxins, gibberellins, and cytochinins) of tomato cuttings. The above results can thus be linked with the role of small peptides because of their hormone-like activities leading to morpho-physiologic and metabolic changes of plants that induced the regulation of plant growth and development.[42] PHs with peptides of low molecular weight exhibit hormone-like activity and hence improve plant growth and fruit setting as previously reported in tomato[15,40,43] and in leafy vegetables crops grown under diverse N regimes, such as spinach, baby lettuce, baby rocket.[1,18,21] In the current study, the highest marketable production was recorded for plants treated with the smallest PH fraction (MDPH3) and under optimal

Table 5. Effect of N level and biostimulant treatment on the different classes of phenolic compounds in tomato fruits

Source of variance	Anthocyanins (mg eq. g^{-1} FW)	Isoflavonoids (mg eq. g^{-1} FW)	Flavanols (mg eq. g^{-1} FW)	Flavonols (mg eq. g^{-1} FW)	Lignans (mg eq. g^{-1} FW)	Other polyphenols (mg eq. g^{-1} FW)	Tyrosols (mg eq. g^{-1} FW)	Phenolic acids (mg eq. g^{-1} FW)	Stilbenes (mg eq. g^{-1} FW)
N level (mM N-NO_3)									
N-14	0.54 ± 0.16b	0.15 ± 0.03b	0.15 ± 0.08b	0.02 ± 0.00b	0.50 ± 0.19	2.60 ± 0.52b	4.54 ± 0.44b	3.98 ± 1.19	0.07 ± 0.02
N-2	0.59 ± 0.16a	0.20 ± 0.04a	0.22 ± 0.12a	0.03 ± 0.01a	0.51 ± 0.18	3.30 ± 0.58a	7.03 ± 1.28a	3.85 ± 1.95	0.07 ± 0.02
Biostimulant (B)									
Control	0.32 ± 0.09b	0.18 ± 0.05	0.15 ± 0.08b	0.02 ± 0.01b	0.38 ± 0.19b	3.03 ± 0.65	5.75 ± 1.96	4.94 ± 1.97a	0.05 ± 0.01c
MDPH	0.64 ± 0.09a	0.16 ± 0.05	0.14 ± 0.11b	0.02 ± 0.01b	0.40 ± 0.07b	2.84 ± 0.63	5.59 ± 1.55	2.84 ± 0.81b	0.07 ± 0.01bc
MDPH1	0.60 ± 0.11a	0.18 ± 0.04	0.19 ± 0.11ab	0.03 ± 0.01ab	0.53 ± 0.24a	2.64 ± 0.48	5.55 ± 1.34	3.63 ± 1.23b	0.07 ± 0.02ab
MDPH2	0.63 ± 0.12a	0.18 ± 0.04	0.20 ± 0.11ab	0.03 ± 0.01a	0.60 ± 0.09a	3.13 ± 0.58	5.95 ± 1.38	4.81 ± 1.48a	0.08 ± 0.02ab
MDPH3	0.64 ± 0.12a	0.16 ± 0.04	0.24 ± 0.11a	0.03 ± 0.01a	0.63 ± 0.09a	3.12 ± 0.79	6.09 ± 1.70	3.38 ± 1.32b	0.08 ± 0.02a
N level × B									
N-14 × Control	0.30 ± 0.07	0.14 ± 0.02	0.14 ± 0.06	0.01 ± 0.00d	0.28 ± 0.06 e	2.78 ± 0.48	5.01 ± 0.19	4.11 ± 1.49abcd	0.05 ± 0.01d
N-14 × MDPH	0.63 ± 0.10	0.12 ± 0.01	0.09 ± 0.07	0.01 ± 0.00d	0.40 ± 0.08cde	2.44 ± 0.53	4.15 ± 0.23	3.07 ± 1.12bcd	0.06 ± 0.02bcd
N-14 × MDPH1	0.55 ± 0.12	0.16 ± 0.04	0.12 ± 0.09	0.02 ± 0.00d	0.70 ± 0.24a	2.47 ± 0.48	4.39 ± 0.56	4.78 ± 0.44ab	0.08 ± 0.02ab
N-14 × MDPH2	0.61 ± 0.13	0.16 ± 0.03	0.15 ± 0.08	0.02 ± 0.00d	0.56 ± 0.05abcd	2.76 ± 0.54	4.66 ± 0.33	4.64 ± 0.42abc	0.07 ± 0.01abcd
N-14 × MDPH3	0.62 ± 0.13	0.16 ± 0.04	0.24 ± 0.05	0.02 ± 0.00d	0.58 ± 0.08abc	2.60 ± 0.60	4.49 ± 0.32	3.32 ± 1.16bcd	0.07 ± 0.02abcd
N-2 × Control	0.34 ± 0.11	0.21 ± 0.04	0.15 ± 0.10	0.02 ± 0.00cd	0.47 ± 0.24bcde	3.28 ± 0.73	6.50 ± 2.62	5.77 ± 2.13a	0.06 ± 0.01d
N-2 × MDPH	0.65 ± 0.09	0.20 ± 0.05	0.19 ± 0.13	0.03 ± 0.01bc	0.40 ± 0.06cde	3.25 ± 0.45	7.04 ± 0.57	2.62 ± 0.25cd	0.07 ± 0.00bcd
N-2 × MDPH1	0.64 ± 0.10	0.19 ± 0.04	0.25 ± 0.08	0.03 ± 0.01abc	0.36 ± 0.07de	2.81 ± 0.43	6.70 ± 0.69	2.47 ± 0.21d	0.06 ± 0.01cd
N-2 × MDPH2	0.64 ± 0.12	0.17 ± 0.04	0.24 ± 0.11	0.03 ± 0.02a	0.63 ± 0.10ab	3.12 ± 0.79	6.09 ± 1.70	3.38 ± 1.32ab	0.08 ± 0.02abc
N-2 × MDPH3	0.67 ± 0.11	0.17 ± 0.03	0.25 ± 0.15	0.04 ± 0.01ab	0.69 ± 0.07a	3.64 ± 0.60	7.68 ± 0.54	3.43 ± 1.55bcd	0.09 ± 0.02a
Significance									
N level	*	***	**	***	ns	***	***	ns	ns
B	***	ns	*	***	***	ns	ns	***	***
N level × B	ns	ns	ns	**	***	ns	ns	**	***

Note: All data are expressed as means ± standard errors; $n = 4$. ns, *, **, ***: non-significant or significant at $P \leq 0.05$, 0.01 and 0.001, respectively. Nitrogen level means (N-14 = 14 mM N-NO_3, N-2 = 2 mM N-NO_3) were compared by *t*-tests. Different letters within each column indicate significant differences according to Tukey's honestly significant differences (HSD) test ($P = 0.05$). MDPH: Malvaceae-derived protein hydrolysate; MDPH1, MDPH2 and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively. Eq: equivalents. FW: fresh weight.

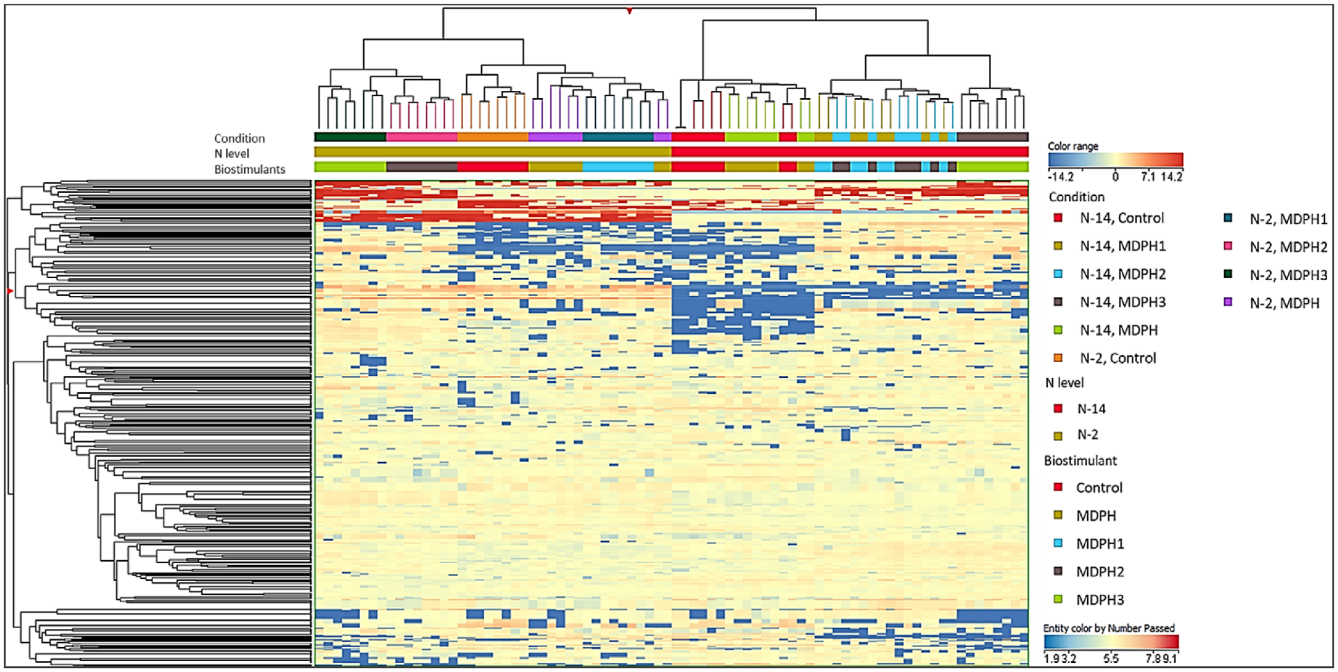

Figure 3. Unsupervised hierarchical cluster analysis for the untargeted phenolic profiling of tomato fruits treated by biostimulants with different molecular weight (MDPH is the Malvaceae-derived protein hydrolysate; MDPH1, MDPH2, and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively) under optimal (N-14 = 14 mM N-NO_3) and suboptimal (N-2 = 2 mM N-NO_3) condition. The heatmap was based on the fold-change values of clustered samples, according to Ward's algorithm and using Euclidean distance.

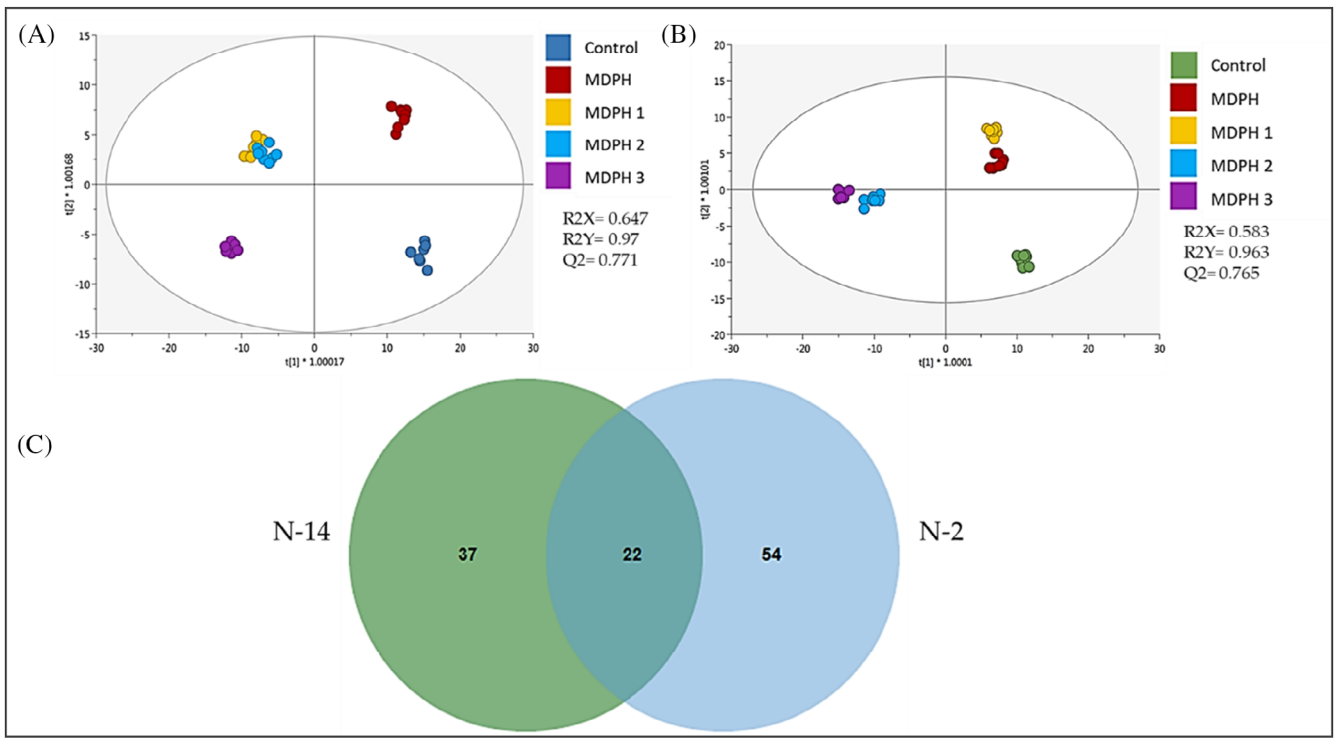

Figure 4. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) score plots built considering the effect of biostimulants (MDPH is the Malvaceae-derived protein hydrolysate; MDPH1, MDPH2 and MDPH3 correspond to molecular fractions >10 kDa, between 1 and 10 kDa, <1 kDa, respectively) under optimal N (A = 14 mM N-NO_3) and suboptimal N conditions (B = 2 mM N-NO_3) as class discrimination parameters. The cumulative goodness parameters of each model, namely R2X, R2Y, and Q2, are provided. Venn analysis (C) of VIP makers extracted by OPLS-DA using fold change under both nitrogen levels.

N availability and was linked to an increase in the number of fruit per plant (Table 1). This increase could be explained by the fact that auxin and the gibberellin-like activity of MDPH3 may have promoted fruit set.[15,44] Indeed, it is known that fruit set initiation is regulated, in terms of pollination and fertilization, by a cross-talk between auxins and gibberellins.[45] In addition to hormone-like effects, another possible mechanism behind the marketable yield increase could be the enhancement of photosynthetic capacity and, therefore, the better translocation of photosynthates to fruit setting.[15,18] So far, studies have shown that PHs can improve the movement of photosynthetates towards the parts of the plant where they are needed, such as fruits. This is achieved by increasing the amount of chlorophyll, and by enhancing the nutrient content of the leaves, especially their N levels.[7] This was the case in the current research because the foliar application of MDPH and its fractions induced a significant increase in chlorophyll a and total chlorophyll, in comparison with the untreated plants, even under suboptimal N conditions (2 mM N-NO_3). The concentration of chlorophyll a, b and total chlorophyll is also an indicator of leaf N content as well as of N utilization by plants because chlorophyll synthesis depends on the availability of amino acids.[46]

Although N fertilizers are needed to maximize crop productivity, excessive application can result in undesirable changes in horticulture commodities, thus leading to a reduction of commercial, nutritional, and functional quality.[20] With regard to soluble solids content, our results were in agreement with Li *et al.*[47] who revealed a decrease of soluble solids content (SSC) in tomato fruit when the N dose increased. However, irrespective of the N fertilization treatment, the effect of PHs positively modulated the synthesis and the accumulation of soluble solids content with the highest value when tomato plants were treated with MDPH fractions. Consistently with our findings, other researchers reported the increase of soluble solids in tomatoes upon treatment with PHs.[15,40,43] The positive effect of MDPHs on SSC could be ascribed to them sustaining C and N metabolism by enhancing photosynthetic CO_2 assimilation and translocation of neo-synthetized soluble sugar to the fruit and thereby improving their quality attributes.[15]

With regard to the organic acids, malate, oxalate, and citrate contribute to modulate freshness and sweetness, which are two important balance factors for the taste-related traits of tomato fruits.[48] The relative amounts of each organic acid in respect of total acidity obtained in PH-treated plants is consistent with the tomato-ripening process and with the improved fruit quality. The MDPH-mediated increase in the concentration of citric acid, a crucial complexing agent for oxidant metals, may also have enhanced its synergistic reducing action in conjunction with ascorbic acid. Oxalic acid is considered an anti-nutrient compound that inhibits the bioavailability of calcium (Ca^{++}) because of its tendency to form insoluble salts with cations and this molecule may therefore increase the risk of kidney stones in the human body.[49] For this reason, the reduction in oxalic acid induced by MDPHs should be considered a positive effect from the point of view of human nutrition. Analogously, tomato fruits firmness was affected by all MDPHs in comparison with untreated controls, at optimal N levels. Our finding was different from those reported by other authors, who recorded that PHs increased tomato fruit firmness.[39,43] However, knowledge about the underlying mechanisms that control fruit firmness still remains incomplete.[50] We suppose that MDPHs can stimulate and coordinate changes in endogenous phytohormone biosynthesis, as previously postulated by Hawkesford *et al.*,[51] who verified a change of cell-wall components leading to better flexibility and plasticity of cell membranes under treatments with algae extract containing hormones. Overall, fruit development is pivotally maintained by a rather complicated and still poorly understood coordinated interaction network between ethylene, gibberellins, and auxins, cytokinins and abscisic acid.[52–55]

Regarding functional quality, the lycopene content was higher in fruits of plants treated with MDPH fractions than in untreated controls under optimal N levels (MDPH2, MDPH3; Fig. 4). This finding is in agreement with previous research reported by several authors.[39,56] Tomato fruit represents an important functional food because the antioxidant molecules exhibit their essential role in the prevention of various human diseases.[25,57] On the other hand, a comprehensive analysis of the untargeted metabolomics has shown that the low N level significantly activated almost all phenolic classes. It has been reported previously that reduced N availability is an efficient strategy to increase the accumulation of phenolic compounds;[58] a similar increase in phenolic compounds, and, in particular, anthocyanins, flavanols, lignans, and stilbenes, was observed for the fruits of plants grown under the PHs treatments in comparison with the control plants, especially MDPH3, indicating the effectiveness of the PH fractionation process. This may have been caused by higher plant uptake of PHs with signaling peptides having lower molecular weight. Moreover, in agreement with lycopene content, phenolic analysis of fruits also indicated that PHs with different molecular weights have affected the profiling under the both optimal and suboptimal N conditions remarkably, which is more evident in the low N treatment as it is confirmed by HCA, OPLS-DA, and Venn analyses. For example, a higher concentration of anthocyanins, flavonols, lignans, tyrosols, and other polyphenols was recorded for MDPH3 under low N levels. This synergistic effect can be attributed, first, to nutrient availability modulation induced by biostimulants[7] and, second, to improved signaling molecules involved in the secondary metabolism.[59,60] Regarding bioactive compounds modulated by biostimulants under low N level, the results showed that the content of flavonols, lignans, and stilbene subclasses has been significantly increased in comparison with plants grown under optimal N level and control, which is in line with recent studies reported the effectiveness of different biostimulants on nutraceutical and biochemical parameters.[61-63] Quercetin, naringenin, rutin, and chlorogenic acids derivatives have been reported to be the main phenolic compounds in tomato plants.[64] In the current study, PHs treatments under low N levels resulted in identifying more discriminant compounds related to categories such as quercetin 3-*O*-glucosyl-rhamnosyl-glucoside, quercetin 3-*O*-(6″-acetyl-galactoside) 7-*O*-rhamnoside, quercetin 3-*O*-(6″-malonyl-glucoside), quercetin 3-*O*-rutinoside, quercetin 3-*O*-galactoside, 6-prenylnaringenin, and luteolin 7-*O*-rutinoside, which were not identified in the optimal N level treatments.

CONCLUSIONS

This study adds useful information regarding the mode of action of PHs. It highlights how the effect can vary depending on the different molecular weights of peptides extracted from the same matrix of a new Malvaceae-derived PH. The peptide length affected plant performance, and this also depended on the N level (optimal or suboptimal). Under optimal N conditions, the smaller fraction (MDPH3, <1 kDa) successfully increased plant biomass, total and marketable yield, and fruit lycopene content, whereas when N was limited it was the MDPH1 (>10 kDa) and MDPH2

(1–10 kDa) fractions that determined the greater accumulation of dry matter in tomato plants. Regardless of N availability, all the fractions affected fruit quality by increasing SSC and modifying the organic acid and phenolic profile. The results confirm the pivotal role of peptide molecular weight in determing the biostimulantc activity of PHs, as previously postulated. In this case, the lower fraction (<1 kDa) had a separated effect in comparison with the other ones under optimal N, while PHs fractions with molecular weight over 1 kDa showed distinct effects under suboptimal N, confirming the effectiveness of fractioning processes.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, Mariateresa Cardarelli, Youssef Rouphael, Youry Pii, Luigi Lucini, Giuseppe Colla; methodology, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Hajar Salehi, Paola Ganugi, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Luigi Lucini, Giuseppe Colla; software, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Youssef Rouphael, Youry Pii., Luigi Lucini, Giuseppe Colla; validation, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Hajar Salehi, Paola Ganugi, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Luigi Lucini, Giuseppe Colla; formal analysis, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Youssef Rouphael, Youry Pii., Leilei Zhang, Giuseppe Colla; investigation, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Hajar Salehi, Paola Ganugi, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Leilei Zhang, Giuseppe Colla; resources, Youssef Rouphael, Youry Pii, Luigi Lucini, Giuseppe Colla; data curation, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Hajar Salehi, Paola Ganugi, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Leilei Zhang, Luigi Lucini, Giuseppe Colla; writing—original draft preparation, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Christophe El Nakhel, Youssef Rouphael, Youry Pii; writing—review and editing, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Luigi Lucini, Seunghyun Choi, Hye-Ji Kim, Giuseppe Colla; visualization, Mariateresa Cardarelli, Angela Valentina Ceccarelli, Hajar Salehi, Paola Ganugi, Christophe El Nakhel, Youssef Rouphael, Youry Pii, Luigi Lucini, Giuseppe Colla; supervision, Mariateresa Cardarelli, Youry Pii, Luigi Lucini, Youssef Rouphael., Giuseppe Colla; project administration, Mariateresa Cardarelli, Youry Pii, Luigi Lucini, Youssef Rouphael, Giuseppe Colla; funding acquisition Youry Pii, Luigi Lucini, Youssef Rouphael, Giuseppe Colla. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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