Research Article

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Hydrolysates from cauliflower and artichoke industrial wastes as biostimulants on seed germination and seedling growth: a chemical and biological characterization

Andrea Salvo,[a] Fabrizio Masciulli,[a] Donatella Ambroselli,[a] Enrico Romano,[a] Cinzia Ingallina,[a] Mattia Spano,[a] Giacomo Di Matteo,[a] Anna Maria Giusti,[b] Antonella Di Sotto,[c] Ester Percaccio,[c] Silvia Di Giacomo,[c,d] Giuliana Vinci,[e] Sabrina Antonia Prencipe,[e] Erica Acciaro,[f] Anatoly P. Sobolev,[f] Lara Costantini,[g] Nicolò Merendino,[g] Roberto Giulianelli,[h] Enio Campiglia[h] and Luisa Mannina[a*]

Abstract

Background: Cauliflower (*Brassica oleracea* L.) and globe artichoke (*Cynara scolymus* L.) are vegetables with a high waste index mainly related to stems and leaves. In this study, enzymatic hydrolysates obtained from these wastes were proposed to be used as plant biostimulants. Life cycle assessment methodology was also applied to evaluate environmental performances related to cauliflower and artichoke byproducts.

Results: Hydrolysates (HYs) were chemically and biologically characterized. Amino acids, organic acids, amines, polyols, mineral elements, phenols, tannins, flavonoids and sulfur compounds were identified and quantified by means of NMR, inductively coupled plasma mass spectrometry and UV–visible analyses. Cauliflower leaf and flower HYs showed the highest concentration of free amino acids, whereas stems showed the highest concentration of Ca. Regarding artichoke, asparagine, glutamine and aspartic acid were exclusively detected in stems, whereas artichoke leaves showed the highest Mg and Mn levels together with the highest antioxidant activity. The HYs diluted in water were tested as biostimulants. The impacts of five concentrations of HYs (0.00, 0.28, 0.84, 2.52 and 7.56 g L^{-1}) on seed germination and early seedling growth of crimson clover, alfalfa, durum wheat and corn were investigated.

Conclusions: The application of artichoke biostimulant (0.28 g L^{-1}) positively influenced the coefficient of velocity of germination in alfalfa, crimson clover and durum wheat, whereas cauliflower biostimulant significantly improved corn germination speed.

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

Keywords: hydrolysates; biostimulants; agricultural waste; NMR analysis; antioxidant activity; LCA analysis

* *Correspondence to: L Mannina, Department of Chemistry and Technology of Drugs, Food Chemistry Laboratory, Sapienza University of Rome, via P. le A. Moro 5, 00185 Rome, Italy. E-mail: luisa.mannina@uniroma1.it*

a *Department of Chemistry and Technology of Drugs, Food Chemistry Laboratory, Sapienza University of Rome, Rome, Italy*

b *Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy*

c *Department of Physiology and Pharmacology 'V. Erspamer', Sapienza University of Rome, Rome, Italy*

d *Department of Food Safety, Nutrition and Veterinary Public Health, Italian National Institute of Health, Rome, Italy*

e *Department of Management, Sapienza University of Rome, Rome, Italy*

f *'Segre-Capitani' Magnetic Resonance Laboratory, Institute for Biological Systems, CNR, Monterotondo, Italy*

g *Department of Ecological and Biological Sciences (DEB), University of Tuscia, Largo dell'Università snc, Viterbo, Italy*

h *Department of Agricultural and Forest Sciences, University of Tuscia, Via San Camillo de Lellis snc, Viterbo, Italy*

INTRODUCTION

Fruit and vegetable supply chains represent some of the most important markets in the world. This sector produces significant quantities of byproducts that are often disposed of as waste rather than being reintroduced for new purposes in the food supply chain.[1] The conventional treatment of agro-food waste has shown economic, social and environmental impacts that make it a global problem. For this reason, the agriculture sector is increasingly engaged in a radical transition towards a circular, solid and resilient system based on production processes that improve agricultural activities' sustainability.[2] In this context, the re-use of food waste is attracting increasing interest as required by the green economy and better environmental preservation.[3] In particular, the management and treatment of waste to obtain new products have become a current topic from economic and environmental points of view,[2] mainly considering that wastes obtained from the agricultural field are a precious source of molecules with both nutritional and bioactive activities.

Among the several applications that have been proposed for the re-use of agricultural wastes, the production of plant biostimulants (PBs)[4] represents an interesting potential one. PBs are effective in promoting the smooth running of plant life processes and ameliorating stress-induced injury by regulating vegetal antioxidant systems[5] or supplying essential or beneficial minerals and compounds.[6] PB applications[7] mainly involve foliar, soil and seed treatments, with the last one being considered a highly eco-friendly method due to the reduced treated surface area[5]: only a small PB amount per hectare is needed compared to those used for soil and foliar treatments by spraying.

Organic PBs are derived or are made from living matter, including humic substances (natural constituents of soil), seaweed extract, biofertilizer (symbiotic bacteria or fungi) and protein hydrolysates (HYs). The last type of PBs are mixtures of amino acids and peptides and their production requires the use of vegetable or animal residue extracts obtained by chemical or enzymatic processes. These formulations have a positive effect on agricultural production due to different bioactive compounds which enhance plant growth by regulating nutrient assimilation, storage, metabolism and radical scavenging.[8,9] In particular, HYs obtained from agro-industrial byproducts are widely recognized for their positive roles in regulating plant responses to environmental stress.[10] Some examples of PBs obtained from HYs include those produced using withered tomato plant,[11] rapeseed husks[12] and legume biomass.[13]

In the work reported in this article, HYs were produced from cauliflower (*Brassica olearacea* var. *botrytis*) and globe artichoke (*Cynara scolymus* L.), typical products of Lazio region (central Italy). These crops produce a large amount of waste and byproducts and are considered among vegetables with the highest waste index.[14] To the best of our knowledge, the chemical composition and the biological activity of HYs obtained from cauliflower and artichoke waste as well as their use as biostimulants have not yet been investigated and their potential as PBs has never been evaluated.

HYs obtained from cauliflower and artichoke were investigated by using untargeted NMR,[15-17] and targeted inductively coupled plasma mass spectrometry (ICP-MS) and UV–visible analyses,[18,19] as well as the potential radical scavenging power being investigated through DPPH and ABTS assays. Biostimulants were developed from HYs and their effect at different concentrations was tested on seeds of crimson clover (*Trifolium incarnatum* L.), alfalfa (*Medicago sativa* L.), durum wheat (*Triticum durum* Desf.) and corn (*Zea mays* L.).

Finally, life cycle assessment (LCA) was also applied as a globally standardized comprehensive framework for sustainability assessment.[20]

MATERIALS AND METHODS

Sampling

Cauliflower (*Brassica olearacea* var. *botrytis*) wastes were supplied by the Azienda Agricola F.lli Calevi Alberto E Stefano SS (Viterbo, Lazio region, Italy). Artichoke (*Cynara scolymus* L.) wastes were supplied by the organic farm Azienda Agricola Sperlonga-SANVIDA (Sperlonga, Lazio region, Italy). Flavourzyme 500MG was obtained from Novozymes China Inc. (Guangzhou, China).

Sample treatment

Cauliflower stems, leaves and flowers, and artichoke stems and leaves were washed with running water to remove all dirty residue.

All matrices were freeze-dried for 3 days using a Buchi Lyovapor L-200 at −55 °C and 8.0×10^3 Pa. The freeze-drying process promotes water removal, thereby reducing the oxidation process. Each sample was homogenized and frozen at −80 °C before enzymatic hydrolysis.

Enzymatic hydrolysis

Distilled water (600 mL) was added to dried waste product (20 g) of each matrix (cauliflower stems, leaves, and flowers, and artichoke stems and leaves). The samples were heated to 90 °C to inactivate endogenous enzymes and, after achieving the boiling point, the system was kept for 10 min under magnetic stirring. After cooling, the pH was adjusted to neutral value by adding 2 mol L^{-1} NaOH in the solution and then 1120 μL of protease solution (1 g matrix/56 μL proteolytic enzyme ratio) (from *Aspergillus oryzae*, Flavourzyme) was added. For enzyme activation, the temperature was maintained at 50 °C for 90 min in an Eco cell oven. The hydrolysis reaction was stopped by raising the temperature to 90 °C for 15 min. The inactivated homogenates were first filtered under vacuum with a Buchner funnel and a 0.45 μm paper filter, then under vacuum with a Buchner funnel and a resin filter. Liquid HY was then lyophilized with a Buchi Lyovapor L-200 at −55 °C and 0.200 mbar until complete loss of water. A 1.0–1.6 g range of yield was obtained by drying 100 mL of the considered HY.

NMR analysis of HY samples

HY samples from waste matrices (cauliflower stems, leaves, and flowers, and artichoke stems and leaves) were dissolved in 750 μL of buffered D_2O (400 mmol L^{-1} phosphate buffer, pH = 7.4) containing 2 mmol L^{-1} 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) as internal standard for chemical shift referencing and quantification.[21] The NMR spectra were recorded at 27 °C with a Bruker AVANCE III HD 600 NMR spectrometer operating at the proton frequency of 600.13 MHz and equipped with a Bruker multinuclear *z*-gradient inverse probehead. Proton spectra were referenced to the TSP signal ($\delta = 0.00$ ppm). The ^{1}H spectra were acquired by co-adding 128 transients with a recycle delay of 7 s and using a 90° pulse of 12–15 μs, 32 K data points and 12 ppm spectral window width. The residual HDO signal was suppressed using a soft pulse

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presaturation scheme (Bruker pulse program zgpr) during the relaxation delay. After the acquisition, proton spectra were zero-filled to 64 K data points, and Fourier-transformed using a 0.3 Hz exponential multiplication factor. Manual phase and baseline correction were performed. For the quantification of metabolites, the selected signals were integrated and the integrals were normalized with respect to the integral of the internal standard (TSP) signal at 0.0 ppm.

ICP-MS analysis of minerals in HY samples

Mineral concentrations from artichoke leaves and stems, and from cauliflower leaves, stems and flowers were estimated by ICP-MS after the preventive digestion of each sample, according to a previously reported methodology.[22]

Spectrophotometric determination of total polyphenols, tannins and flavonoids in HY samples

These analyses were performed according to the literature.[21] The total amounts of polyphenols/tannins and flavonoids were determined from the calibration curves of gallic acid ($y = 47\,698x + 0.01084$; $R^2 = 0.99$) and quercetin ($y = 27\,495x + 0.002971$; $R^2 = 0.99$), respectively, and expressed as grams of gallic acid equivalents (GAE) and quercetin equivalents (QE) per kilogram of sample.

Spectrophotometric determination of sulfur compounds in HY samples

Sulfur compounds were determined spectrophotometrically, by exploiting their ability to be oxidized under an alkaline environment by ferricyanide, whose absorbance can be measured at 420 nm.[23] To perform the assay, the sample (50 µL), potassium ferricyanide (12 mmol L^{-1}; 50 µL) and sodium hydroxide (1 N; 100 µL) were mixed under an alkaline environment (pH = 12). Then the absorbance of potassium ferricyanide was measured at 420 nm using a microplate reader (Epoch microplate spectrophotometer, BioTeK). Suitable controls, including potassium ferricyanide in the absence of the sample (negative control) and progressive concentrations of sinigrin (positive control), were included; moreover, sample in the absence of potassium ferricyanide was tested, to reveal the presence of possible interfering compounds in the extract. The total amount of glucosinolates was determined from the calibration curve of sinigrin ($y = -2.496x + 0.2517$; $R^2 = 0.98$) and expressed as grams of sinigrin equivalents (SINE) per kilogram of sample.

Radical scavenging activity of HY samples

The radical scavenging activities of the samples were determined with the DPPH and ABTS methods, carried out according to previously standardized microplate methods[21] and calculated as percentage difference from the control. The experiments were repeated at least twice, and in each experiment each concentration was tested at least in triplicate.

Data from the antioxidant activity assays were expressed as mean ± SE of at least three experiments with at least three technical replicates ($n = 9$) and analyzed by GraphPad Prism (Version 4.00) software (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison post test, was used to highlight possible differences among the treatments, while Student's *t*-test was used to determine a significant difference between leaf and stem HYs for each matrix. The concentration–response curves were calculated using the Hill equation, according to previous studies.[21]

Biostimulant preparation from HYs

Two biostimulants, namely a cauliflower mixture (leaf, stem and flower HYs) and an artichoke mixture (leaf and stem HYs), were prepared according to the proportions reported in Table 1. The wastes ratio was maintained for each treatment in order to ensure reproducibility in crop trials. Thus, the biostimulants can be easily reconstituted adding the appropriate amounts of water.

To test the effectiveness of artichoke and cauliflower biostimulants, four different concentrations of biostimulants, namely 0.28, 0.84, 2.52 and 7.56 g L^{-1} (low dose (LD), medium-low dose (MD), high dose (HD) and very high dose (VHD), respectively), were prepared. The effectiveness of the artichoke and cauliflower biostimulants were compared with distilled water used as control (CtrlW) and a commercial biostimulant (Comm.Biost),[24] based on *Ascophyllum nodosum* and used by dissolving 15 mL of it in 1 L of distilled water.[25]

Germination test on seeds of: *Trifolium incarnatum* L., *Medicago sativa* L., *Triticum durum* Desf. and *Zea mays* L

The germination test was conducted in the seed laboratory at the University of Tuscia (UNITUS), Viterbo, Italy. To evaluate the effect of HYs from artichoke and cauliflower as biostimulants for seed germination, the following crop species were tested: crimson clover (*Trifolium incarnatum* L.), alfalfa (*Medicago sativa* L.), durum wheat (*Triticum durum* Desf.) and corn (*Zea mays* L.). Seeds of the four species were taken from authorized and certified dealers. Sets of 25 seeds of corn and durum wheat and 50 seeds of alfalfa and crimson clover were used for each treatment with different doses.[26,27] The seeds were soaked in the respective biostimulant for 24 h. Then they were placed on filter paper in sterile Petri dishes, imbibed and put in a germination chamber with automatic control of light and temperature, with photoperiod 16 h darkness/8 h light at a constant temperature of 20 ± 2 °C.[28] Throughout the germination period the seeds were rehydrated with additional biostimulant to prevent drying when necessary. All treatments were performed in three replicates. Seed germination was recorded daily for 7 days, leaving the seedling to develop until day 10, when measurements were made on the seedlings.[26,28]

Seed germination and seedling growth measurements

The parameters to assess seed germination are described as follows. Percentage of germination was recorded every 24 h to assess the number of newly germinated seeds and after 7 days to calculate the total germination percentage (Gtot%). The emergence of radicle (>2 mm) was used as an indicator of germination.[29] Coefficient of velocity of germination (CVG)[30] was used to give an indication on the speed of germination as follows: $CVG = 100 \times (N_1 + N_2 + \ldots N_n)/(N_1T_1 + N_2T_2 + \ldots N_nT_n)$; N = number of germinated seeds per day; T = number of days from sowing corresponding for each N. The seedlings were harvested 10 days after the germination test had started. The

Table 1. HY portions (leaves, stems and flowers) used for biostimulant preparation

HY portion	Cauliflower waste (%)	Globe artichoke waste (%)
Leaves HY	42.5	50.8
Stems HY	22.0	49.2
Flowers HY	35.5	—

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biomass was weighed first as fresh biomass, then after being in an oven at 70 °C for 48 h, as dry biomass. The maximum length of shoot and roots was measured with a graduated ruler to the nearest 1.0 mm and the number of roots for each seedling was recorded. Total seedling length was calculated as root length + shoot length. The seed vigor index (SVI) was calculated as Gtot% multiplied by total seedling length divided by 100.[31]

The germination test was conducted according to a randomized block with eight treatments and three replications for each crop species and biostimulant concentration. Data were subjected to one-way ANOVA. The mean values were tested for significance with Tukey's HSD test. Statistical difference was set at $P < 0.05$. Percentage data were arcsine-transformed for analysis, whereas they were presented in tables as non-transformed means. For statistical analysis, the software R version 4.2.1 was used.

Life cycle assessment

According to ISO 14040, LCA consists of four distinct phases: (i) goal and scope definition, (ii) life cycle inventory analysis and (iii) life cycle impact assessment are compulsory; (iv) interpretation of results is optional (ISO 14040, 2006). Subsequent sections elaborate on each phase. The analysis of all environmental impacts was conducted using SimaPro v 9.5.0.2 software. Details are reported in the supporting information.

RESULTS AND DISCUSSION

Hydrolysate samples

Artichoke and cauliflower wastes were subjected to enzymatic hydrolysis and analyzed using different methodologies, the results of which are here separately discussed.

Metabolite profile by ^{1}H NMR analysis

The NMR analysis of cauliflower and artichoke HYs was based on the methodology previously reported for crude extracts of the same byproducts.[32] Amino acids, organic acids, amines and polyols were identified and quantified (Table 2). In the case of cauliflower HYs, the content of almost all free amino acids, except glutamine, was higher in flowers than in stems, with aspartic acid, glutamic acid and methionine being the most abundant. In the case of artichoke HYs, the content of amino acids in leaves was slightly higher than in stems (except for lysine, threonine and glutamic acid). Moreover, asparagine, glutamine and aspartic acid were detectable only in stems. Lactic acid was the most abundant organic acid in all HYs except in artichoke stems, followed by citric and malic acids. Unexpectedly, mannitol, not detected in crude extracts,[32] was found in all cauliflower HY samples, mainly in stems. Artichoke HYs also contain inositol isomers (*scyllo*- and *chiro*-inositols), the amounts of which were similar to those found in crude extracts.[32]

Inorganic elements by ICP-MS analysis

The amounts of inorganic elements in hydrolyzed artichoke leaves and stems and in hydrolyzed cauliflower leaves, stems and flowers are reported in Table S6. Data are expressed in mg kg^{-1} DW ± SD. Thirty mineral elements were determined, spanning from nontoxic (i.e. Ba, Ca, K, Na, Fe, Mg, Mn, Zn, etc.) to potentially toxic ones (i.e. As, Cr, Ni, V, etc.). On average, Ca, K and Mn were the elements present at the highest concentration. In all samples, the levels of Hg and Cd were below the limit of instrumental detection. Levels of Cr (range: 0.000–0.036 mg kg^{-1}) and Pb (range: 0.005–0.049 mg kg^{-1}) were below the limits set by the European Regulation[33] and/or below the limit of instrumental detection. For the other inorganic elements that are generally considered potentially toxic, the safety limits are not fixed.

Total polyphenols, tannins, flavonoids and glucosinolates by spectrophotometry

HYs obtained from the leaves of both cauliflower and artichoke contained the highest amounts of total polyphenols and tannins, which were two- to threefold higher than those of the stem and flower samples (Table 3). Polyphenols were mainly concentrated in the HYs from the leaves of both samples, whereas lower amounts were detected in those from stems of both cauliflower and artichoke, and flowers of cauliflower. Conversely, low levels of sulfur compounds were determined in the protein HYs from cauliflower leaves, stems and flowers; as expected, these compounds were lacking in the artichoke samples (Table 3).

Antioxidant activity

The ability of HYs to neutralize radical scavengers could be an important feature to favor plant development. Indeed, several environmental factors (e.g. drought, metal toxicity, pollutants, UV-B, pesticides, pathogen infection) can induce oxidative stress in plants, so affecting multiple biological processes via reactive oxygen species generation, leading to cell plant death.[34]

Under our experimental conditions, the tested HYs were able to neutralize DPPH radicals, except for the sample obtained from cauliflower stems, for which a weak activity was revealed (Fig. 1). The HY from artichoke leaves was the most active, followed by that from cauliflower flowers; with these samples being able to induce an about 80% inhibition of DPPH at a concentration of 500 μg mL^{-1}, despite a lower than 20% inhibition being achieved with other samples. This trend was confirmed by the IC_{50} values as presented in Table S7. Regarding HYs from cauliflower stems, the IC_{50} value was not evaluable since at the highest tested concentration the achieved inhibition was lower than 80%. As expected, the positive control, that is, Trolox, exhibited a more potent scavenging effect, the IC_{50} value being 133- to 500-fold lower than those of the HYs from artichoke and cauliflower leaves (Table S7).

When the antioxidant activity was assessed towards ABTS, all the tested protein HYs exhibited radical scavenging properties. HY from artichoke leaves was the most effective, being able to induce a 50% ABTS neutralization at a concentration of 200 μg mL^{-1}, followed by that from cauliflower leaves and flowers (almost 40% and 30% inhibition at 200 μg mL^{-1}, respectively) (Fig. 2). This trend was confirmed by the IC_{50} values as displayed in Table S7. As expected, the positive control Trolox exhibited a more potent scavenging effect, the IC_{50} value being 90- to 140-fold lower than those of the HYs from artichoke and cauliflower leaves.

It is known that small peptides, arising from the hydrolyzation process, can exhibit antioxidant activities.[35] Particularly, a high degree of hydrolysis is associated with marked antioxidant properties, likely due to the presence of low-molecular-weight peptides, although the correlation between molecular weight and antioxidant activity has to be clarified.[36,37] Moreover, the time and hydrolysis conditions can strongly affect the antioxidant power.[14] In line with this evidence, Yathisha *et al.*[38] reported that the peptic HY of cauliflower leaves has been endowed with ABTS radical scavenging activity, with an IC_{50} of 0.6 mg mL^{-1}, this value being halved after pancreatic digestion. Moreover, Caliceti *et al.*[14] have highlighted that time and hydrolysis conditions can strongly affect the antioxidant power of samples and found that 3–5 h

Table 2. Metabolite content (g kg^{-1} DW) in HYs of cauliflower and globe artichoke by ^1H NMR analysis

		Cauliflower HYs			Globe artichoke HYs	
	δ^1H (ppm)	Leaves	Stems	Flowers	Leaves	Stems
Amino acids						
Alanine	1.49	2.17 ± 0.17^a	1.68 ± 0.08^b	4.62 ± 0.28^c	6.79 ± 0.34^a	1.31 ± 0.07^b
Arginine	3.24	2.96 ± 0.18	—	—	—	—
Asparagine	2.96	2.27 ± 0.16^a	1.47 ± 0.12^b	1.06 ± 0.07^c	—	2.80 ± 0.27
Aspartic acid	2.80	5.32 ± 0.27^a	1.98 ± 0.14^b	9.21 ± 0.92^c	—	0.99 ± 0.11
GABA	3.01	1.94 ± 0.21^a	1.40 ± 0.14^b	3.85 ± 0.31^c	0.90 ± 0.07^a	0.55 ± 0.07^b
Glutamine	2.46	1.87 ± 0.19^a	3.05 ± 0.27^b	5.59 ± 0.62^c	n.d.	3.05 ± 0.31
Glutamic acid	2.35	4.91 ± 0.34^a	2.42 ± 0.14^b	12.40 ± 0.63^c	2.42 ± 0.24^a	2.07 ± 0.17^a
Histidine	8.13	0.89 ± 0.07^a	0.23 ± 0.03^b	1.09 ± 0.13^a	n.d.	n.d.
Isoleucine	1.01	1.10 ± 0.13^a	0.20 ± 0.02^b	1.24 ± 0.10^a	0.56 ± 0.06^a	0.41 ± 0.02^b
Leucine	0.96	1.89 ± 0.17^a	0.43 ± 0.04^b	1.74 ± 0.16^a	0.92 ± 0.07^a	0.67 ± 0.07^b
Lysine	3.03	2.41 ± 0.12^a	0.47 ± 0.06^b	1.94 ± 0.19^a	0.28 ± 0.03^a	0.74 ± 0.06^b
S-Methyl-L-cysteine-*S*-oxide (methiin)	2.84	4.96 ± 0.45^a	3.22 ± 0.26^b	14.16 ± 0.99^c	—	—
Phenylalanine	7.43	1.96 ± 0.16^a	0.35 ± 0.02^b	1.52 ± 0.14^c	1.36 ± 0.11^a	0.57 ± 0.07^b
Threonine	1.34	2.13 ± 0.09^a	1.15 ± 0.13^b	1.64 ± 0.18^c	1.11 ± 0.10^a	0.89 ± 0.11^a
Tryptophan	7.73	0.50 ± 0.07^a	0.11 ± 0.01^b	0.24 ± 0.03^c	0.52 ± 0.06^a	0.20 ± 0.03^b
Tyrosine	6.91	1.36 ± 0.15^a	0.34 ± 0.03^b	1.16 ± 0.10^a	0.80 ± 0.12^a	0.32 ± 0.03^b
Valine	1.05	1.91 ± 0.16^a	0.60 ± 0.08^b	2.76 ± 0.29^c	2.27 ± 0.25^a	1.78 ± 0.14^b
Organic acids						
Citric acid	2.55	10.81 ± 0.54	—	n.d.	3.83 ± 0.46^a	3.35 ± 0.27^a
Formic acid	8.46	0.03 ± 0.01^a	—	0.064 ± 0.020^b	0.11 ± 0.03^a	0.033 ± 0.010^b
Fumaric acid	6.52	0.19 ± 0.04^a	—	0.66 ± 0.08^b	0.079 ± 0.02^a	0.16 ± 0.04^b
Lactic acid	1.34	41.69 ± 2.92^a	74.17 ± 6.70^b	87.81 ± 9.64^b	21.22 ± 2.12^a	0.13 ± 0.02^b
Malic acid	4.29	2.69 ± 0.13	—	n.d.	1.69 ± 0.14^a	12.34 ± 1.36^b
Quinic acid	1.87	—	—	—	6.81 ± 0.55^a	5.96 ± 0.78^a
Succinic acid	2.41	1.24 ± 0.14^a	3.45 ± 0.28^b	—	4.30 ± 0.43^a	1.18 ± 0.12^b
Miscellaneous						
Mannitol	3.68	14.25 ± 1.14^a	50.13 ± 2.51^b	16.12 ± 0.97^a	—	—
scyllo-Inositol	3.36	—	—	—	1.27 ± 0.10^a	0.39 ± 0.05^b
chiro-Inositol	3.58	—	—	—	4.76 ± 0.29^a	3.24 ± 0.26^b
Choline	3.21	1.37 ± 0.15^a	0.51 ± 0.04^b	2.86 ± 0.31^c	0.44 ± 0.05^a	0.60 ± 0.07^b

In each column, mean values (± SD) are reported together with the results of ANOVA applied to the leaves *versus* stems and flowers (for cauliflower) comparison for each crop separately. Different superscript letters (a *versus* b) indicate statistically significant ($P \leq 0.05$) differences between leaf and stem HYs, whereas the same letters indicate no significant difference.

Table 3. Total content of polyphenols, tannins, flavonoids and sulfur compounds per mg of HYs from cauliflower (*Brassica oleracea* L.) and artichoke (*Cynara scolymus* L.) leaves and stems

HY	Polyphenols (g GAE kg^{-1})	Tannins (g GAE kg^{-1})	Flavonoids (g QE kg^{-1})	Sulfur compounds (g SINE kg^{-1})
Cauliflower leaves	$9.54 \pm 0.14^{***}$	2.48 ± 0.17	0.90 ± 0.02	0.014 ± 0.004
Cauliflower stems	4.81 ± 0.09	$1.49 \pm 0.12^{***}$	bld	bld
Cauliflower flowers	2.96 ± 0.14	$1.73 \pm 0.14^{\S}$	bld	bld
Artichoke leaves	14.05 ± 0.17	5.83 ± 0.07	0.96 ± 0.03	—
Artichoke stems	$5.89 \pm 0.08^{***}$	$2.13 \pm 0.11^{***}$	bld	—

Total polyphenols and tannins were determined as gallic acid equivalents (GAE), flavonoids as quercetin equivalents (QE).
*** $P < 0.001$, denotes a significant difference of stems *versus* leaves for each crop (Student's *t*-test).
§ $P < 0.05$, denotes a significant difference of flowers *versus* leaves of cauliflower (Student's *t*-test).[a] bld, below the limit of detection; —, not determined.

hydrolysis time using alcalase was the best condition to obtain remarkable antioxidant HYs from ribbon fish.

Additionally, the cauliflower and artichoke HYs are characterized by the presence of bioactive compounds that can contribute to their antioxidant properties against DPPH and ABTS radicals. Artichoke samples were the most effective scavenging agents, although with a higher potency towards ABTS than DPPH. Such properties have been already reported for both cauliflower and

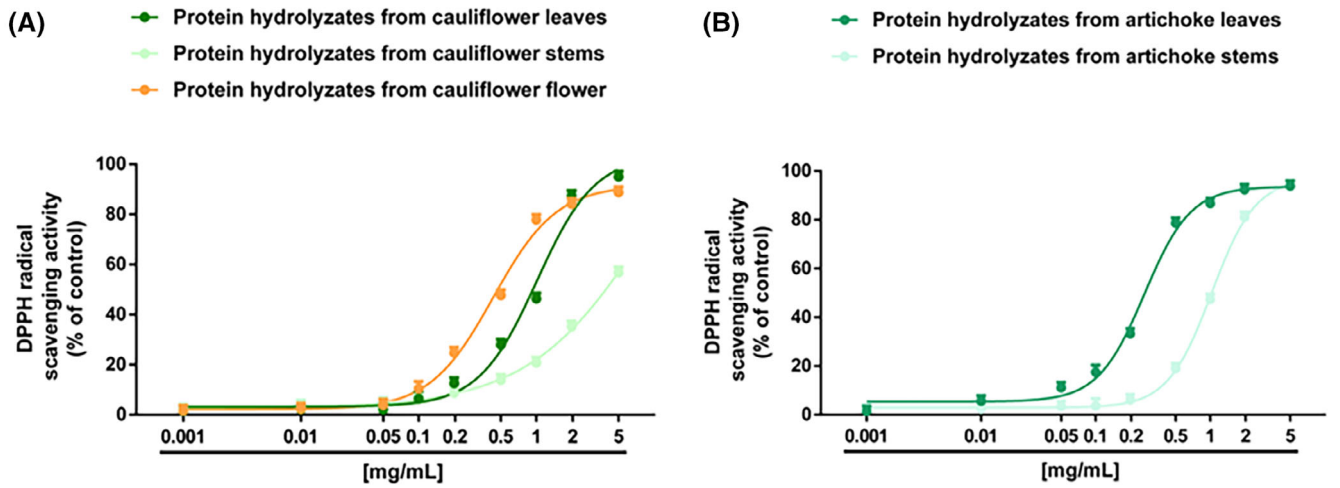

Figure 1. DPPH radical scavenging activity of cauliflower waste HYs and artichoke waste HYs. Data are displayed as mean ± SD ($n = 9$).

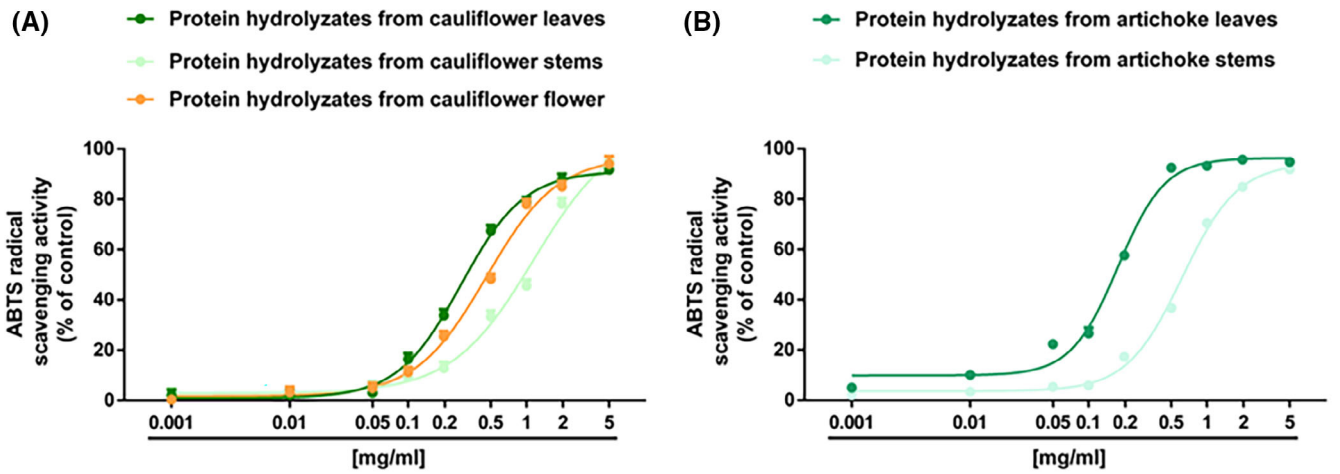

Figure 2. ABTS radical scavenging activity of cauliflower waste HYs and artichoke waste HYs. Data are displayed as mean ± SD ($n = 9$).

artichoke waste and for different classes of polyphenols and glucosinolates.[39-43]

Altogether the obtained results suggest an interest in the protein HYs from cauliflower and artichoke as sources of bioactive molecules useful in different application fields.

Biostimulants

Effects of biostimulants on seed germination and seedling growth

To assess the effects of cauliflower and artichoke biostimulants on seed germination and seedling growth, four different solutions (0.28, 0.84, 2.52 and 7.56 g L^{-1}) were tested on the seeds of four plant species: alfalfa, crimson clover, durum wheat and corn. The results were compared with those obtained for seeds germinated in distilled water or using a commercial biostimulant. The main parameters of seed germination and seedling growth for alfalfa, crimson clover, durum wheat and corn after the application of different biostimulant concentrations are presented in Tables 4–7, respectively.

Generally, the highest concentrated cauliflower biostimulant (7.56 g L^{-1}) caused a decrease of Gtot, with respect to control and commercial biostimulant, except in corn seeds where no statistical differences were detected (Table 7). However, at the highest concentration, both cauliflower and artichoke biostimulants decreased Gtot, especially in alfalfa and durum wheat (Tables 4 and 6), whereas, with respect to control, corn seeds were negatively affected by the treatment with the artichoke biostimulant with the highest concentration (Table 7). On the contrary, the speed of germination, indicated by the CVG, was moderately affected by the treatments: many treatments showed CVG values statically similar to those of the control and the commercial biostimulant. However, a low concentration of artichoke biostimulant seems to exert a positive effect on the CVG in alfalfa, crimson clover and durum wheat, whereas a low concentration of cauliflower biostimulant tended to improve the germination speed in corn with respect to control. Similarly to germination parameters, the seedling growth characteristics were negatively affected by cauliflower biostimulant at the highest concentration. It was associated with the lowest values of root length, sprout length, total length and biomass of the seedlings in most of the plant species tested. With respect to control, the treatment with 7.56 g L^{-1} cauliflower biostimulant significantly decreased the biomass (dry weight) by 30%, 79%, 54% and 48% and the seedling total length by 50%, 19%, 26% and 29% in alfalfa, crimson clover, durum wheat and corn, respectively. On the contrary, the treatment with the lowest concentration of artichoke biostimulant (0.28 g L^{-1})

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Table 4. Effect of cauliflower and artichoke biostimulants on alfalfa seed germination and seedling growth

Treatment	Biostimulant concentration (g L^{-1})	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	86.7 ± 2.9 a	48.80 ± 2.08 ab	2.77 ± 0.18 a	1.60 ± 0.04 ab	4.38 ± 0.20 ab	1.59 ± 0.03 a	3.80 ± 0.29 ab
Comm.Biost	—	86.3 ± 1.9 a	45.63 ± 4.53 abc	2.98 ± 0.16 a	1.63 ± 0.02 a	4.61 ± 0.15 a	1.64 ± 0.05 a	3.99 ± 0.18 a
Artichoke	0.28	87.3 ± 1.2 a	56.42 ± 3.57 a	3.07 ± 0.05 a	1.74 ± 0.01 a	4.81 ± 0.06 a	1.59 ± 0.05 a	4.21 ± 0.11 a
Artichoke	0.84	82.7 ± 0.7 a	55.04 ± 4.01 a	2.51 ± 0.13 ab	1.57 ± 0.05 ab	4.08 ± 0.13 ab	1.55 ± 0.04 ab	3.37 ± 0.12 abc
Artichoke	2.52	82.0 ± 1.2 a	44.39 ± 7.32 abc	2.31 ± 0.50 ab	1.52 ± 0.02 ab	3.84 ± 0.52 abc	1.50 ± 0.05 ab	3.14 ± 0.38 abc
Artichoke	7.56	78.0 ± 1.2 a	44.90 ± 4.46 abc	1.25 ± 0.21 bc	1.48 ± 0.08 ab	2.73 ± 0.29 bc	1.40 ± 0.12 abc	2.13 ± 0.24 bc
Cauliflower	0.28	85.3 ± 1.3 a	42.07 ± 5.83 abc	2.50 ± 0.03 ab	1.51 ± 0.01 ab	4.01 ± 0.03 ab	1.42 ± 0.11 abc	3.42 ± 0.04 abc
Cauliflower	0.84	82.7 ± 1.3 a	31.30 ± 2.90 bc	2.24 ± 0.06 abc	1.45 ± 0.06 ab	3.69 ± 0.11 abc	1.31 ± 0.05 abc	3.05 ± 0.05 abc
Cauliflower	2.52	77.3 ± 3.5 a	26.78 ± 1.15 bc	1.97 ± 0.33 bc	1.37 ± 0.09 ab	3.34 ± 0.32 abc	1.22 ± 0.04 bc	2.56 ± 0.17 bc
Cauliflower	7.56	46.7 ± 8.2 b	22.78 ± 6.49 c	0.99 ± 0.63 c	1.22 ± 0.20 b	2.21 ± 0.82 c	1.12 ± 0.08 c	1.17 ± 0.60 d

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

Table 5. Effect of cauliflower and artichoke biostimulants on crimson clover seed germination and seedling growth

Treatment	Biostimulant concentration (g L^{-1})	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	88.7 ± 1.8 a	26.92 ± 1.81 ab	1.13 ± 0.08 ab	1.87 ± 0.09 bc	3.01 ± 0.15 bc	1.72 ± 0.04 a	2.66 ± 0.09 bc
Comm.Biost	—	87.3 ± 1.3 a	24.65 ± 3.15 ab	1.35 ± 0.05 ab	2.24 ± 0.19 abc	3.59 ± 0.24 ab	1.75 ± 0.05 a	3.13 ± 0.16 ab
Artichoke	0.28	86.7 ± 0.9 a	29.97 ± 1.40 ab	1.60 ± 0.08 a	2.70 ± 0.23 a	4.28 ± 0.29 a	1.83 ± 0.09 a	3.71 ± 0.25 a
Artichoke	0.84	90.7 ± 4.4 a	30.82 ± 2.15 a	1.28 ± 0.16 ab	2.12 ± 0.08 abc	3.40 ± 0.24 abc	1.81 ± 0.06 a	3.08 ± 0.25 ab
Artichoke	2.52	86.3 ± 3.2 a	29.02 ± 1.09 ab	1.27 ± 0.26 ab	1.67 ± 0.21 bc	2.94 ± 0.47 bc	1.57 ± 0.06 ab	2.53 ± 0.38 bc
Artichoke	7.56	84.0 ± 1.2 ab	25.65 ± 0.65 ab	1.06 ± 0.06 ab	1.94 ± 0.02 bc	3.00 ± 0.07 bc	1.68 ± 0.02 ab	2.52 ± 0.05 bc
Cauliflower	0.28	89.0 ± 1.0 a	25.43 ± 0.48 ab	1.33 ± 0.02 ab	2.27 ± 0.13 ab	3.60 ± 0.14 ab	1.68 ± 0.03 ab	3.20 ± 0.16 ab
Cauliflower	0.84	86.3 ± 1.2 a	21.00 ± 2.47 b	1.42 ± 0.09 ab	1.99 ± 0.06 bc	3.40 ± 0.09 abc	1.64 ± 0.02 ab	2.94 ± 0.09 ab
Cauliflower	2.52	85.3 ± 0.7 ab	31.31 ± 2.35 a	1.24 ± 0.06 ab	1.79 ± 0.02 bc	3.03 ± 0.06 bc	1.40 ± 0.09 b	2.58 ± 0.04 bc
Cauliflower	7.56	76.7 ± 1.8 b	28.94 ± 1.85 ab	0.86 ± 0.05 b	1.58 ± 0.14 c	2.44 ± 0.10 c	0.36 ± 0.07 c	1.87 ± 0.11 c

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

Table 6. Effect of cauliflower and artichoke biostimulants on durum wheat seed germination and seedling growth

Treatment	Biostimulant concentration (g L^{-1})	Seed germination	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	92.0 ± 2.0 ab	13.46 ± 1.41 ab	10.87 ± 1.40 ab	12.64 ± 0.56 a	23.51 ± 1.91 ab	25.76 ± 0.68 a	21.11 ± 1.42 ab
Comm.Biost	—	96.0 ± 2.3 a	15.59 ± 0.47 a	11.53 ± 0.25 ab	13.34 ± 0.50 a	24.87 ± 0.35 a	26.85 ± 1.24 a	23.88 ± 0.68 a
Artichoke	0.28	92.7 ± 1.8 ab	15.70 ± 1.03 a	11.87 ± 0.58 a	11.21 ± 0.23 a	23.08 ± 0.78 ab	27.30 ± 0.21 a	21.42 ± 1.13 ab
Artichoke	0.84	82.7 ± 0.7 bc	12.56 ± 1.20 ab	9.27 ± 0.58 ab	12.68 ± 0.75 a	21.96 ± 1.26 ab	23.53 ± 1.77 ab	18.16 ± 1.13 b
Artichoke	2.52	80.0 ± 4.6 cd	11.52 ± 1.98 ab	9.07 ± 0.50 ab	11.70 ± 0.65 a	20.77 ± 0.50 ab	24.14 ± 2.51 ab	16.63 ± 1.20 bc
Artichoke	7.56	76.0 ± 2.3 d	10.62 ± 0.85 bc	9.36 ± 0.73 ab	12.17 ± 0.58 a	21.53 ± 1.28 ab	24.11 ± 1.74 ab	16.32 ± 0.76 bc
Cauliflower	0.28	90.0 ± 2.0 ab	15.66 ± 0.75 a	10.87 ± 1.38 ab	12.36 ± 0.48 a	23.23 ± 1.10 ab	23.73 ± 1.14 ab	20.94 ± 1.39 ab
Cauliflower	0.84	74.3 ± 1.2 d	9.02 ± 0.97 bc	10.13 ± 0.28	12.06 ± 0.58 a	22.19 ± 0.68 ab	20.87 ± 0.82 ab	16.49 ± 0.32 bc
Cauliflower	2.52	58.0 ± 3.5 e	8.15 ± 0.94 bc	8.57 ± 0.33 ab	10.58 ± 1.52 a	19.16 ± 1.19 ab	17.37 ± 1.06 bc	11.15 ± 1.11 cd
Cauliflower	7.56	60.0 ± 2.0 e	5.92 ± 0.64 c	7.65 ± 0.72 b	9.81 ± 1.71 a	17.46 ± 2.16 b	11.93 ± 1.50 c	10.56 ± 1.60 d

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

tended to positively affect the development of seedlings, providing an increase with respect to control and to commercial biostimulant for root and sprout length in alfalfa and crimson clover, and for dry biomass in durum wheat and crimson clover even though the values did not differ significantly from each other. Instead, the solution of cauliflower biostimulant with the lowest

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Table 7. Effect of cauliflower and artichoke biostimulants on corn seed germination and seedling growth

Treatment	Biostimulant concentration (g L^{-1})	Gtot (%)	CVG	Seedling root length (cm)	Seedling sprout length (cm)	Seedling total length (cm)	Seedling biomass dry weight (mg)	SVI
CtrlW	0.00	93.3 ± 3.5 a	8.97 ± 0.74 ab	15.90 ± 1.21 ab	12.97 ± 0.65 ab	28.87 ± 1.79 abc	67.52 ± 0.61 abc	26.92 ± 1.80 b
Comm.Biost	—	81.3 ± 1.3 bc	5.90 ± 0.33 b	16.47 ± 1.07 ab	14.20 ± 0.10 a	30.67 ± 1.02 ab	73.42 ± 1.15 a	24.97 ± 1.25 b
Artichoke	0.28	94.7 ± 1.3 a	8.37 ± 0.53 ab	15.20 ± 0.65 ab	12.67 ± 0.35 ab	27.87 ± 0.98 abc	59.09 ± 3.70 bcd	26.36 ± 0.63 b
Artichoke	0.84	90.7 ± 1.3 ab	8.31 ± 0.33 ab	12.00 ± 1.22 b	9.60 ± 0.40 b	21.60 ± 1.48 bc	45.97 ± 3.53 cd	19.54 ± 1.05 c
Artichoke	2.52	89.3 ± 1.3 abc	8.66 ± 0.87 ab	11.30 ± 2.15 b	9.26 ± 1.31 b	20.57 ± 3.43 c	45.75 ± 6.75 cd	18.34 ± 2.94 c
Artichoke	7.56	79.3 ± 1.2 c	10.65 ± 0.34 a	11.55 ± 0.63 b	9.43 ± 0.49 b	20.97 ± 1.11 c	44.26 ± 1.68 cd	16.67 ± 0.12 c
Cauliflower	0.28	93.3 ± 2.7 a	9.91 ± 0.85 a	20.47 ± 2.35 a	13.97 ± 0.77 a	34.43 ± 3.09 a	71.26 ± 5.47 ab	32.17 ± 3.26 a
Cauliflower	0.84	93.3 ± 2.7 a	11.42 ± 0.98 a	17.73 ± 0.49 ab	12.53 ± 0.78 ab	30.26 ± 0.89 ab	66.14 ± 2.30 abc	28.23 ± 0.96 ab
Cauliflower	2.52	89.3 ± 3.5 abc	9.50 ± 1.14 ab	12.47 ± 1.79 ab	10.10 ± 1.17 b	22.57 ± 2.90 bc	50.10 ± 9.17 bcd	20.32 ± 3.20 c
Cauliflower	7.56	90.3 ± 1.5 abc	7.52 ± 1.17 ab	11.15 ± 0.71 b	9.44 ± 0.80 b	20.59 ± 1.17 bc	35.32 ± 1.97 e	18.62 ± 1.27 c

In each column, mean values (± SD) followed by different letters indicate statistically significant ($P \leq 0.05$) differences (Tukey's test). Effects of control and commercial biostimulant are also reported.

concentration (0.28 g L^{-1}) seemed to positively affect the root length, sprout length and biomass in corn. The SVI, which combines the percentage of germination and the seedling total length, provided an indication of the overall effect of the biostimulant on the germination phase. This indicator confirmed that the cauliflower biostimulant with the highest concentration (7.56 g L^{-1}) negatively affected the seed germination in all plant species. With respect to control, it significantly decreased SVI by 69%, 50% and 31% for alfalfa, durum wheat and corn, respectively, whereas in the case of crimson clover, a 30% SVI reduction was not statistically significant (Table 5). On the contrary, the treatment with the most diluted solution of cauliflower biostimulant (0.28 g L^{-1}) positively affected the SVI in corn providing an increase with respect to control and biostimulant by 20% and 29% (Table 7). Instead, the lowest concentration of artichoke biostimulant (0.28 g L^{-1}) tended to positively affect the SVI in crimson clover, providing an increase compared to control by 39% and to commercial biostimulant by 19%, although significant differences were observed only in the case of control (Table 5).

From an agronomic point of view, these two biostimulants seem to have a positive effect on seed germination and seedling growth only at low concentrations, showing an inhibitory effect at higher ones. This detrimental effect can be due to a large amount of phenolic compounds present in cauliflower and artichoke waste; in fact indeed inhibitory effects of high concentrations of various protein HYs on plant growth are already reported in the literature[44,45] and have been observed also by other authors. Muscolo and Sidari[46] have reported that phenolic compounds are potential inhibitors of nitrogen uptake and their toxicity may have interfered with the biostimulating effect in the presence of high concentrations. It is noteworthy that the commercial biostimulant did not show any significant effect, although several authors have reported that extract of *Ascophyllum nodosum* can have a positive effect increasing the germination and seed vigor of annual crops (herbaceous) such as barley, tomato, pepper and eggplant.[47,48] According to Ali *et al.*[49] the positive effects of the seaweed products are dependent on the type of the seaweed resource, quality and composition of the extract, as well as method, concentration and frequency of application. Therefore, this study confirms that no biostimulant has a generalizable effect which is clearly species-specific and product-specific; what we know about one biostimulant or about one plant does not directly transfer to another plant species. Moreover, since seaweed extracts are complex composts, made from natural raw materials, they cannot have a standardized composition and this could lead to different effects over time. Nevertheless, it has been shown that higher dosage of amino acids, humic acid or salicylic acid could have adverse effects on many leafy vegetable crops,[3,6,50,51] thus confirming that plant sensitivity to biostimulants is variable on a case-to-case basis.

Life cycle impact assessment

A sustainability assessment of cauliflower and artichoke production allocated to industrial waste was carried out through the application of LCA, using the ReCiPe Midpoint 2016 method. Results for all impact categories considered are detailed in the supporting information (Tables S4 and S5; Figs S1 and S2) showing the main impact categories involved in the crop life cycle; in the case of cauliflower, water used for irrigation and fuel consumption turned out to have a relevant impact on the production process whereas in the case of artichoke production, polyester mulching film and diesel burned by agricultural machinery as well the use of synthetic fertilizers were found to be the most impacting inputs.

For both crop cycles, it is noteworthy that industrial waste is responsible for an average of 57% of total greenhouse gas emissions for cauliflower production and 36% for artichoke production. In these regards, the possibility of recycling these industrial wastes was considered for a scenario analysis, thus evaluating possible mitigating strategies during the crop life cycle.

Two recycling scenarios of crop industry, namely biostimulant formulation and composting substrates, Figs 3 and 4, were investigated. In three of the five most affected categories, biostimulant formulation from waste is of advantage, mitigating the impacts associated with global warming (−143 029 kg CO_2 eq), fossil resource scarcity (-1.43×10^8 kg oil eq) and ozone formation, human health (-2.30×10^2 kg NO_x eq). Nevertheless, even though the formulation of biostimulants particularly affects the impact categories TA and MRS (7.72×10^3 kg SO_2 eq and 7.24×10^5 kg Cu eq, respectively), it is still an advantageous recycling scenario for agro-industrial artichoke and cauliflower waste compared with the composting scenario, which negatively impacted three of five categories. In particular, the TA and MRS impact could be related to the production of electricity and the related combustion of fossil fuels required for the formulation of biostimulants on a laboratory scale. In these regards, it is worth

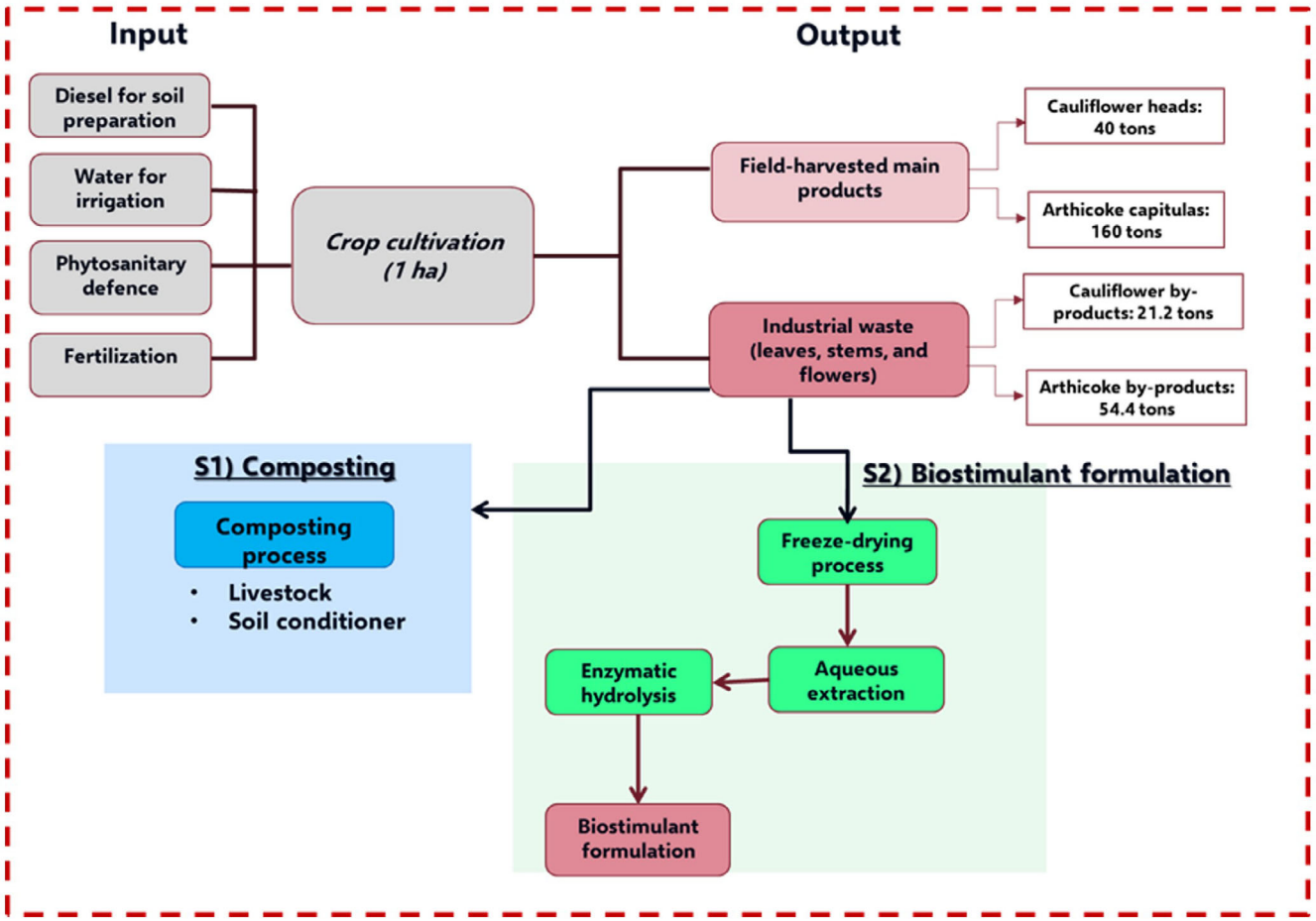

Figure 3. System boundaries of the analyzed crop cycles considering (S1) crop byproducts as composting substrates and (S2) crop byproducts for biostimulant formulation.

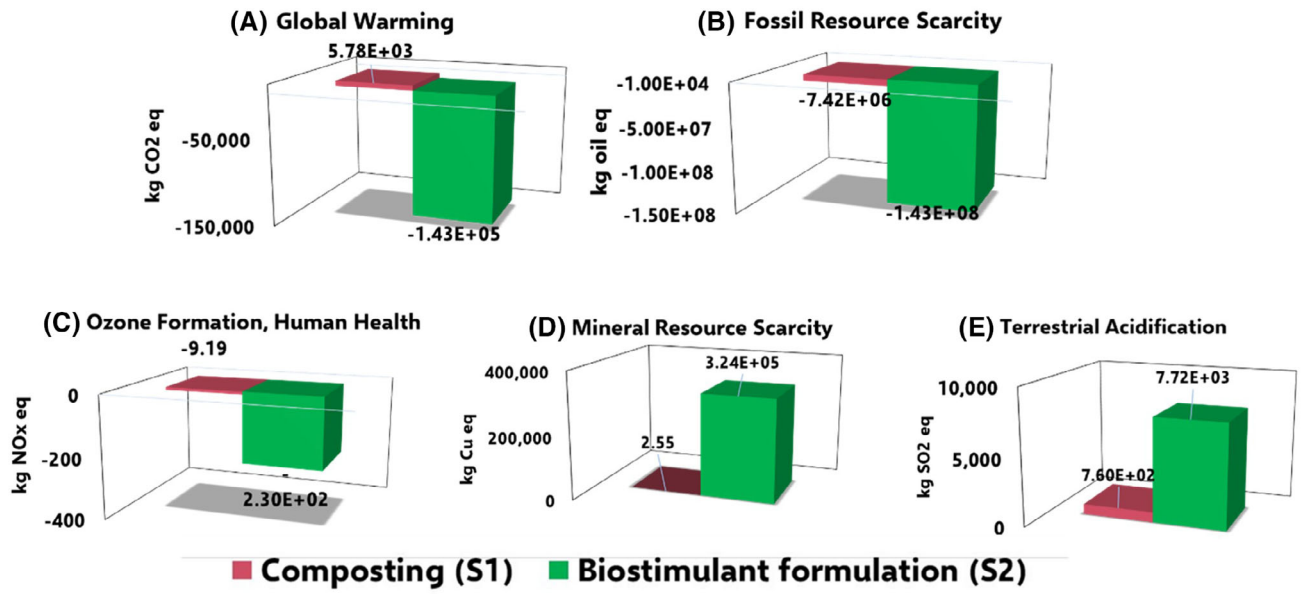

Figure 4. Recycling scenarios for industrial waste: composting (S1) and biostimulant formulation (S2). Global warming (A), fossil resource scarcity (B), ozone formation, human health (C), mineral resource scarcity (D) and terrestrial acidification (E) impact categories.

highlighting the opportunity of valorizing agricultural waste, through its conversion into biostimulants, thus representing a mitigating strategy for the whole product life cycle as well as it being able to provide nutrient-rich compost for soil health, cost savings and sustainable agriculture.[52]

CONCLUSIONS

The obtained results highlighted the presence of several compounds involved in plant development in the protein HYs from leaves, flowers and stems of cauliflower (*Brassica oleracea* L.) and from leaves and stems of artichoke (*Cynara scolymus* L.). These properties led to a consideration of cauliflower and artichoke byproducts and their HYs as eligible raw matrices for several applications, not only as plant biostimulants but also as potential matrices to isolate and concentrate bioactive compounds. Their use as biostimulants appears to have a positive impact on seed germination and seedling growth only at low concentrations, whereas at higher concentrations, they exhibit an inhibitory effect. This detrimental effect can be due to large amounts of compounds present in cauliflower and artichoke waste, mainly amino acids. However, this study provided evidence that the effect of plant-based biostimulants on the seed germination process is species specific, and therefore further studies are necessary to explain the mechanism of action involved in seed germination and seedling growth.

Finally, LCA results show the critical points of the two life cycles giving the inspiration to find applicable and concrete solutions, since biostimulant formulations from wastes are advantageous to mitigate the impacts associated with global warming, fossil resource scarcity, ozone formation and human health.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors declare no conflict of interest for the article.

SUPPORTING INFORMATION

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

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