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Biostimulants from waste: humic-like substances from municipal organic fractions enhance growth, nitrogen acquisition and antioxidant activity of rocket (*Eruca sativa* Mill.) plants

Alice Boarino[1*], Michela Schiavon[1], Andrea Ertani[1], Federica Epifani[1], Daniela Vindrola[1] and Elio Padoan[1]

Abstract

Background The integration of mineral fertilizers with biostimulants offers a valuable solution to improve nutrient use efficiency and reduce environmental impact, especially considering the increasing prices of synthetic fertilizers and of the growing ecological concerns. This work explores the valorisation of low-value materials, derived from the organic fraction of municipal solid waste (OFMSW), to produce potential biostimulants.

Results OFMSW was processed using different treatments and extraction methods to obtain several products containing humic-like substances. Three of these extracts, selected through germination tests, were evaluated for their biostimulant effects on rocket (*Eruca sativa* Mill.) plants grown under soilless conditions. All treatments resulted efficient in enhancing leaf and root development and significantly increasing nitrogen acquisition and assimilation by the plant. The biostimulant mechanism of the humic-like materials was investigated under both high and low nitrogen conditions, not only considering biomass production and nitrogen uptake, but also evaluating nitrate content in the leaves, activity of enzymes involved in N assimilation, and total antioxidant activity.

Conclusions The results confirm the potential of waste-derived humic-like products to improve plant growth and nutrient use efficiency, supporting their use as effective, sustainable biostimulants in line with circular economy principles.

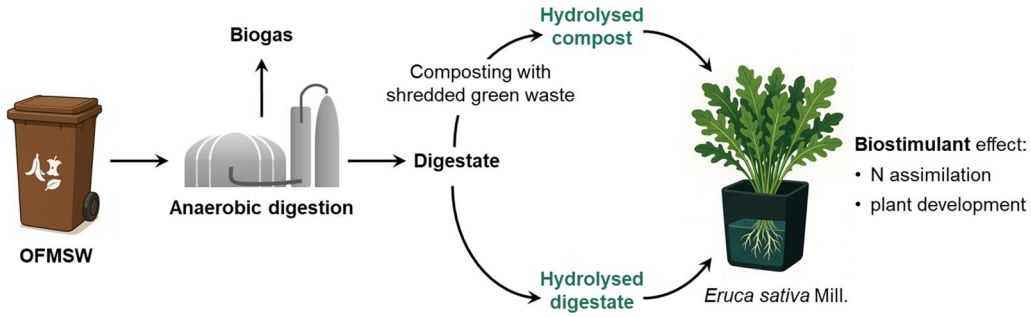
Keywords Humic acids, Organic fraction of municipal solid waste, Anaerobic digestate, Compost, Rocket, Nitrogen, Antioxidants, Hydroponics

*Correspondence:
Alice Boarino
alice.boarino@unito.it

Full list of author information is available at the end of the article

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Graphical abstract



Background

In recent years, the cost of mineral fertilizers has risen significantly due to increased extraction and processing expenses [1]. Global demand for key nutrients such as nitrogen, phosphorus, and potassium has surged, placing pressure on finite natural resources [2]. This demand, combined with energy-intensive manufacturing processes, has led to elevated production costs [3].

Fluctuations in energy markets, particularly natural gas, further contribute to fertilizer price volatility [3]. These rising costs directly impact farm input budgets, altering the economic viability of many agricultural operations [4]. For small and medium-sized farms, the increased financial burden can severely constrain profitability [5]. As fertilizer becomes more expensive, farmers may reduce application rates, risking lower crop yields [6]. Considering rising fertilizer costs and increasing environmental concerns, integrating mineral fertilizers with biostimulants has emerged as a promising strategy to enhance nutrient efficiency and reduce ecological impact. Biostimulants are substances able to improve crop quality and yield at small amount and regardless of their nutrient content. By definition, biostimulants can promote plant growth and health by enhancing nutrient use efficiency, increasing the availability of otherwise inaccessible soil nutrients, improving plant tolerance to abiotic stresses, and ameliorating certain rhizospheric soil properties [7, 8].

One of the main categories of biostimulants is humic substances (HS) [8], organic compounds generated from plant, animal and microbial decomposition, which significantly contribute (up to 80%) to soil organic matter. They can be fractionated in components that have been typically classified based on their solubility: humin, the insoluble fraction; humic acids, which are soluble in alkaline medium but precipitate at pH below 2; and fulvic acids, which remain soluble across all pH conditions [9]. More recently, studies have shown that HS originate from the self-assembly (supramolecular association) of relatively small, heterogeneous organic molecules held together by intermolecular hydrophobic interactions and hydrogen bonds [10, 11].

In soil, HS play a key role in environmental processes that support soil health and fertility. In particular, they regulate soil nutrient bioavailability by forming complexes with several ions [12, 13], and exert biostimulant effects in plants by promoting their stress resilience, nutrient acquisition and nutrient use efficiency [14], through stimulation of primary and secondary metabolic pathways [15–17]. In addition, HS contribute to alter plant nutrient status perception and modulate signalling pathways involved in nutrient sensing, thus leading to enhanced expression and activity of root H^+-ATPases and nutrient transporters [17–21].

The biological activity of HS is influenced by multiple factors, including their origin, dosage, content of bioactive molecules and moieties, molecular weight of the humic fraction, and mode of application (foliar or root-based) [22]. The source is particularly important, as it determines both the structural characteristics and the abundance and nature of active functional groups. Dosage is also critical, as the efficacy of HS often follows a bell-shaped response curve, where biological activity increases with dose up to an optimal point before declining [23]. Furthermore, HS bioactivity can be partially attributed to their content of plant hormones and to the hormone-like activity of functional components containing aliphatic carbon, carboxylic carbon, and phenolic (aromatic) groups [21, 23–25], the hydrophobic nature and the molecular distribution.

Investigating the biostimulant potential of humic-like substances derived from organic wastes could be relevant in the context of sustainable agriculture. However, significant knowledge gaps remain regarding their molecular composition and specific mechanisms of action in plants. In this study, humic-like materials (HLMs) were derived from the anaerobic digestate of the organic fraction of municipal solid waste (OFMSW) and tested as biostimulants. This type of biomass waste is generated year-round in large quantities (59 million tons, i.e. 132 kg/inhabitant

in the EU only in 2022 [26]) and is generally disposed through landfilling and incineration, both of which emit greenhouse gases (GHG) and pose serious environmental challenges [27]. Anaerobic digestion offers a more sustainable alternative method for OFMSW disposal, that aligns with circular economy principles [28–30]. This process converts organic wastes into biogas, a green energy source, and digestate, a by-product rich in plant nutrients. Digestate is generally separated into a liquid and a solid fraction, to facilitate the transportation, storage and handling of this by-product [31, 32]. The liquid portion is normally re-utilized inside the anaerobic digestion plant or sent to a wastewater treatment plant to be disposed of, while the solid is often stabilized by composting and utilized as soil amendment [33]. Herein, the digestate solid fraction was post-treated with different methods to be used as a source of HLM. Several extraction processes were applied to the solid before or after composting. The achieved extracts were characterized in terms of chemical composition and applied to rocket (*Eruca sativa* Mill.), a widely marketed leafy vegetable that can be cultivated in soil and in soil-less system, to assess their biostimulant properties.

Materials and methods

Production of humic-like materials from OFMSW

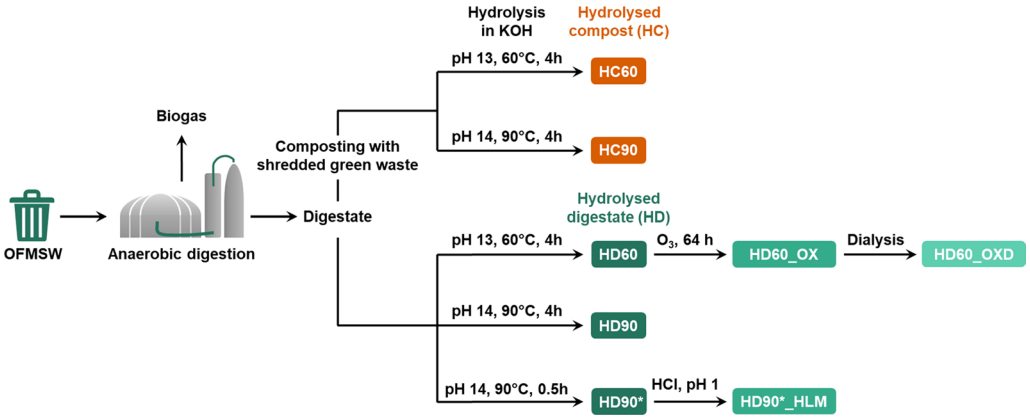
The humic-like products used in the current study were obtained from anaerobic digestate of OFMSW sampled from the ACEA Pinerolese Industriale S.p.A plant, located in Pinerolo (Turin, Italy), and compost made of OFMSW digestate and shredded green waste produced at the same site. For the production of compost, the solid fraction of digestate from the anaerobic biogas plant and the shredded green waste, previously grinded to get small fractions, are mixed at a volume ratio of 1:2. The composting process consisted of two phases. The first one is the bio-oxidation phase, characterized by the intense degradation of the most easily degradable organic components by bacteria, keeping temperature between 60 and 65 ℃. The second one is the curing phase, characterized by several transformation processes of the organic substance whose final expression is the formation of humic substances, at a temperature range comprised between 40 and 45 ℃. At the end the composting process, lasting at least 90 days, the compost was sieved to obtain a fine product compliant to the Italian legislation.

The hydrolyzed digestate (HD) and hydrolyzed compost (HC) were extracted using a potassium hydroxide (KOH) solution following different methodologies, described below and summarized in Scheme 1.

HD60 and HC60. The digestate and the compost were hydrolysed in KOH aqueous solution according to a previously reported process [34]. In brief, the biomass (digestate or compost) was mixed with a 0.1 M KOH solution at pH 13, at 4 V/w water/solid ratio. The mixture was mechanically stirred and electrically heated at 60 ℃ for 4 h and then was allowed to settle to separate the supernatant liquid phase from the insoluble residue. The liquid was then centrifuged at 3000 rpm for 20 min and ultrafiltrated through a 5 kDa cutoff polysulphone membrane (Idea Engineering s.r.l., Italy). The membrane retentate was dried at 60 ℃ to yield the solid HD60 from the digestate and HC60 from the compost.

HD60_OX. The HD60 product was dissolved in KOH aqueous solution at pH 10 in a 2 L flask and oxidized by bubbling a 4 mol/mol % ozone–oxygen stream through the solution using an ozonizer Model MBS (Panciroli, Italy), operated with oxygen at 2 L min^{-1} for 64 h as reported in Montoneri et al. [35]. The flask content was then centrifuged at 3000 rpm for 20 min to separate the solid material from the liquid phase. The solid phase was finally dried at 60 ℃ to yield the insoluble ozonized residue HD60_OX.

HD60_OXD. This product was obtained by dialyzing HD60_OX through ultrafiltration membranes and consists of the fraction containing salts and organic molecules with a molecular weight below 0.2 kDa [35, 36].



Scheme 1 Post-treatments of OFMSW digestate solid fraction and achieved products

In detail, HD60_OX was treated by sequential vertical flow filtration, using a 400 mL Amicon Stirred Cell operating under 3 bars N_2 pressure. Filtration was carried through polysulphone membrane with a 0.2 kDa cut off and the permeate was dried to 60 ℃ to yield HD60_OXD.

HD90 and HC90. The same procedure described for the preparation of HD60 and HC60 was repeated, using a KOH aqueous solution at pH 14 and heating the biomass at 90 ℃ instead of 60 ℃. The digestate and the compost were hence hydrolysed at pH 14 and 90 ℃ for 4 h, to yield the solid HD90 and HC90, respectively.

HD90.* The same procedure described for the preparation of HD90 was applied, heating for 30 min instead of 4 h. The digestate was hence hydrolysed at pH 14 and 90 ℃ for 30 min, to yield the solid HD90* [37].

*HD90*_HLM.* The HD90* was diluted to yield a total solid concentration of 1 g/L with deionized water and then 10% HCl was added to lower the pH until 1 and to precipitate the humic-like fraction of the hydrolysed extract, while salts and the fulvic-like fraction of the hydrolysate remained in solution. The extract was centrifuged and the liquid discarded. The solid was then lyophilized to be stored.

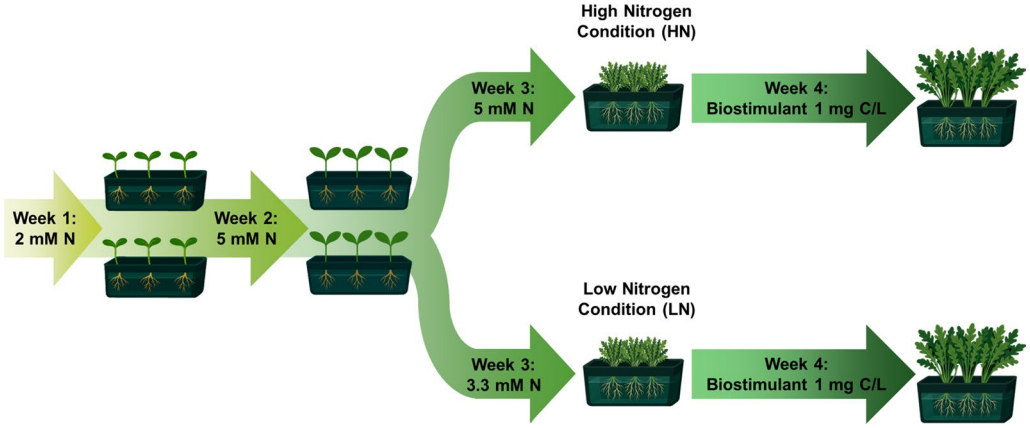
Characterization of extracted products

The pH in each product was determined on a suspension achieved by shaking 10 g of fresh sample in 100 mL of deionized water for 15 min. Total solids (TS) and volatile solids (VS) were quantified by drying the samples at 105 ℃ overnight and at 650 ℃ for 4 h, respectively. The content of carbon (C), nitrogen (N) and sulfur (S) was quantified on samples ground to 0.5 mm by dry combustion (UNICUBE, Elementar Analyses System GmbH, Germany). The content of the other elements was determined on 0.5 g of sample dried, ground and sieved at 0.5 mm. The samples were first heated at 450 ℃ for 4 h and then soaked with 10 mL of HCl 1N and 0.5 mL of HNO_3 1N and heated again to 100 ℃ for 2 h. Phosphorus (P) was quantified by ammonium molybdate assay as reported by [38]. Calcium (Ca), magnesium (Mg) and potassium (K) were analyzed by atomic absorption spectroscopy (F-AAS, AAnalyst 400, PerkinElmer, USA), while all other elements were determined by inductively coupled plasma mass spectrometry (ICP-MS, NexION® 350D, PerkinElmer, USA). Fourier Transform Infrared (FTIR) spectra were recorded in attenuated total reflectance (ATR) mode, using a Spectrum 100 (Perkin Elmer, USA) instrument with 32 scans and a resolution of 4 cm^{-1}.

Seed germination tests were conducted on all products using *Lepidium sativum* L. and *Cichorium intybus* L. The seeds were placed in deionized water for 2 h and then transferred into petri dishes coated with qualitative filter paper soaked with 1.2 mL of product, diluted to reach a C concentration of 0.1, 1 or 10 mg/L. Five replicates containing 10 seeds each were prepared for each dose of product, and a negative control consisting of deionized water was tested in parallel. The petri dishes were sealed with parafilm to maintain constant moisture, covered with aluminum foil, and incubated in a dark thermostatic chamber at 25 ℃ for 48 (*Lepidium sativum* L.) and 72 h (*Cichorium intybus* L.). The seeds were considered germinated in calculations if a minimal radicle was visible (1 mm).

Experimental design

Based on the characterization data and on the results of preliminary biological tests, three potentially promising products (HC90, HD60 and HD60_OXD) were selected and tested on rocket. First, rocket (arugula) seeds (*Eruca sativa* Mill. var. Coltivata E0618, Blumen Group, Italy) were sterilized using 70% (v/v) ethanol for 30–60 s, followed by treatment with 5% (v/v) sodium hypochlorite (NaClO) for 30 min. During sterilization, seeds were kept in agitation on a platform shaker and subsequently rinsed five times with distilled water for 10 min each. The seeds were germinated for 12 days on half-strength Murashige and Skoog (MS) agar medium [39] in a growth chamber equipped with LED set to a photoperiod of 14 h light/10 h dark, day/night air temperatures of 26/21 ℃, relative humidity of 70/85%, and a photosynthetic photon flux density (PPFD) of 280 μmol m^{-2} s^{-1}. Twelve-day-old seedlings were then transferred to plastic containers, each filled with 3.2 L of a 1:5 strength Hoagland nutrient solution to facilitate acclimatation to the hydroponic setup. This solution contained 2 mM N and was applied throughout the first week of growth, with renewal every two days (Scheme 2). During the second week, a 1:2 strength Hoagland nutrient solution was used, with an increased N concentration of 5 mM. From the third week onward, for a duration of two weeks, the actual experimental treatment was initiated. Trays were divided into two experimental groups: one exposed to a high nitrogen concentration (5 mM N) and the other to a reduced nitrogen concentration (3.3 mM N). The N reduced nutrient solution was prepared by replacing KNO_3 with an equimolar amount of KCl, thereby maintaining the same potassium concentration. The higher N concentration was chosen to promote optimal growth of rocket in a soilless system while limiting excess nitrate accumulation, whereas concentrations below 5 mM could restrict rocket plant development. During the fourth week of growth, the three selected biostimulant products were applied individually to the trays. Each tray received the same dose of the selected product, corresponding to a final concentration of 1 mg C/L. Products were applied once only, with the treatment lasting four days. Throughout the experiment, all nutrient solutions



Scheme 2 Representation of the experimental plan overtime

were continuously aerated. The entire setup was placed in a controlled growth chamber (as above) with a temperature control (24 ℃ during the day and 22 ℃ at night). Plants were harvested at the end of the experimental period, corresponding to day 38 from sowing. At harvest, samples were collected for subsequent biochemical analyses on both fresh and dried rocket tissue. Each tray contained three plants; after separating roots and leaves, the biomass was divided as follows: one plant was placed in aluminum foil pouches and dried at 60 °C, while the other two were stored in plastic tubes and frozen at − 80 °C.

The three selected products were applied individually and in identical doses to both experimental groups, high nitrogen (HN) and low nitrogen (LN), in order to evaluate their potential biostimulant effects. Control plants, also grown under HN or LN conditions, were not treated with any product. Each treatment and control condition were performed in triplicate (n = 3 trays per treatment).

Element quantification in plant tissues

The carbon, nitrogen and sulfur contents were quantified as previously described, on samples milled to 0.5 mm, by dry combustion (UNICUBE, Elementar Analyses System GmbH, Germany). Phosphorus concentration in plant samples (100 mg) was determined by ammonium molybdate assay [38] on samples mineralized with a mixture of perchloric and sulfuric acid 1:5 v/v applied and heated at 150 ℃ for 24 h. Phosphorus was then quantified spectrophotometrically at λ = 650 nm.

Determination of nitrate content

Nitrate concentration in leaves was analyzed on the fresh material. For each plant, 100 mg of fresh tissue was ground in liquid nitrogen and suspended in 10 mL of deionized water. Suspensions were incubated for 30 min at 80 ℃ and then centrifuged at 5000 rpm for 15 min. The extract was filtered, and the nitric N concentration was determined spectrophotometrically by the Griess reaction [40]. Nitrogen uptake efficiency (NUptE) was calculated as the ratio between N accumulated in plants and N applied, while nitrogen use efficiency (NUE) was calculated as the ratio between biomass produced and N applied [41].

Quantification of proteins and N assimilation enzyme activity

Frozen samples (200 mg) were ground in a mortar with liquid nitrogen and extracted with 100 mM phosphate buffer (pH 7.8) containing polyvinylpyrrolidone (PVP) (10 g/L) in the ratio of 1:10. Protein content was quantified using the Bradford's method [42].

The extraction of N assimilation enzymes and the determination of their activity were performed according to the protocols reported by Ertani et al. [43]. Leaf tissues (1 g) were ground in a mortar and added with 100 mM Hepes–NaOH solution at pH 7.5, 5 mM $MgCl_2$ solution, and 1 mM dithiothreitol (DTT) solution. The ratio of plant material to mixture solution was 1:3. The extract was filtered and clarified by centrifugation at 20,000 g for 15 min, and the supernatant was used for enzymatic analysis. All steps were carefully performed at 4 ℃. Glutamate synthase (GOGAT) assay contained 25 mM Hepes–NaOH (pH 7.5), 2 mM L-glutamine, 1 mM R-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2EDTA, and enzyme extract. GOGAT activity was measured spectrophotometrically by monitoring NADH oxidation at λ = 340 nm. For glutamine synthetase (GS) assay, the mixture contained 90 mM imidazole–HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM $KAsO_4$, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine, and the enzyme extract. The enzymatic reaction was developed colorimetrically for 15 min at 37 ℃. The γ-glutamyl hydroxamate was determined by the addition of 250 µL of a mixture (1:1:1) of 10% (w/v) $FeCl_3{\cdot}6H_2O$ in 0.2 M HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl. The optical density was recorded at λ = 540 nm. The analyses

were conducted on three biological replicates (one replicate = 1 plant).

Antioxidant activity

The total antioxidant activity was evaluated by measuring the ferric-reducing antioxidant power (FRAP). For the assay, the FRAP Assay Kit (Merk) was used according to Benzie and Strain [44]. Samples (0.5 g) were extracted in methanol 80% (v/v) (ratio 1:10) and centrifuged for 10 min at 10,000 g. Then, 100 µL of the methanol extract was added to 1900 µL of FRAP reagent. After leaving the mixture at 20 ℃ for 4 min, the absorbance was determined at λ = 593 nm. Calibration was performed against a standard curve (0–1200 mg/mL ferrous ion). FRAP values are presented as milligrams per kilogram of Fe^{2+} Eq (ferrous ion equivalents).

Statistics

For each measurement, an analysis of variance (one-way ANOVA) was performed using the SPSS software version 26.0 (SPSS, Chicago, IL), followed by Tuckey HSD test to determine which means differed significantly at $p < 0.05$. All data are expressed as means ± standard error.

Results

Characterization of extracted products

The main features of the extracted products are summarized in Table 1. Reasonably, all materials extracted with KOH exhibited an alkaline pH (9.3–12.8), except for HD90*_HLM, which had a pH < 1 due to the final extraction step involving HCl.

Volatile solids, which represent a measure of the organic matter content in the sample, were similar in both hydrolyzed composts (68% in HC60 and 67% in HC90), suggesting that the different extraction conditions did not significantly influence their organic content. In contrast, the hydrolysis method impacted on the volatile solids in HD60 (73% VS) and HD90 (48% VS), indicating that the higher pH and temperature promoted organic matter degradation.

On the other hand, process duration did not substantially influence degradation, as proven by the volatile solid content in HD90* (51% VS), which underwent the same extraction as HD90, but for 0.5 h instead of 4. HD90*_HLM, mainly composed of organic molecules after precipitation, was reasonably the richest in volatile solids (97% VS), while HD60_OX and HD60_OXD showed the lowest VS content (30 and 20% VS, respectively), because the oxidation process had converted most of the organic matter into CO_2. Regarding the C and N contents, both were lower in the products extracted at pH 14 and 90 °C compared to those obtained at pH 13 and 60 ℃. The C/N ratio was particularly high in HD60_OX and HD60_OXD, likely due to their higher content in heteroatoms-containing molecules, such as proteins, which are more recalcitrant to oxidation than other molecules such as polysaccharides. The potassium content was higher in HC90 and HD90, extracted at pH 14, than in HC60 and HD60, extracted at pH 13, because the change in pH required a consistent addition of KOH in the extractive solution.

The content of all other elements in the hydrolyzed products was less influenced by the extraction process,

Table 1 Characterization of products extracted from OFMSW digestate. (TS = total solids; FM = fresh matter; VS = volatile solids)

	HC60	HC90	HD60	HD90	HD90*	HD90*_HLM	HD60_OX	HD60_OXD
pH	9.7	9.5	10.0	10.3	12.8	< 1	9.3	10.1
TS (% FM)	92.0 ± 0.1	86.2 ± 0.3	89.0 ± 0.1	90.1 ± 0.1	90.5 ± 0.2	89.3 ± 0.1	93.5 ± 0.8	94.8 ± 0.7
VS (% TS)	68.0 ± 0.1	67.4 ± 0.3	72.7 ± 0.2	48.0 ± 0.3	50.9 ± 0.1	96.5 ± 0.1	29.9 ± 0.1	20.2 ± 0.1
C (% TS)	47.3 ± 0.0	38.9 ± 0.3	50.2 ± 0.3	25.1 ± 0.7	34.1 ± 3.0	57.6 ± 0.1	17.4 ± 0.4	15.2 ± 0.2
N (% TS)	5.41 ± 0.06	4.91 ± 0.05	7.26 ± 0.05	1.85 ± 0.09	4.33 ± 0.36	6.90 ± 0.01	1.50 ± 0.12	1.35 ± 0.21
C/N	8.7	7.9	6.9	13.6	7.9	8.3	11.6	11.3
P (% TS)	0.23 ± 0.02	0.87 ± 0.72	0.88 ± 0.08	1.13 ± 0.08	0.14 ± 0.03	0.42 ± 0.01	0.29 ± 0.03	0.11 ± 0.03
K (% TS)	0.52 ± 0.02	4.20 ± 0.15	0.46 ± 0.02	6.36 ± 2.03	1.00 ± 0.07	0.15 ± 0.01	< 0.1	< 0.1
S (% TS)	0.85 ± 0.04	0.93 ± 0.03	1.31 ± 0.07	0.26 ± 0.12	1.32 ± 0.70	1.24 ± 0.32	0.05 ± 0.00	0.08 ± 0.01
Ca (% TS)	2.35 ± 0.49	3.91 ± 0.88	2.11 ± 0.38	5.21 ± 0.77	1.37 ± 0.13	0.02 ± 0.01	0.92 ± 0.06	0.03 ± 0.01
Fe (% TS)	0.44 ± 0.11	1.37 ± 0.02	0.23 ± 0.13	0.45 ± 0.03	0.24 ± 0.01	0.08 ± 0.01	0.11 ± 0.01	0.02 ± 0.01
Mg (mg/kg TS)	2675 ± 140	6005 ± 1543	2072 ± 23	5876 ± 4155	1011 ± 96	382 ± 20	965 ± 21	387 ± 9
Pb (mg/kg TS)	81 ± 25	48 ± 4	21 ± 16	14 ± 2	26 ± 1	13 ± 0.4	17 ± 4	12 ± 1
Zn (mg/kg TS)	284 ± 19	548 ± 9	272 ± 382	217 ± 14	30 ± 3	14 ± 1	29 ± 11	13 ± 1
Cu (mg/kg TS)	66 ± 30	124 ± 13	115 ± 71	41 ± 1	7 ± 0.1	32 ± 2	130 ± 7	36 ± 1
Cd (mg/kg TS)	8.8 ± 0.3	1.8 ± 0.2	7.4 ± 0.1	0.3 ± 0.1	16.7 ± 3.0	5.7 ± 0.5	10.8 ± 2.4	8.3 ± 0.3
Cr (mg/kg TS)	< 1	62 ± 1	< 1	20 ± 1	< 1	14 ± 1	< 1	< 1
Ni (mg/kg TS)	15 ± 5	66 ± 1	4 ± 2	17 ± 1	< 1	< 1	< 1	< 1
Mn (mg/kg TS)	346 ± 4	411 ± 11	153 ± 1	202 ± 12	68 ± 9	19 ± 1	65 ± 5	16 ± 1

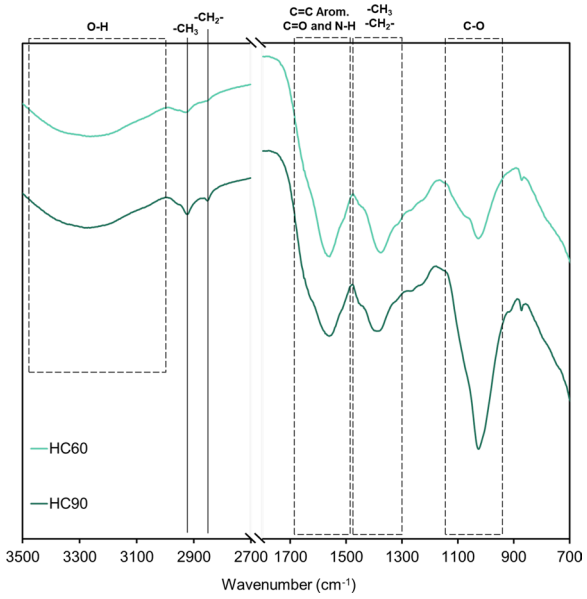


Fig. 1 FTIR spectra of HC60 and HC90

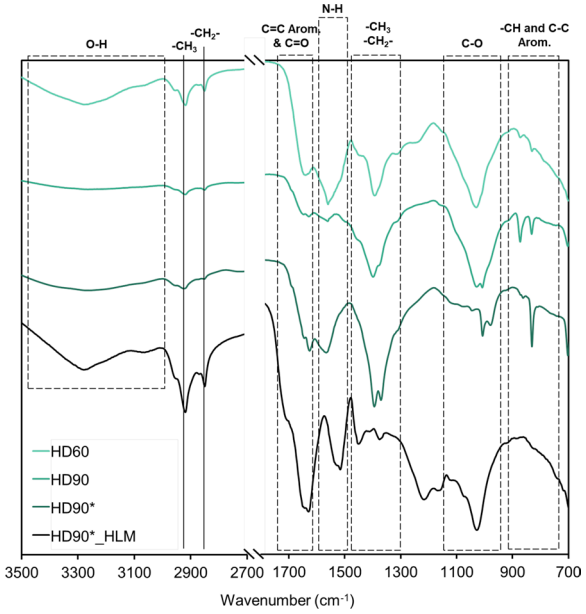


Fig. 2 FTIR spectra of HD60, HD90, HD90* and HD90*_HLM

and more by the heterogeneity and variability of the OFMSW that was used as feedstock material for anaerobic digestion. For example, HD60, HD90* and HD90*_HLM exhibited elevated sulfur content, while HC90 and HD90 contained the highest levels of iron and calcium. HD60, HD90* and HD60_OXD were characterized by high sodium concentrations, while HD90* and HD90*_HLM were high in magnesium concentration.

With regard to macronutrients (Ca, S, Mg) and micronutrients (Fe, Mn, Cu, Zn, Ni) contents in the humic-like substances, their concentrations are in line with literature studies and so are the potentially toxic elements (PTE) [45]. These elements are unlikely to affect plant performance as these products are diluted ~ 400,000-fold to a target of 1 mg C/L, making their nutritional contribution negligible. Similarly, PTE concentrations at the low application rates used to obtain biostimulant activity remain well below safety limits.

The structural differences of the extracted products were analyzed by FTIR spectroscopy. Figure 1 compares the FTIR spectra of the compost-derived extracts, revealing distinct signals associated with humic-like substances. In detail, the broad absorption band observed at 3000–3500 cm^{-1} corresponds to the stretching of O–H bonds present in alcohol and phenol groups, i.e., functional groups found in polysaccharides like cellulose and in humic substances. The sharper peaks in the 2850–2950 cm^{-1} range are attributed to the stretching vibrations of $–CH_2$ and $–CH_3$ groups, characteristic of aliphatic molecules like waxes and lipids [46, 47]. The strong band between 1500 and 1750 cm^{-1} reflects the presence of C=O stretching in carboxylic acid groups (1650–1750 cm^{-1}), typical of humic and fulvic acids, as well as C=C stretching of aromatic molecules (1500–1650 cm^{-1}), commonly found in lignin residues or humified organic matter [47, 48]. This region may also include overlapping signals from N–H bending, related to proteins and amides [46]. Finally, the prominent band observed at 950–1100 cm^{-1} corresponds to the stretching of C–O bonds associated with alcohols, esters and ethers, which are functional groups widely present in humic substances [21, 49, 50]. The spectra of HC60 and HC90 show overall similarity, except for a stronger presence of aliphatic and carbonyl groups as well as of C–O bonds in HC90, highlighting that the different temperature might have led to the extraction of different compounds.

The spectra of hydrolyzed digestates reported in Fig. 2 clearly show a reduction in the intensity of bands associated with O–H, C=O and C–O stretching, which is reasonable since these materials were extracted right after anaerobic digestion, a process which typically yields fewer oxidized compounds. In contrast to the digestates, the compost previously went through an aerobic decomposition process, which promoted the formation of more oxidized compounds like carboxylic acids and phenols. In addition, the spectra of hydrolyzed digestate shows more pronounced signals of $–CH_2$ and $–CH_3$, indicative of aliphatic molecules recalcitrant to the hydrolysis in anaerobic digestion, such as waxes and fatty acids. Among the hydrolyzed digestates, HD90*_HLM exhibits the most intense absorption bands related to aliphatic and carbonyl groups, as well as aromatic C=C and C–O bonds. This indicates a relatively higher content of humic-like substances, such as humic and fulvic acids, as compared to the other digestate extracts.

Figure 3 depicts the FTIR spectra of the digestate extracts oxidated with ozone. In these spectra there is an evident reduction of the C–O stretching signal, while the band corresponding to C=O and aromatic C=C is significantly enhanced compared to the spectra reported in Fig. 2.

The effects, either promoting or phytotoxic, of the products previously characterized were evaluated in germination tests on *Lepidium sativum* L. and *Cichorium intybus* L. at three concentrations, 0.1 mg C/L, 1 mg C/L, e 10 mg C/L. The germination indexes, determined by measuring the root growth from the tested seeds, are reported in Supporting Information, Figure S1. Although in some cases the humic-like substances yielded higher germination indices at the highest concentration, 1 mg C/L was considered the most adequate for further tests on rocket. While the products at 10 mg C/L did not impair seed germination, they may exert phytotoxic effects on plants cultivated under hydroponic conditions [23]. Three best candidates were identified for showing higher germination index for both watercress and chicory at 1 mg C/L than the other samples within three different groups. Among the compost-derived extracts, HC90 demonstrated the highest efficacy, outperforming HC60. HD60 exhibited superior performance relative to HD90 and HD90* among the digestate extracts. Post-treatment of HD60 yielded two products, among which HD60_OXD showed superior efficiency to HD60_OX. Therefore, HC90, HD60 and HD60_OXD were selected for further investigation as potential biostimulants for rocket plants grown in hydroponic.

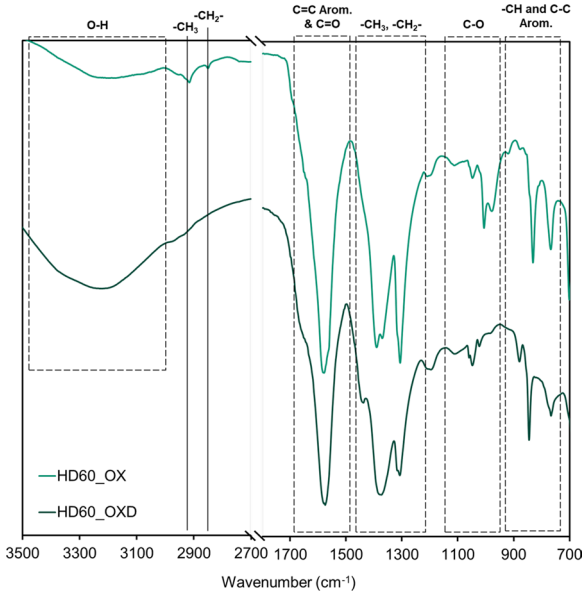


Fig. 3 FTIR spectra of HD60_OX and HD60_OXD

Characterization of plants

Effect of humic-like products on plant growth

The effects of HC90, HD60 and HD60_OXD on rocket leaf and root biomass are presented in Fig. 4. The data indicate that all three products significantly enhanced both leaf (**C**) and root (**D**) biomass under high and low N conditions. Under high N availability, in particular, their application led to increases in leaf fresh weight ranging from 41 to 61% compared to the control plants. Nevertheless, significant root stimulation was observed only with HC90 and HD60, which increased root growth by 106% and 164%, respectively. Under low N supply, all products promoted leaf biomass production, with HD60_OXD causing the most pronounced effect, an increase by 102% compared to the control. In terms of root biomass, HD60_OXD again had the strongest effect, enhancing growth by 83% relative to untreated plants. The effect of the three humic-like products on rocket dry weight closely mirrored the trends observed for fresh weight (Supporting Information, Figure S2).

Effect of humic-like products on plant nutrient content, N acquisition and assimilation

Regardless of N supply or humic-like product application, the concentrations of major nutrients (C, N, P, and S) in rocket leaves showed no significant variation (Supporting Information, Table S1). However, both N uptake efficiency (NUptE) and N use efficiency (NUE) increased following the supplementation of humic-like products (Fig. 5). HC90 and HD60 effectively enhanced NUptE and NUE, promoting comparable increases under both high (approximately 42% and 66%, respectively) and low (approximately 60% and 50%, respectively) N conditions. In contrast, HD60_OXD significantly improved both NUptE and NUE, by over 80%, but only under low N availability. In untreated plants, both NUptE and NUE were higher under low N supply compared to high N.

In addition to total N, nitrate content was also measured in rocket leaves, as it represents an important quality parameter for this crop (Fig. 6). According to EU regulations, nitrate levels must not exceed 6000–7000 mg NO_3^- per kg of fresh weight. Although humic-like product application increased NUptE, this was not accompanied by a significant rise in nitrate concentration, which remained consistently below the recommended threshold.

Consistent with the increase in NUE, we observed the stimulation of N metabolism triggered by humic-like products, as evidenced by enhanced activity of N assimilation enzymes (GS and GOGAT) and increased total protein content in rocket leaves (Fig. 7). Regarding the GS enzyme, its activity was higher in untreated plants grown under low N compared to those grown under high N. All humic-like products further increased GS activity under

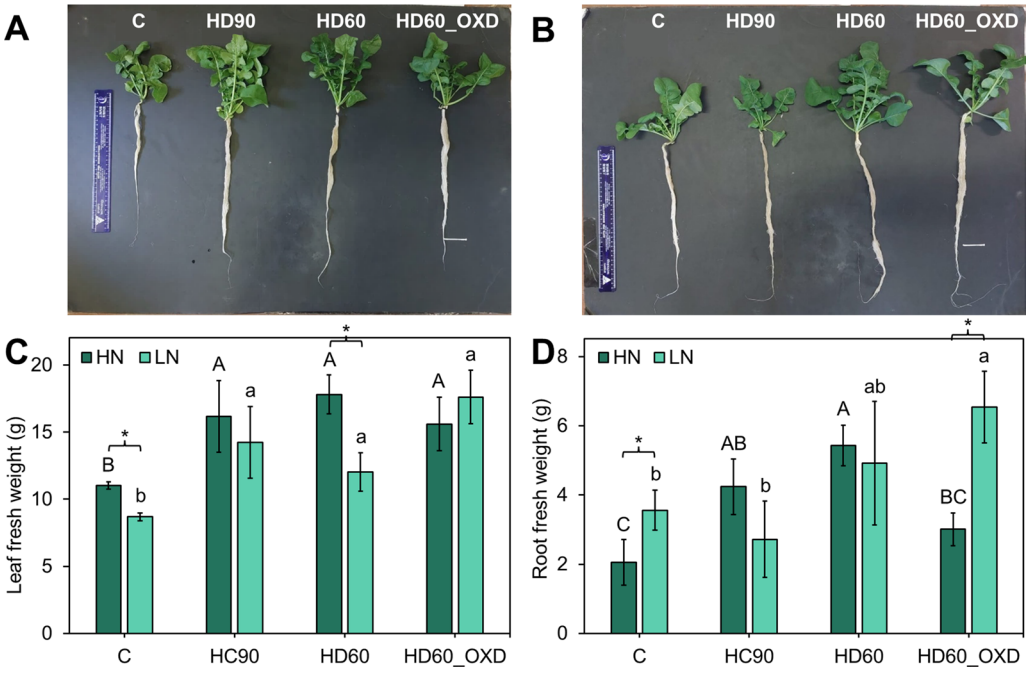


Fig. 4 Pictures of plants grown in (**A**) high and (**B**) low nitrogen concentration. Fresh weight of (**C**) leaves and (**D**) roots of rocket fed with different products in high (HN, dark green) and low (LN, light green) nitrogen concentration. Results and error bars correspond to the average and standard error of measurements performed on three replicates. Uppercase letters indicate significantly different results achieved under high N condition; lowercase letters indicate significantly different results achieved under low N condition; asterisks indicate significantly different results achieved with the same product under high and low N condition

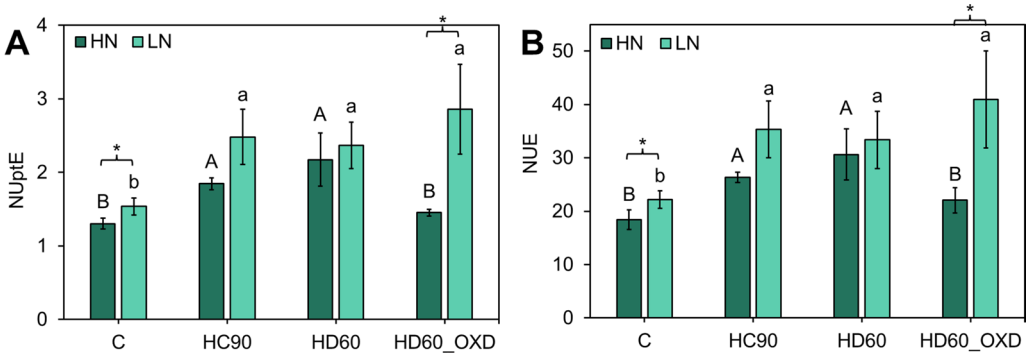


Fig. 5 **A** Nitrogen uptake efficiency (NUptE) and **B** nitrogen use efficiency (NUE) of rocket fed with different products in high (HN, dark green) and low (LN, light green) nitrogen concentration. Uppercase letters indicate significantly different results achieved under high N condition; lowercase letters indicate significantly different results achieved under low N condition; asterisks indicate significantly different results achieved with the same product under high and low N condition

high N, with the most pronounced effect occurring with HD60. Under low N conditions, GS activity showed a significant increase (about 80%) only in plants treated with HC90 compared to the untreated control plants, while no significant differences were observed for the other two products. With respect to the GOGAT enzyme, no differences in activity were found between untreated plants under low and high N, nor did humic-like product application affected its activity under high N. However, under low N conditions, all products enhanced GOGAT activity by similar increases (32–42%).

Under high N availability, all humic-like products significantly increase protein content compared to the untreated controls. Conversely, under low N conditions, protein levels did not differ significantly among treatments or compared to the untreated controls, suggesting that the effectiveness of the treatments in promoting protein accumulation depended on adequate nitrogen availability.

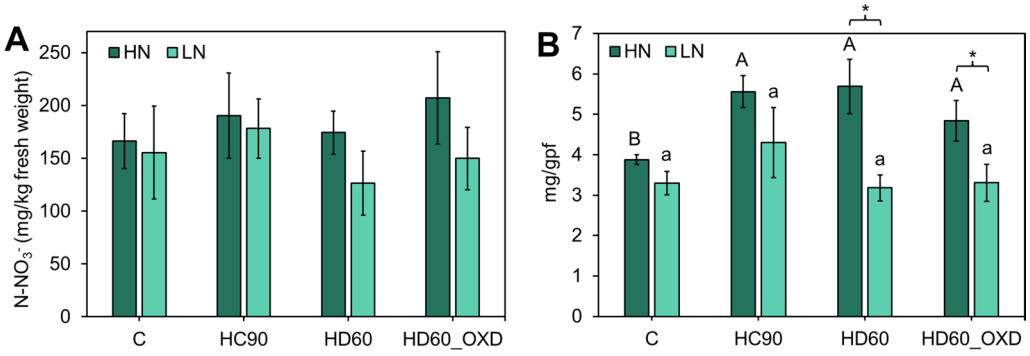


Fig. 6 **A** Nitrate content in leaves of rocket fed with different products in high and **B** protein content in the leaves of rocket fed with different products in high (HN, dark green) and low (LN, light green) nitrogen concentration. In panel A results are not statistically different. In panel B uppercase letters indicate significantly different results achieved under high N condition; lowercase letters indicate significantly different results achieved under low N condition; asterisks indicate significantly different results achieved with the same product under high and low N condition

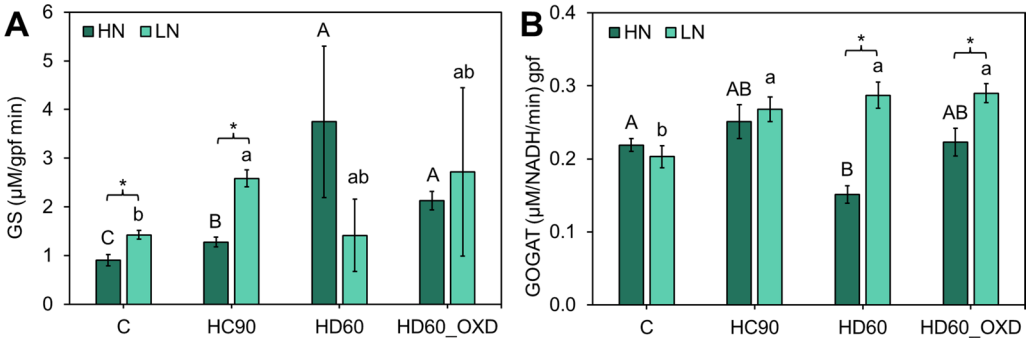


Fig. 7 Activity of **A** GS and **B** GOCAT in the leaves of rocket fed with different products in high (HN, dark green) and low (LN, light green) nitrogen concentration. Uppercase letters indicate significantly different results achieved under high N condition; lowercase letters indicate significantly different results achieved under low N condition; asterisks indicate significantly different results achieved with the same product under high and low N condition

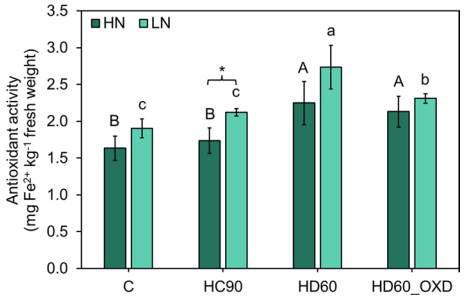


Fig. 8 Antioxidant activity in the leaves of rocket fed with different products in high (HN, dark green) and low (LN, light green) nitrogen concentration. Uppercase letters indicate significantly different results achieved under high N condition; lowercase letters indicate significantly different results achieved under low N condition; asterisks indicate significantly different results achieved with the same product under high and low N condition

Effect of humic-like products on total plant antioxidant activity

The application of humic-like products enhanced antioxidant activity in rocket leaves, particularly under low N conditions (Fig. 8). Under high N supply, increased antioxidant activity was observed with HD60 and HD60_OXD treatments, whereas HC90 showed no significant effect. In contrast, under low N availability, all three humic-like products stimulated antioxidant activity, with HD60 producing the most pronounced effect, resulting in a 44% increase.

Discussion

The organic fraction of municipal solid waste is a source of organic material homogeneously distributed throughout the EU and, still, underutilized, as less than half of the total is composted or digested, while the majority is not differentiated and ends-up in landfills and incinerators [26]. The most useful technology for OFMSW treatment is the integration of anaerobic digestion and composting, to overcome most of the disadvantages posed by each treatment alone [51]. With this technology, both digestate and compost produced from OFMSW could be used as biofertilizers and soil amendments in agriculture [52], although their use is very limited due to a lack of confidence in potential consumers and, thus, a very low value of the products [53].

In this study, we aimed to go over the use of these materials as soil amendments or biofertilizers, whose economic value is also often lower than the cost of

production, to extract HLM to be used as biostimulants [36, 54], valuable materials also recently included in the new EU fertilizer regulation [55].

Biostimulant products or substances with biostimulant activity are widely used in horticulture in addition to fertilizers, with the aim of improving nutrient use efficiency, yield and product quality. In the context of sustainable agriculture, the use of such products can help reduce the amount of synthetic fertilizers applied during crop cultivation, while promoting plant growth, nutrient uptake, primary and secondary metabolism, and tolerance to abiotic stress. In the current study, in addition to the chemical characterization of starting materials (humic-like substances) derived from the transformation of organic urban wastes, the effects of these materials were evaluated on rocket plants cultivated in a floating growing system under high and low N input conditions. The aim was to assess whether their application could improve plant performance and nutrition even under low N input.

Germination tests confirmed that the extracts containing the humic-like substances did not exert phytotoxic effects at the tested concentrations, indicating that the starting materials had been adequately processed and did not contain phytotoxic compounds (e.g., excess organic acids or ammonia) or toxins. These preliminary tests also helped identify the most promising substances for use in subsequent trials on plants.

Plants grown under high N supply produced greater shoot and root biomass compared to those grown under low N input and exhibited a lower root-to-shoot ratio (0.18 vs 0.40, based on fresh weight), confirming that the applied N concentrations led to significant differences in productivity and resource allocation patterns. When rocket plants were further treated with the three humic-like products, an overall increase in both leaf and root growth was observed, although the effectiveness varied depending on the specific product, N condition applied and plant organ. For instance, while all products enhanced rocket leaf biomass, HD60_OXD was particularly effective in stimulating root growth only under N limitation, whereas the other two products promoted root growth primarily under high N conditions. These findings suggest that the distinct properties of the tested humic-like products influenced their biostimulant activity under different nutritional regimes, possibly due to differences in chemical composition or mode of action. In support of this, previous studies underscored the importance of the chemical characteristics (acidity, abundance and type of functional groups) of HS or HS-like products to induce biostimulatory effects on plant growth [15, 56, 57]. The oxidation of HS could stimulate plant growth more effectively than their non-oxidized counterparts for several reasons, related to changes in chemical structure, biological activity and interaction with plants. In particular, the oxidation introduces or increases the abundance of oxygen-containing functional groups, such as carboxylic acid (-COOH) and phenolic (-OH) groups in HS structure, which can enhance the reactivity and solubility of HS, making them more biologically active. Humic substances can enhance plant growth through various mechanisms, including the stimulation of root development, which increases the nutrient uptake surface area, and enhancement of cell division activity in meristematic tissues [15, 58]. These processes are highly influenced by the physicochemical characteristics of the humic and humic-like substances [21], and in some cases, by their inherent hormone-like activity [15, 59].

The limited effectiveness of HC90 and HD90 in promoting root growth under low N input may suggest that, under reduced N availability, the humic-like substances in these products reduced carbon allocation for root biomass production and instead likely activated alternative mechanisms for nutrient acquisition, such as the upregulation of nitrogen transporter expression, as previously observed [60, 61]. This interpretation is supported by the significant increase in N uptake efficiency (NUptE index) observed in both HN and LN plant groups following application of the humic-like products under low-N conditions. These findings suggest that the biostimulant action of HS on N acquisition operates at multiple levels and possibly involves distinct physiological processes targeting the root system.

However, despite plant growth being enhanced as a result of increased N acquisition efficiency following humic-like product supplementation, the N concentration in plant tissues did not show a corresponding rise. This can be explained by what is commonly referred to as a "dilution effect". As the plant produces more biomass, the absorbed N is distributed across a larger volume of tissue. Although the total amount of N taken up by the plant may have increased, the relative concentration (%N on a dry weight basis) could remain unchanged or even decrease because of this expanded biomass. Therefore, the apparent lack of increase in N percentage that we observed did not necessarily indicate a lack of N uptake or assimilation. Rather, it reflected the plant's improved efficiency in using the N to support greater growth, with the nutrient being diluted in the larger biomass pool.

Given the tendency of rocket to accumulate nitrates in its tissues, one of the biochemical analyses performed on leaves was to determine nitrate content, as high levels of these compounds can pose health risks to humans. Nitrate is the prevalent form of N absorbed and assimilated by most horticultural crops, particularly leafy vegetables. Its accumulation in leafy vegetables depends on N input and on other factors such as the plant species, the form of nitrogen applied, the environmental conditions (*e.g.*, light intensity, temperature) and agricultural

practices [62]. Under high N input conditions, nitrate uptake can exceed the assimilation capacity of the plant, resulting in nitrate accumulation in plant tissues [63, 64]. The results obtained in our study showed that in leaves of both HN and LN plant groups, nitrate concentration was below the maximum permissible limits established by the European Union for *Eruca sativa* Mill. (6000–7000 mg NO_3/kg). These results can be explained in two ways. First, the enhanced development of the root system promoted by the applied products likely improved nitrogen acquisition efficiency from the nutrient solution and its translocation to the shoots, followed by dilution in the tissues of plants due to increased biomass production. Secondly, nitrate balance control may have been partly due to increased N assimilation promoted by the tested products, as the current literature indicates that HS can stimulate N metabolism by enhancing the expression and activity of enzymes involved in N assimilation, thus increasing the NUE [21].

To support this latter hypothesis, the activity of enzymes involved in N assimilation, GS and GOGAT, was analyzed. The enzyme activity generally increased following product application, with a stronger response in either HN or LN plants depending on the case, compared to untreated plants. Increased activity of these enzymes typically indicates higher assimilation of N into organic compounds, such as amino acids and proteins. Protein content, in particular, increased following humic-like product application, especially in plants grown with a high N input. Several studies have reported increased protein content due to HS and humate application, attributed to stimulation of N metabolism [23, 65, 66]. The observation that under low N input no substantial change in total protein content was observed despite increased activity of N assimilation enzymes might indicate that under N limitation, plants tended to prioritize the synthesis of essential N-containing compounds, such as free amino acids (e.g., glutamine and glutamate) involved in transport and signaling, structural proteins, often insoluble and associated with the cell wall, and N-secondary metabolites with defensive and antioxidant roles. As a result, even though GS and GOGAT activity increased, the assimilated N was not necessarily directed toward the synthesis of soluble proteins [67].

The enhanced synthesis of secondary metabolites with antioxidant functions is a well-documented response to both biotic and abiotic stresses [68, 69]. Nitrogen limitation, a common abiotic stress condition for plants, can trigger oxidative stress in plants, making the increased production of enzymatic and non-enzymatic antioxidant molecules crucial for mitigating or preventing oxidative damage [70]. Brassicaceae species, including rocket, are known to contain a wide variety and quantity of substances with antioxidant activity, such as ascorbic acid and polyphenols. Therefore, the total antioxidant activity of rocket leaves was analyzed using the FRAP assay, which evaluates the capacity of cell non-enzymatic antioxidants (e.g., glutathione, ascorbic acid, phenolic compounds) to reduce Fe^{3+} to Fe^{2+} [71]. The total antioxidant activity was found to be generally increased by the application of tested products, particularly by product HD60, in both HN and LN plant groups. These results align with previous reports showing that, beyond their effects on primary metabolism, HS can significantly modulate the secondary metabolism involved in the production of antioxidant compounds, particularly phenolic acids and phenylpropanoids [24]. Notably, HS applied at the same concentration used in this study (1 mg C/L), have been shown to enhance the activity of the enzyme phenylalanine ammonia-lyase (PAL), a key enzyme in the biosynthetic pathway of phenolic compounds.

Overall, the results obtained in this study highlight the effective biostimulant activity of the extracted materials, offering promising prospects for their application in sustainable agriculture. However, further research is needed to confirm their efficacy on a broader scale. Given the inherent variability and heterogeneity of OFMSW over time, the composition of the hydrolyzed extracts may fluctuate, potentially affecting their performance and safety. This biomass waste can contain inorganic and organic contaminants, whose concentrations may vary between batches depending on the source, collection practices, and seasonal factors. Therefore, additional studies should be conducted using materials derived from different OFMSW batches to ensure that the resulting biostimulants are not only effective but also safe for plants, the environment, and human health.

Conclusions

The main objective of this experimental study was to explore the reuse of low-value materials, such as urban waste and by-products, as potential sources of biostimulants. Among the materials derived from OFMSW using different extraction methods, three humic-like products were selected based on germination tests and evaluated for their possible biostimulant effects on rocket plants. All treatments promoted leaf and root growth, with variable effects depending on the specific product, nitrogen availability, and plant organ. These results suggest that the distinct properties of each product may influence their biostimulant activity under different nutritional conditions, likely related to differences in their chemical composition or mode of action. The observed increase in nitrogen uptake efficiency in both high- and low-nitrogen plants is consistent with a potential role of humic substances in nitrogen metabolism, possibly involving specific root-level physiological processes. Additional analyses, such as nitrate content, assessment of nitrogen

assimilation enzymes, and measurement of antioxidant activity in leaves, helped to provide insights into the mechanisms underlying this biostimulant effect. Overall, these results indicate promising biostimulant activity and suggest that humic-like substances extracted from OFMSW could contribute to enhanced N assimilation and plant development, aligning with previous observations and providing a basis for further research into their mechanisms of action. The study points out the potential of waste-derived biostimulants to support more sustainable agricultural practices if validated in broader trials, thus potentially contributing to improved crop performance and reduced environmental impact.

Abbreviations

OFMSW	Organic fraction of municipal solid waste
HS	Humic substances
HLM	Humic-like materials
GHG	Greenhouse gasses
TS	Total solids
VS	Volatile solids
HD	Hydrolyzed digestate
HC	Hydrolyzed compost
NUptE	Nitrogen uptake efficiency
NUE	Nitrogen use efficiency
GOGAT	Glutamate synthase
GS	Glutamine synthetase
MS	Murashige and Skoog

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-025-00901-z.

Supplementary Material 1. (DOCX 182 kb)

Author contributions

AB: data interpretation and writing original draft. MS: supervision, conceptualization, investigation, methodology, data interpretation and writing original draft. AE: supervision, investigation, methodology, review and editing. FE: methodology and investigation. DV: methodology. EP: supervision, conceptualization, investigation, methodology, data interpretation and writing original draft. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors listed have read the complete manuscript and have approved submission of the paper.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Agricultural, Forest and Food Sciences, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco, Italy

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