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Effects of vegetal- versus animal-derived protein hydrolysate on sweet basil morpho-physiological and metabolic traits

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ABSTRACT

Despite scientific evidence supporting the biostimulant activity of protein hydrolysates (PHs) derived from vegetal or animal sources, the morpho-physiological and biochemical mechanisms underlying the biostimulant action of PHs from plant biomass or animal by-products are still poorly explored. Accordingly, we performed a greenhouse experiment for assessing the morphological, physiological and biochemical responses of sweet basil (*Ocimum basilicum* L.) to three nitrogen equivalent rates (0.05, 0.15, and 0.25 g N/kg) of an animal-derived protein hydrolysate (A-PH) and a vegetal-derived protein hydrolysate (V-PH). The V-PH and A-PH applications determined a quadratic-dose response regarding the number and area of leaves and the shoot fresh and dry weight, with the best results obtained using V-PH at the N equivalent rates of 0.05 and 0.15 g N/kg. Improvement of shoot fresh weight with V-PH foliar application at the rate of 0.15 g N/kg was associated with a higher leaf CO_2 assimilation and water use efficiency, with a concomitant higher uptake and translocation of K, Mg, and S in leaf tissue. The excessive accumulation of Na, Cl, and some amino acids (e.g., proline) under A-PH applications above 0.05 g N/kg induced a rapid decrease in plant photosynthetic performance, growth, and biomass production. The plants treated with A-PH at a higher dosage appeared to activate an alternative pathway involving the synthesis of alanine and GABA for storing excess ammonia, buffering cytoplasmic acidosis, and counteracting the negative effects of Na and Cl at toxic levels. The above findings demonstrated the potential benefits of protein hydrolysate application in agriculture, especially of vegetal-derived PHs, and highlighted the need to understand dose-dependent effects in order to optimize crop response.

1. Introduction

The global food production system highly contributes to global warming, land-use change, and pollution of water bodies and soils through excess fertilizers and pesticide runoff (Springmann et al., 2018). In particular, greenhouse horticulture is the most resource-intensive agriculture system, using the highest inputs of fertilizers per unit area (Colla et al., 2011). However, since the exponential growth of population and the improved quality of life are predicted to double the world's demand for food by 2050, it is necessary to secure food supply while adopting more sustainable agricultural practices (Searchinger et al., 2018). Plant biostimulants represent a promising strategy to guarantee food safety without burdening the environment (Calvo et al., 2014). Currently, vegetable- and animal-derived protein hydrolysates (V-PHs and A-PHs, respectively) represent an interesting category of biostimulants for agriculture (Colla et al., 2015). Their composition is influenced by the source of proteins (plant or animal origin) and the hydrolytic processes (chemical-thermal and/or enzymatic hydrolysis) utilized (Ertani et al., 2009, 2013; Colla et al., 2017a). Enzymatically produced V-PHs are mainly characterized by signaling peptides as bioactive compounds, whereas free amino acids are the main bioactive molecules in chemically-produced A-PHs. A-PHs are used for more than 50 years because of their ability to enhance plant growth and mitigate the effects of environmental stresses. A-PHs, like Siapton®, have a high content of free amino acids, with specific proline/hydroxyproline ratios. They improve the physiology of the crop by increasing the natural plant

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defenses and result effectively in regulating crop response to abiotic stress conditions (Colla et al., 2017a). They also improve nitrogen use efficiency by activating N metabolism enzymes. However, the effects of A-PHs seem effective and advantageous only when they are supplied at low doses, while repeated or high foliar treatment doses can elicit toxic effects (Liu and Lee, 2012). Some A-PH components might be responsible for the dose-dependent toxicity. In particular, since chemical production hydrolysis uses alkalis or acids, it increases chloride content and other salts in A-PHs, at much higher doses than those of V-PHs.

Moreover, A-PHs contain high levels of thermostable amino acids like glycine, alanine, proline, hydroxyproline, and hydroxylysine. Exogenous glycine supply to roots can inhibit root growth and decrease nitrate uptake in *Brassica campestris* (Han et al., 2018). These effects, particularly that of root growth inhibition, have been ascribed to the increase of ethylene caused by the glycine dependent-enhancement of the enzymes involved in its synthesis. Muller and Touraine (1992) showed that 18 h pre-treatment with alanine could inhibit N uptake by 50 % in soybean seedlings. Exogenous proline can also impart toxic effects if supplied at excessive concentrations and/or over-accumulated (Hayat et al., 2012). Among these negative effects, it was possible to observe accumulation of toxic concentrations of chloride in tomato leaves (Heuer, 2003) and inhibition of *Arabidopsis* hypocotyls organogenesis *in vitro* (Hare et al., 2001) under 10 mM proline supply, and toxic effects and poor plant growth in rice seedlings under 40–50 mM proline (Roy et al., 1993). The proline's high dose-dependent toxic effects can derive from the feedback inhibition exerted by proline itself on Δ^1-pyroline-5-carboxylate synthetase (P5CS), the enzyme involved in its cytosolic synthesis from glutamate. In fact, the functioning of this enzyme and proline synthesis have been proved to finely tune, by an unknown mechanism independent of hormones, on several processes involved in growth and development (e.g., root elongation, flowering time, pollen fertility, and embryo formation; Trovato et al., 2018).

The content of hydroxyproline in animal collagen is around 100–130 residues/1000 amino acid residues in meat because it plays a fundamental and unique role in maximizing cross-linking and improving the functional properties of collagen (Berg and Prockop, 1973; Muralidharan et al., 2013). Hydroxyproline has been demonstrated to act as proline (Kuznetsov and Shevyakova, 1997) and glycine betaine (Carillo et al., 2008) biosynthesis inhibitor. Hydroxylysine, further contribues to the formation of collagen cross-links (Asghar and Henrickson, 1982), has also been proved to be able to inhibit glutamine synthetase isoforms GS1 and GS2 in maize (Acaster and Weitzman, 1985) and pea leaves (Leason et al., 1982). Moreover, during the chemical hydrolysis of proteins at high temperature, the A-PHs undergo the destruction of thermolabile amino acids, among which minor amino acids like tryptophan, this latter playing an important role in plant physiology as a precursor of auxin synthesis.

On the other hand, V-PHs are emerging as a valid alternative to A-PHs. V-PHs can be produced from plant-derived protein sources such as residual crop biomasses or agricultural by-products, therefore representing an eco-friendly and economical solution to reduce waste, minimize reliance on landfill and pollution, and increase recycling in compliance with the principles of the circular economy (Colla et al., 2017b). Moreover, no phytotoxicity problems have ever emerged upon foliar treatments with V-PHs alone, such as Trainer®, even at doses much higher than those recommended by manufacturers (Cerdán et al., 2009; Colla et al., 2014; Kim et al., 2019). V-PHs consist mainly of a mixture of amino acids (e.g., aspartic acid, glutamic acid, and essential amino acids), oligopeptides and polypeptides, (Colla et al., 2014), which can enhance resource use efficiency in horticultural crop production (Colla et al., 2017a, 2017b; Rouphael et al., 2018), C and N metabolism (Ertani et al., 2009, 2013; Colla et al., 2015), productivity (Colla et al., 2017a, 2017b; Rouphael et al., 2017), product quality (Ertani et al., 2014; Colla et al., 2017a, 2017b) and tolerance to abiotic stresses (Ertani et al., 2013; Lucini et al., 2015, 2018). The beneficial effects of V-PHs have been attributed to bioactive peptides with hormone-like activities (auxin and gibberellins) able to modify root growth and architecture (e.g., density, length, and the surface of lateral roots) and shoot growth, thus enhancing nutrients uptake and crop yield (Ertani et al., 2009; Li et al., 2016; Colla et al., 2017a, 2017b). V-PH peptides and amino acids like glutamic acid and aspartic acid can also form complexes with mineral nutrients making them more available for root uptake and enhancing microbial activity (Colla et al., 2015, 2017a, 2017b). Besides, V-PHs root drenching can increase iron uptake and transport in plants and chlorophyll concentration (Cerdán et al., 2013).

Although plant responses to A- and V-PHs have been evaluated in several studies, few of them have compared the effects of the two types of PHs on plant growth and metabolism in the same conditions (Cerdán et al., 2009, 2013; Polo and Mata, 2018), and none of them has been performed applying equivalent values of N from both A-PHs and V-PHs. Thus, we have analyzed the morphological, physiological, and metabolic responses of sweet basil to three nitrogen equivalent rates (0.05, 0.15, 0.25 g N/kg) of two widely used commercial PHs, Siapton® (A-PH) and Trainer® (V-PH). Several parameters were evaluated: growth, leaf gas exchange, photosynthetic pigments, and photosystem efficiency, mineral profile, sugars, proteins, free amino acids, polyphenols, and antioxidant capacity. Tools of multivariate statistics, already proven to be effective in studies on effects of plant biostimulants in horticultural species, were applied to achieve a comprehensive and integrated interpretation of data. (Carillo et al., 2019a, b).

2. Materials and methods

2.1. Tested crop and greenhouse conditions

Sweet basil (*Ocimum basilicum* L.) cultivar Gecom (Genovese type; SAIS, Cesena, Italy) was grown in a protected greenhouse of the Horticulture section, University of Naples Federico II, Italy. The experiment was conducted on sandy loam soil: 75 % sand, 17 % silt, and 8% clay. Before transplanting, the soil was analyzed to assess its chemical characteristics. The soil had an electrical conductivity of 600 μs cm^{-1}, a pH of 7.1, and an organic matter of 1.3 % (w/w). The nitrate, ammonium, available phosphorus and exchangeable potassium (mg/kg) were 100, 10, 45, and 900, respectively.

2.2. Experimental design, protein hydrolysates sources and characteristics, and cultural management practices

A randomized complete-block scheme was applied with a total of 7 treatments, replicated three times, accounting for a total of 21 experimental units (each replicate/experimental unit having an area of 2 m^2). Sweet basil was hand transplanted on March 28, 2018, at a plant density of 33 plants/m^2. In addition to the control treatment, both vegetal (V) and animal (A) protein hydrolysates (PH) were applied at three N equivalent rates 0.05, 0.15, 0.25 g N/kg, which correspond to 1.0, 3.0, and 5.0 g/kg for V-PH and 0.6, 1.8 and 2.9 g/kg for A-PH, respectively. In all treatments, a total amount of 100 kg N/ha was applied in two identical doses as nitrate ammonium (34 % of N) at 15 and 31 days after transplanting (DAT). The amount of N applied with PHs was considered negligible, being up to a maximum of 0.8 % on the total N applied at the highest PH rate (0.25 g/kg N equivalent). Since P and K had a high content in the soil, they were not further supplied by fertilization. Standard greenhouse management procedures were adopted for the control of pathogens, pests, and weeds.

The commercial biostimulant Trainer®, a legume-derived protein hydrolysate (V-PH), was manufactured by Hello Nature, Rivoli Veronese, Italy. It was obtained through enzymatic hydrolysis and contained 190 g/kg of organic carbon and 50 g/kg of organic nitrogen, 310 g/kg of peptides, small amount of free amino acids, and no phytohormones. The detailed composition of the V-PH was reported by Rouphael et al. (2017) and Paul et al. (2019).

The commercial Siapton® (Isagro S.p.A., Milano, Italy) biostimulant

was a collagen-derived protein hydrolysate (A-PH), obtained in high-pressure thermal reactors (140 °C for 30 min at 3.6 atm). It contained 250 g/kg of organic carbon and 85 g/kg of organic nitrogen, 543.5 g/kg of total amino acids, and 79 g/kg of free amino acids. The detailed composition was reported by Polo et al. (2006).

The V- and A-PH treated basil plants were uniformly sprayed four times (27, 34, 41, and 48 DAT) during the cultivation cycle at 7-days intervals starting on April 23 (27 DAT).

2.3. Harvesting, biometric measurements, and sampling

The harvest of basil plants was done on May 16, 50 DAT, and the following biometric data were determined: number and area of leaves, fresh and dry shoot (leaves plus stems) biomass. A Li-Cor3000 electronic area meter (Li-Cor, Lincoln, NE, USA) was used to assess the leaf area, whereas dry shoot biomass was determined by placing the plant material in an oven at 75 °C for three days until achieving constant weight. For pigments (chlorophyll and carotenoids) and quality (total phenols and antioxidant activities) analyses, basil leaves were shock frozen in liquid N and then stored at −80 °C until further use.

2.4. Leaf colorimetric measurements, SPAD index, and leaf gas exchange

Prior to harvest (49 DAT, May 15), a Minolta Chroma meter, CM-2600d (Minolta Camera Co. Ltd., Osaka, Japan) was used to evaluate the colorimetric indices (L*, a*, b*) on the top leaf blade of 15 fully expanded leaves per experimental unit/replicate. On the same date, a handheld Minolta Chlorophyll Meter SPAD-502 (Minolta Camera Co. Ltd., Osaka, Japan) was used to measure the green index (SPAD) of the third fully expanded leaves from the top in 10 plants per replicate. Leaf gas exchange measurements were performed in leaves from the same phenological stage were also determined by a portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., UK). For each experimental unit, four measurements were made in the 11:00−13:00 timeslot. The following physiological parameters were recorded: i) net CO_2 assimilation rate, ii) stomatal resistance, and iii) transpiration rate. The physiological water use efficiency was calculated as the ratio between the net CO_2 assimilation rate and the transpiration rate.

2.5. Mineral composition analysis

The total N of basil leaf tissue was assessed according to the Kjeldahl method (Bremner, 1965). Basil concentrations of potassium, phosphorus, calcium, magnesium, sulfur, chloride, and sodium were separated and quantified as previously described in detail by Rouphael et al. (2017) by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA).

2.6. Soluble carbohydrates and starch analysis

Soluble carbohydrates were determined after ethanolic extraction and hydrolysis of starch to glucose in the pellets by a coupled enzymatic assay described by Carillo et al. (2019b). The absorbance was recorded at 340 nm by a FLX-Xenius spectrophotometer (SAFAS, Monaco).

2.7. Total chlorophyll and carotenoids analysis

Total chlorophylls, as the sum of chlorophyll *a* and chlorophyll b, and total carotenoids after methanolic extraction (Annunziata et al., 2012), were estimated spectrophotometrically at 665, 652, and 470 nm by FLX-Xenius microplate reader (SAFAS, Monaco) according to Wellburn (1994).

2.8. Antioxidant activities and polyphenols analysis

The hydrophilic antioxidant fraction (HAA) and the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) antioxidant activity of lyophilized basil leaves were quantified by UV–vis spectrophotometry at 505 and 734 nm, respectively by a FLX-Xenius spectrophotometer (SAFAS, Monaco), according to Fogliano et al. (1999) and Pellegrini et al. (1999), respectively.

The total polyphenols content, after methanolic extraction, was determined by the Folin–Ciocalteu method as reported by Carillo et al. (2019b), measuring the absorbance at 760 nm by a FLX-Xenius spectrophotometer.

2.9. Proline, free and total amino acids analysis

Primary amino acids after a 60 % (v/v) ethanolic extraction performed according to Carillo et al. (2012) were determined, after pre-column derivatization with o-phthaldialdehyde (OPA), by high-performance liquid chromatography (HPLC) according to Woodrow et al. (2017). Proline was determined in the same ethanolic extracts by mixing an aliquote of 50 µL with 100 µl of a reaction mix consisting of ninhydrin 1% (w/v), acetic acid 60 % (v/v), ethanol 20 % (v/v), and ddH_2O 20 % at 95 °C for 20 min and reading the absorbance at 520 nm using a FLX-Xenius spectrophotometer (SAFAS, Monaco) according to (Woodrow et al., 2017).

2.10. Statistics and principal component analysis

All data underwent one-way analysis of variance (ANOVA) using the software package SPSS 13. The means were separated by Duncan's test ($p = 0.05$). Regression analysis was conducted to identify relationships between the biometric parameters and the PH sources and doses. A principal component analysis was performed on all analyzed parameters to determine the dominant parameters that mainly discriminated between the two PH sources and the three application doses using Minitab® 18 software (Ciarmiello et al., 2015).

3. Results

3.1. Implications of protein hydrolysate sources and rates for morpho-physiological responses, starch, and soluble carbohydrate content

The significant relationships between the biometric parameters: leaf number, total leaf area, fresh and dry shoot biomass, and biostimulant rate expressed in g/kg of N equivalent were best described by non-linear functions for both V-PH and A-PH (Fig. 1). Except for the dry shoot biomass, the remaining three functions exhibited a coefficient of determination $R^2 \geq 0.95$ (Fig. 1). The quadratic functions reported in Fig. 1 showed that V-PH applications allowed to reach a higher leaf number per plant (87.9 vs. 77.0 at 0.1 g/kg of N equivalent rate for V-PH and 0.09 g/kg of N equivalent rate for A-PH, respectively), total leaf area (1006.1 vs. 882.7 cm^2 $plant^{-1}$ at 0.13 g/kg of N equivalent rate for V-PH and 0.1 g/kg of N equivalent rate for A-PH, respectively), shoot fresh biomass (59.2 vs. 51.8 g $plant^{-1}$ at 0.15 g/kg of N equivalent rate for V-PH and 0.1 g/kg of N equivalent rate for A-PH, respectively), and shoot dry biomass (6.4 vs. 6.1 g $plant^{-1}$ at 0.13 g/kg of N equivalent rate for V-PH and 0.08 g/kg of N equivalent rate for A-PH, respectively), in comparison to A-PH treatments. The above findings demonstrated that V-PH biostimulant was able to stimulate the vegetative growth much more than A-PH (Fig. 1). Interestingly, leaf traits and shoot biomass in A-PH treated plants showed a more pronounced decline after reaching the maximum value than in V-PH treated plants (Fig. 1).

The different changes induced by the V-PH application rates between 0.1 to 0.15 g N/kg in the vegetative growth (higher leaf number and area and fresh shoot biomass) were also shown at a physiological level (Table 1). The highest fresh shoot biomass observed with V-PH at 0.15 g N/kg seems to be associated with a higher photosynthetic activity: significantly ($p < 0.001$) higher net CO_2 assimilation rate and instantaneous water use efficiency (WUE_i; Table 1). Neither PH sources nor

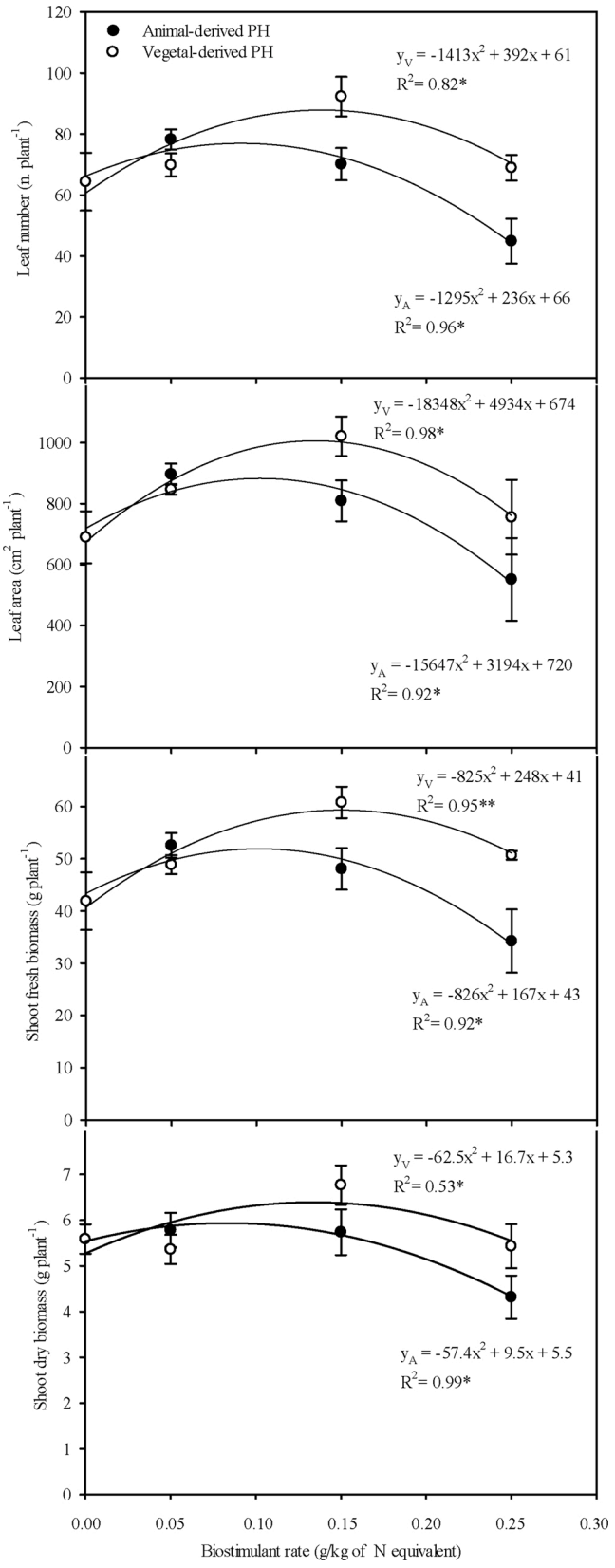

Fig. 1. Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0, 0.05, 0.15, or 0.25 g N/kg)] on leaf number, leaf area, shoot fresh biomass and shoot dry biomass of sweet basil plants. Mean values ± standard errors.

Table 1
Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)] on net assimilation rate (A_{CO2}; (μmol CO_2 m^{-2} s^{-1}), transpiration rate (E; mol H_2O m^{-2} s^{-1}), and instantaneous water use efficiency (WUEi; μmol CO_2 mol^{-1} H_2O) in basil leaves.

Treatment	A_{CO2}	E	WUEi
Control	8.21d	2.60	3.18b
A-0.05 N	10.87b	3.90	2.85b
A-0.15 N	9.60bc	3.49	2.81b
A-0.25 N	9.41cd	4.09	2.35b
V-0.05 N	10.52bc	3.62	2.92b
V-0.15 N	12.57a	2.76	4.65a
V-0.25 N	9.80bc	3.16	3.13b
Significance	***	ns	***

ns(non significant), ***($p < 0.001$). Different letters within each column reveal significant differences among treatments according to Duncan's test $p = 0.05$.

application rates influenced the transpiration (average 3.37 mol H_2O m^{-2} s^{-1}; Table 1). Similarly, fructose (average 0.38 mg g^{-1} fw) and starch (average 8.4 mg g^{-1} fw) content were not affected by the tested factor as reported in Table 2, except for fructose at V-0.15 g N/kg, which significantly ($p < 0.05$) decreased. Finally, the lowest glucose and sucrose content in sweet basil leaves were recorded in A-PH and V-PH at 0.15 g/kg of N equivalent, respectively (Table 2).

3.2. Implications of protein hydrolysate sources and rates for leaf mineral composition, colorimetric indices, pigments, and qualitative parameters

PHs treatments (sources and rates) did not significantly influence P (average 1.5 g kg^{-1} dw) and Ca (average 9.4 g kg^{-1} dw) concentrations in leaf tissue (Table 3). The N concentration in basil leaves was positively affected by both V-PH and A-PH application, especially at 0.15 and 0.25 N equivalent treatments (Table 3). Basil-treated plants with V-PH had the highest K concentration in leaf tissue at V-0.15 N and the lowest in A-0.15 N ones (Table 3). Moreover, data observed on leaf Mg concentration confirmed the trend recorded for the K content with the highest values observed in plants treated with V-PH 0.15 N (Table 3). The Na and Cl concentrations were significantly affected by the biostimulant rate, as reported in Table 3. Leaf sodium concentration was significantly ($p < 0.01$) increased by 54 % compared to the untreated control when the foliar applications of the A-PH were delivered at 0.25 g N/kg. Interestingly, Na concentration in leaves was not significantly changed by V-PH applications at any rate. Chloride concentration in leaves increased from 20.5 to 30.3 g/kg dw in A-PH treatments as the biostimulant rate increased from 0.05 to 0.25 N equivalent, respectively, whereas a slight accumulation of chloride was observed even when V-PH was applied to basil (average 17.9 g/kg dw; Table 3), being significant only at V-0.15 N.

The Hunter color parameters, in particular the lightness (L*) and greenness (-a*), were significantly influenced by the biostimulant

Table 2
Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)] on carbohydrate content in basil leaves.

Treatment	Carbohydrate content (mg g^{-1} fw)			
	Glucose	Fructose	Sucrose	Starch
Control	0.86ab	0.50	0.10ab	9.32
A-0.05 N	0.69abc	0.41	0.10ab	9.05
A-0.15 N	0.37c	0.27	0.08abc	8.03
A-0.25 N	0.90ab	0.32	0.06bc	8.24
V-0.05 N	0.51bc	0.37	0.11a	9.17
V-0.15 N	0.58abc	0.38	0.05c	7.78
V-0.25 N	0.95a	0.39	0.06bc	7.25
Significance	*	ns	*	ns

ns(non significant), ***($p < 0.05$). Different letters within each column reveal significant differences among treatments according to Duncan's test $p = 0.05$.

Table 3
Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)] on mineral content in basil leaves.

Treatment	Mineral content (g kg^{-1}dw)							
	N	P	S	K	Ca	Mg	Na	Cl
Control	29.9d	1.44	0.76d	34.9bc	10.03	5.11c	0.13bc	13.5d
A-0.05 N	34.6bc	1.20	1.21ab	34.0bc	9.87	5.92bc	0.13bc	20.5c
A-0.15 N	35.9abc	1.46	0.86cd	32.1c	9.10	5.99b	0.16ab	23.5b
A-0.25 N	38.9a	1.68	0.93bcd	35.5bc	9.31	5.49bc	0.20a	30.3a
V-0.05 N	33.3b	1.71	1.04bcd	37.3ab	9.38	5.60bc	0.15bc	17.4cd
V-0.15 N	37.9ab	1.45	1.38a	40.2a	8.51	6.80a	0.14bc	18.6c
V-0.25 N	36.4abc	1.47	1.11abc	36.8ab	9.42	5.94bc	0.11c	17.7cd
Significance	***	ns	**	**	ns	**	**	***

^{ns}(non significant)· **($p < 0.01$), *** ($p < 0.001$). Different letters within each column reveal significant differences among treatments according to Duncan's test $p = 0.05$.

application rates (Table 4). The lowest lightness and the highest a* color coordinate were recorded in untreated control plants, whereas PH-treated basil gave the highest lightness and lowest a* values with no differences between the two PHs sources (Table 4). The PH treatments had no significant influence on b* value. Neither PH sources nor application rates had a significant effect on the total chlorophyll and carotenoid contents (average 2.80 and 0.62 μg g^{-1} fw, respectively; Table 4). In the current experiment, the total polyphenols, ABTS, and hydrophilic antioxidant activities ranged from 9.93 to 13.53 μg gallic acid eq. g^{-1} dw, from 2.43 to 3.90 mmol trolox/100 g dw and from 19.89 to 22.93 mmol ascorbic acid/100 g dw respectively, but the differences were not significant (Table 4).

3.3. Implications of protein hydrolysate sources and rates for amino acids profile

The amino acid profile as a function of the three N equivalent rates (0.05, 0.15 0.25 g N/kg) of the two PH biostimulants is reported in Table 5. Glu, Ala and Gln were quantitatively the major amino acids, representing, on average, 38.7 %, 15.0 %, and 8.9 % of total amino acids in basil plants, respectively. Essential and branched-chain amino acids accounted for 10.8 % and 4.8 % of total amino acids on average (Table 5). An increase of total amino acids was recorded by enhancing A-PH application rates (+75 %, 54 %, and 102 % in the three increasing N equivalent rates, respectively). A-PH, independently to N equivalent rates, determined a significant increase of Arg (avg. + 56 %), Asn (avg. + 94 %), Asp (avg. + 128 %), Glu (avg. + 62 %), Leu (avg. + 23 %), and Val (avg. + 14 %). On the contrary, 0.05 and 0.25 N equivalents of A-PH but not A-PH 0.15 N increased the contents of Ala (+151 % and 121 %, respectively), Asn (+97 % and 121 %, respectively), GABA (+180 % and 115 %, respectively) and Gln (+294 % and 590 %, respectively). At the higher N rate of A-PH 0.15 and 0.25, the concentrations of Gly, Ser, and MEA were increased (+50, 75, and 67 % on average, respectively); while Pro was strongly increased (+355 %) in comparison with untreated control only under the highest A-PH rate (0.25 g/kg N equivalent). The V-PH treatment increased the total amino acid content only at 0.15 N and 0.25 N rates (+40 % and 44 %, respectively). The amino acids significantly increased when V-PH 0.15 N was applied to basil were Asn (+82 %), Asp (+106 %), Glu (+50 %), and Gln (+274 %); while those that significantly increased under V-PH 0.25 N were Ala (+73 %), Asn (+74 %) and GABA (+155 %) (Table 5).

3.4. Principal component analysis and heat map

The first four principal components (PCs: PC1, PC2, PC3 and PC4) were related with Eigen values > 1 and explained 89 % of the total variance (PC1 = 37.0 %, PC2 = 26.2 %, PC3 = 13.3 % and PC4 = 12.6 %; data not shown). PC1 was positively correlated to total proteins, amino acids, asparagine, Cl, serine, glutamine, MEA, glutamate, and N. PC1 were also negatively correlated to fructose, a* parameter, phenols, sucrose, and starch (Fig. 2). Moreover, PC2 was positively correlated to Mg, ACO_2, shoot FW, L*, S, LA, LN, WUEi, and shoot DW, negatively correlated to Ca, HAA, total chlorophylls, starch, sucrose, BCAAs, and glucose (Fig. 2). The score plot of the PCA divided the seven treatments along PC1 with A-0.25 N having the highest total amino acid, and total protein amino acids, Cl, MEA, glycine, proline, and BCAAs in the positive side in the lower right quadrant, and Control and V-0.05 N on the negative side, in the lower and upper left quadrant, respectively, clustered with carbohydrates and phenols. On the positive side of PC2, V-0.15 N in the upper left quadrant close to the y-axis was clustered together with shoot FW, shoot DW, LA, LN, A_{CO2}, WUEi, L*, K, Mg, and S. Whereas all the other treatments were clustered close to the axis of origin (Fig. 2).

4. Discussion

Intensive land use and increasing use of chemical fertilizers and pesticides are causing a decline in fertility of soils and crop yield. In

Table 4
Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)] on leaf colorimetry parameters, total chlorophyll content, carotenoids, total phenols, and antioxidant capacity in basil leaves.

Treatment	Leaf colorimetry			Total chlorophyll (μg g^{-1} fw)	Carotenoids (μg g^{-1} fw)	Total phenols (μg gallic acid eq. g^{-1} dw)	Antioxidant capacity	
	L*	a*	b*				ABTS (mmol Trolox eq. 100 g^{-1} dw)	Hydrophilic (mmol ascorbate eq. 100 g^{-1} dw)
Control	41.0b	−10.1b	15.7	2.94	0.57	13.05	2.43	22.93
A-0.05 N	44.2ab	−15.2a	20.8	2.93	0.57	10.91	2.92	22.29
A-0.15 N	45.0a	−13.9a	18.7	2.45	0.63	13.53	2.27	20.48
A-0.25 N	43.4ab	−15.0a	20.8	3.20	0.64	9.00	2.91	21.15
V-0.05 N	43.5ab	−14.4a	18.9	2.55	0.64	11.77	2.65	19.89
V-0.15 N	46.1a	−14.5a	20.2	2.66	0.65	12.06	3.22	20.53
V-0.25 N	45.3a	−12.7a	19.3	2.92	0.62	9.93	3.90	22.10
Significance	*	**	ns	ns	ns	ns	ns	ns

^{ns}(non significant)· *($p < 0.05$), ** ($p < 0.01$). Different letters within each column reveal significant differences among treatments according to Duncan's test $p = 0.05$.

Table 5
Effects of animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)] on total and single amino acid content in basil leaves.

Amino acids (μmol g^{-1} fw)	Treatment							Significance
	Control	A-0.05 N	A-0.15 N	A-0.25 N	V-0.05 N	V-0.15 N	V-0.25 N	
Ala	0.760d	1.907a	0.896cd	1.679ab	0.706d	0.826d	1.314bc	***
Arg	0.133b	0.215a	0.205a	0.202a	0.100b	0.173a	0.186a	***
Asn	0.072bc	0.142a	0.118ab	0.159a	0.061c	0.131a	0.125a	**
Asp	0.195c	0.392ab	0.549a	0.394ab	0.196c	0.401ab	0.322bc	**
MEA	0.181c	0.229abc	0.290ab	0.315a	0.130c	0.200bc	0.222abc	**
Phe	0.137a	0.159a	0.149a	0.135a	0.072b	0.148a	0.140a	***
GABA	0.178c	0.498a	0.265bc	0.383ab	0.218bc	0.212c	0.454a	***
Gly	0.112bc	0.137ab	0.171a	0.164a	0.080c	0.106bc	0.087c	***
Glu	2.119b	3.303a	3.573a	3.396a	2.309b	3.169a	2.925ab	**
Gln	0.219c	0.862b	0.491bc	1.512a	0.257c	0.819b	0.624bc	***
Ile	0.123ab	0.143a	0.135a	0.123ab	0.077bc	0.071c	0.101abc	*
His	0.007b	0.010b	0.007b	0.008b	0.044a	0.009b	0.007b	***
Leu	0.147c	0.177ab	0.183a	0.184a	0.132c	0.145c	0.151bc	**
Lys	0.059	0.069	0.069	0.065	0.053	0.061	0.058	ns
Met	0.028a	0.004cd	0.008bc	0.007bc	0.002d	0.007bc	0.011b	***
Orn	0.098abc	0.118a	0.119a	0.106ab	0.075c	0.090abc	0.079bc	*
Pro	0.279b	0.322b	0.298b	1.268a	0.200b	0.325b	0.372b	***
Ser	0.234bc	0.371ab	0.420a	0.397a	0.213c	0.300abc	0.317abc	*
Tyr	0.063abc	0.077a	0.073b	0.017d	0.042c	0.048c	0.050bc	***
Thr	0.036bc	0.059b	0.039bc	0.088a	0.023c	0.094a	0.039bc	***
Trp	0.048a	0.014bc	0.020b	0.021b	0.007c	0.018b	0.009c	***
Val	0.100a	0.105a	0.117a	0.121a	0.039b	0.090a	0.098a	**
Total	5.329d	9.313b	8.193bc	10.745a	5.036d	7.442c	7.691c	***

[ns](non significant)' *($p < 0.05$), ** ($p < 0.01$), ** ($p < 0.001$). Different letters within each row reveal significant differences among treatments according to Duncan's test $p = 0.05$.

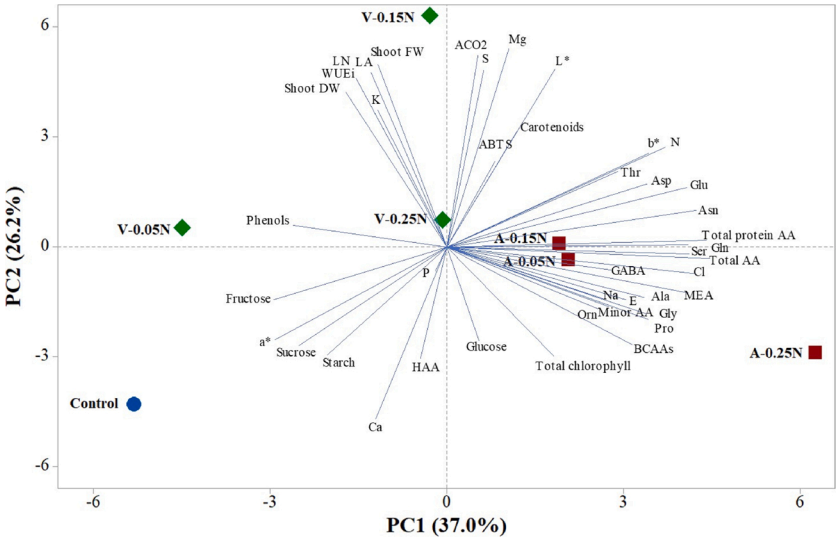

Fig. 2. Loading plot and scores of the PCA of morpho-physiological and biochemical traits of greenhouse basil in response to animal (A) and vegetal (V) derived protein hydrolysates at different N equivalent rates [0.0 (control), 0.05, 0.15, or 0.25 g N/kg)]. LN, leaf number; LA, leaf area; WUEi, instantaneous water use efficiency; DW, dry weight; FW, fresh weight; ACO2, net assimilation rate; E, transpiration rate; AA, amino acids; ABTS; HAA, hydrophilic antioxidant fraction; BCAAs, branched-chain amino acids.

addition, the growing incidence of environmental stresses on agricultural crops is worsening this phenomenon. However, while production declines, the food demand is increasing due to the constant growth in the world population and the standard of living in many countries worldwide. Therefore, to meet the food needs, it is necessary to adopt agricultural models based on a sustainable intensification of production and increase crop production while reducing the use of chemical inputs to reduce pollution and human health risks. In this context, the use of plant biostimulants, including protein hydrolysates (PHs), can sustainably increase crop resources use efficiency (RUE), reduce the incidence of abiotic adversities, and improve the quality of products (Van Oosten et al., 2017; Bulgari et al., 2019; Rouphael and Colla, 2020; Del Buono, 2021). The biostimulant beneficial effects can also help maintaining reasonable yield and profit margins for farmers (Van Oosten et al., 2019). Hence, for increasing the knowledge on the possible mechanisms of biostimulant action in regulating RUE, we analyzed the morpho-physiological and biochemical responses of basil crop to three nitrogen equivalent rates (0.05, 0.15, and 0.25 g N/kg) of two PH widely used biostimulants, Siapton® (A–PH) and Trainer® (V–PH). Our study shows that the treatment with V–PH at a rate between 0.1 to 0.15 g/kg N equivalent caused an increase in the number and area of leaves, leaf fresh and dry weight, resulting in benefit for the treated plants. Similarly, Trainer® application enhanced the length of shoots in gibberellin-dwarf pea plants, evidencing a gibberellin-like activity (Colla et al., 2014). Moreover, Colla et al. (2014) showed that the same biostimulant induced elongation of corn coleoptile segments exerting an auxin-like activity probably due to the signaling peptides and high tryptophan content (Rouphael et al., 2018). At the same concentration of V–PH, A_{CO2} and WUEi were also highly improved. The above findings can be explained by V-PH beneficial effect on stomatal conductance and,

consequently, on net CO_2 assimilation rate as suggested by Paul et al. (2019). Moreover, it is important underline that the improved photosynthetic efficiency and WUE_i were concomitant with a V-PH mediated increase of K, Mg, and S. It has been reported that the enhanced ability of uptaking nutrients in plants treated with the V-PH Trainer® may be due to a remodulation of root growth and shape favoring minerals absorption and transport and known as "*nutrient acquisition response*" (Colla et al., 2014; Rouphael et al., 2017; Carillo et al., 2019a, b). The nutrients K and Mg critically contribute to activation of several enzymes, modulation of the cation-anion balance, photosynthetic efficiency and long--distance transport of photosynthates. In fact, if their content is low in the mesophyll tissue, photosynthetic carbon assimilation is strongly affected (Hawkesford et al., 2012; Tränkner et al., 2018). In particular, K has a crucial role in plant cell osmotic balance and turgor-driven processes such as stomatal movement and thus, the diffusion of CO_2 from ambient air into chloroplasts (Tränkner et al., 2018). Indeed, V-PH-dependent increase of K could be responsible for more responsive stomata that could better sustain photosynthetic activities stimulating plant growth and yield (Rouphael et al., 2017). Whereas Mg is also essential for chlorophyll synthesis, regulation of photophosphorylation, Calvin cycle, ROS generation, and protein synthesis (Cakmak and Yazİcİ, 2010; Dias et al., 2017). Accordingly, Dias et al. (2017) found that photosynthetic efficiency and WUE increased under higher Mg supply. However, the improvements in morphological and physiological parameters could also be partly due to the concomitant increase of S. In fact, this mineral nutrient is critical for preserving chlorophyll content, thylakoids structure, and RUBISCO enzyme content and activity in mesophyll tissues and in particular in expanding young leaves (Resurreccion et al., 2001). Moreover, V-PH at 0.15 g/kg of N equivalent also induced an increase of threonine, an amino acid essential as a precursor for the biosynthesis of isoleucine, a branched-chain amino acid (BCCA) involved in the tissue protection from ROS by a still unknown mechanism (Joshi et al., 2010; Woodrow et al., 2017). Under V-PH 0.05 N and 0.25 N, no significant variations of the various components analyzed were observed, apart from an increase of starch and sucrose under 0.05 N, and a significant increase of GABA and a decrease of starch. Under V-PH 0.05 N probably the N content was too low for inducing starch and sucrose hydrolysis and oxidation of derived sugars to produce precursors for amino acid synthesis (Rigano et al., 1996). While at the highest V-PH concentration, the excess of amino acids presents in the plant extract and the derived NH_4^+ induced the activation of the synthesis of GABA that could temporarily act as a nitrogen storage system to reduce the excess of cytosolic ammonium (Carillo, 2018). The treatment with A-PH exerted positive effects on agronomic traits of basil crop only when it was supplied at a rate ranging from 0.09 to 1.3 g/kg of N equivalent, even if the maximum increase of leaf area and number, shoot FW and DW and A_{CO2} were lower than that caused by V-PH. An increase of A-PH concentration leads to an enhancement of N uptake with a concomitant decrease of glucose and an increase of proteins and free amino acids. In particular asparagine, aspartate, glutamate, and glutamine increased. This was probably due not only to the direct supply of amino acids present in A-PH biostimulant, but also to an A-PH dependent stimulation of nitrate uptake and re-translocation from roots to shoots, and an increase of enzymes involved in nitrate reductive assimilation in leaves, such as nitrate reductase (NR) and glutamine synthetase (GS), accelerating the conversion of nitrate into amino acids (Ertani et al., 2009). However, the treatment of basil plants with A-PH 0.25 N caused a decrease in the number and area of leaves, fresh and dry biomass, with a concomitant increase of sodium, chloride, and proline. The above findings can be explained by the origin and production process of A-PH, which is based on the use of inorganic compounds in the hydrolytic process, which highly increase the content of sodium, chloride, and other salts. The higher leaf contents of Na and Cl treated with A-PH at 0.25 N can explain the decrease of leaf photosynthesis compared to the other A-PH treatments at a lower rate (0.05 and 0.15 g/kg of equivalent N). In fact, high toxic levels of Na and Cl can impair the K uptake and the quantum yield of photosynthetic electron transport, thus determining several plant physiological disorders (Carillo et al., 2019a, b). Moreover, A-PHs have high contents of thermostable amino acids like glycine, alanine, and proline, in addition to hydroxyproline and hydroxylysine, which at high concentrations may inhibit root elongation, N uptake and thus negatively affecting plant growth and productivity (Hayat et al., 2012; Han et al., 2018; Trovato et al., 2018). The treatment with A-PH 2.5 N highly increased the N content of basil leaves. However, since the impossibility to synthesize further proline functioning as N temporary storage, the shortage of ATP caused by the decrease of photosynthetic rate and the toxic effects caused by excess ammonia, basil plants under V-PH 2.5 N stored N as alanine, in addition to glutamine. Indeed, the synthesis of alanine by the cycle coupling NADH-dependent glutamate synthase (GOGAT) and alanine aminotransferase (AlaAT) saves ATP (Diab and Limami, 2016). In addition, since the shortage of ATP caused by the decrease of photosynthetic rate can cause cytoplasmic acidosis because of the impairment of proton pumping ATPase (H^+-ATPase), both the proton-consuming decarboxylations of malate to pyruvate to synthese alanine and of glutamate to produce GABA, exert a pivotal function for buffering cytosolic pH (Carillo, 2018; Carillo et al., 2019b). Moreover, GABA can efficiently scavenge ROS protecting macromolecules and photosynthetic membranes under excess of Na and Cl (Molina-Rueda et al., 2015).

5. Conclusions

The urgent need to decrease synthetic N fertilizers inputs while sustainably increasing RUE and crop growth and yield is the most pressing challenge facing modern agriculture. Biostimulants are at the moment one of the most eco-friendly strategies to sustainably increase horticultural production. Our study applied equivalent values of N from both A-PH- and V-PH-biostimulants to basil plants to compare their effects on plant growth and performance. Vegetal-derived PH increased photosynthesis and color status, ions content, yield, and quality in basil plants as previously demonstrated in perennial wall rocket and spinach (Carillo et al., 2019b; Caruso et al., 2019), especially when supplied at a rate ranging from 0.1 to 0.15 N depending on the crop trait considered. Because the amount of N applied with PHs was negligible, being up to a maximum of 0.8 % on the total N applied at the highest PH rate (0.25 g/kg N equivalent), the positive results in biostimulant-treated plants were probably due to the presence in the V-PH Trainer® of amino acids and especially small peptides acting as signals molecules with an auxin- and/or gibberellin-like activities that increased plant growth by mean of a "nutrient acquisition response" that improved nutrients uptake, translocation, and assimilation. While the changes in the physiological development of basil plants induced by A-PH were less effective than those exerted by V-PH, and even negative when A-PH 0.25 N was used. In fact, this latter induced a general reduction in plant growth and yield and needed the synthesis of alanine and GABA for storing excess ammonia, buffering cytoplasmic acidosis, and counteracting the negative effects due to the presence of toxic levels of Na and Cl. Therefore, we can conclude that the use of PH biostimulants in agriculture can be a valid supplement to agricultural practices for enhancing crop productivity and quality traits. Moreover, this is the first time A-PH and V-PH biostimulants at equivalent N rates have been compared, providing results useful for designing new and effective protocols for horticulture directly translatable into the field.

CRediT authorship contribution statement

Youssef Rouphael: Conceptualization, Data curation, Investigation, Methodology, Software, Writing - original draft, Writing - review & editing. **Petronia Carillo:** Data curation, Investigation, Writing - original draft, Writing - review & editing. **Francesco Cristofano:** Data curation, Investigation, Writing - review & editing. **Mariateresa Cardarelli:** Data curation, Investigation, Writing - review & editing.

Giuseppe Colla: Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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