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Potential use of *Sargassum muticum* as source of plant biostimulants after three different drying methods

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Abstract

Seaweed derived biostimulants are gaining attention as an important tool in sustainable agriculture. This offers a unique opportunity to alleviate the environmental impact of *Sargassum muticum* (Ochrophyta, Phaeophyceae) as an invasive species by finding new applications for its biomass. In this sense, incorporating green extraction technologies is fundamental to ensure environmental-friendly goals. This research was initiated in an attempt to contribute to an integral valorization system of *S. muticum* biomass, exploring the biostimulant potential of the solubles obtained from pressed solids, through an autohydrolysis treatment. In addition, we compared the effect of three different liquid phase drying techniques (spray-drying, freeze-drying and convective air-drying). Low stress drying techniques as spray-drying showed better conservation of bioactive compounds and biostimulant potential. A bioassay with *Nastrium officinale* showed no phytotoxic effects despite high electric conductivity in most of the extracts and concentrations. Dried extracts showed mainly an amorphous structure but occasional crystal formation when spray-dried at low temperatures (T_{out} = 50 ºC) and after convective air drying (T = 40 ºC). Significant increases in root development were achieved at a concentration of 5 mg L^{-1} of spray-dried extracts and 50 mg L^{-1} in case of freeze-dried extracts. Munoo-Liisa vitality index showed best results with 50 mg L^{-1} of freeze-dried extracts. This study provides important information about the influence of different drying techniques on the physicochemical properties and biostimulant potential of *S. muticum* aqueous extracts and contributes to the integral valorization of its biomass.

Keywords *Sargassum muticum* · Biostimulant · Autohydrolysis · Freeze drying · Spray drying · Air convective drying

Introduction

Rising food demand and the conventional approach of global food economy have led to a massive an unsustainable intensification of agriculture, with harmful impacts on the environment and human health (Jackson et al. 2009; Stehle and Schulz 2015; Kazimierczak et al. 2016). Moreover, in a context of climate change, rising resource scarceness and rapid global population growth, production systems based on non-renewable resources will not be sustainable in the long-term (Gomiero 2018; Pretty et al. 2018). Hence, the transition to a more sustainable agriculture should be a priority (Wanger et al. 2020); and is in fact aligned with the 12th Sustainable Development Goal of the UN's 2030 Global Agenda.

Nevertheless, to maintain or even increase crop productivity by reducing the use of synthetic fertilizers and pesticides, is one of the major concerns and challenges for a realistic transition (Pretty et al. 2018). Therefore, the quest for new sustainable strategies and green inputs will be essential to ensure global food security in the future. In this sense, the potential of natural products as seaweed represent a great opportunity towards sustainable food systems (Pacheco et al. 2021).

Brown algae (Phaeophyceae) traditionally have been used as fertilizers in many coastal agrosystems due to their high concentrations in macro-elements as Ca, P and specially K (Hong et al. 2007; Bahcevandziev and Pereira 2021), showing a historical background as sustainable input. Their fertilizing benefits are well-documented, but the amount of seaweed needed for large-scale application could be a constraint. More recently, algal extracts are gaining attention as formulations with strong biostimulant properties, used at low concentrations but showing a huge effect on plant metabolism;

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stimulating plant growth, germination rate, root system development, improvement of nutrient use efficiency and abiotic stress tolerance (De Pascale et al. 2017; Pacheco et al. 2021).

Sargassum muticum, the Japanese wireweed, is a brown seaweed originally from the northwest Pacific region. Now spread worldwide, it is one of the most-studied invasive marine species (Fernández 2020). Due to the difficulties of its eradication by conventional methods, valorization of *S. muticum* biomass has been proposed to encourage control strategies as seasonal harvesting (Kraan 2008; Milledge et al. 2016). There have been many studies on the potential of *S. muticum* as a phytoremediation agent in wastewater systems (Rubin et al. 2005; López-García et al. 2012), as fertilizer (Sharma et al. 2012), as well as a source of food, feed, nutraceuticals, pharmaceuticals and cosmetic products (Milledge et al. 2016), but its biostimulating properties in plants are less studied. Until now, only raw liquid extracts were tested for this purpose (Silva et al. 2019; Flórez-Fernández et al. 2021).

Sargassum spp. contain a complex mixture of potentially bioactive components such as steroids, terpenoids, flavonoids, phytohormones and polysaccharides as alginate or fucoidan (Yende et al. 2014), with variable proportions depending on the species and season of harvest (Balboa et al. 2016). Thus, the physiological mechanisms triggered in crop plants are difficult to establish. Generally, experiments include whole extracts instead of individual components because of its synergistic action (Ertani et al. 2018; Drobek et al. 2019). Composition of these extracts is species-dependent, making it important to study the interspecific variability and applicability of such products. Biostimulant effects can vary among crop-plant species due to different response thresholds to the bioactive components. It is also known that high concentrations of algal extracts can lead to negative effects on plant growth (Colla et al. 2015). Hence, to analyze not only the response but also the composition of algal extracts, is an underpinning part for further development of biostimulant formulations. This topic has been less studied and will be discussed in this manuscript.

Although information about extraction technologies for agricultural purposes is not often available as companies keep it as industrial secrets (Di Filippo-Herrera et al. 2019), it is well-known that conventional algal extractions include solvents such as hexane, methanol or ethanol, in combination with water. They are used within a variable range of physicochemical conditions, including acidic digestion (Hernández-Herrera et al. 2014; Castellanos-Barriga et al. 2017), alkaline extraction (Hernández-Herrera et al. 2016; Di Filippo-Herrera et al. 2019), with (Sharma et al. 2012) or without pressure and high temperature (Silva et al. 2019). These methods are not in accordance with green or circular economy goals: they are expensive and generate waste by-products which are difficult to manage because of their volatility, flammability and toxicity (Cheng et al. 2021). Pure aqueous extracts, with neutral pH, can be obtained by techniques such as ultrasound-assisted extraction, subcritical-water extraction or pressing (Flórez-Fernández et al. 2021; Torres et al. 2021). To the best of our knowledge, extracts obtained by these alternative green extraction procedures were rarely studied for the development of biostimulant products.

The multistage processing of *S. muticum*, within a biorefinery model as proposed by Flórez-Fernández et al. (2021) is an adequate technique for an integral and zero-waste valorization. In this sense, our study could be incorporated as part or extension of this model, focusing on the valorization of the solid residue from already pressed algae, through autohydrolysis extraction (Fig. 1). Autohydrolysis is a subcritical water extraction technique, which occurs under high pressure and high temperature, causing a strong decrease in water polarity. It enables water to act as a non-polar solvent, without the problems of organic solvents (Cheng et al. 2021).

Liquid extracts are difficult to handle, to store and to conserve. Drying is an effective method to obtain a better manageable product with less volume and longer shelf-life. Convective air drying exposes the algae to a constant moderately high temperature up to 75 °C and can be completed after 100 min (Chenlo et al. 2018). Freeze drying or lyophilization is a slow process of sublimation of the liquid extracts over multiple days, until only solid material remains. Spray-drying is a technique which relies on the atomization of a liquid sample into a hot gas current, obtaining in a few hours a fine powder as final product. Nevertheless, no literature is available about the consequences of drying on biostimulant potential. Thus, our aim was to study these drying techniques and to compare its effect on the physicochemical characteristics of green *S. muticum* extracts in order to elucidate the relationship with biostimulant features.

Materials and methods

Raw materials

Sargassum muticum sampling was performed by hand in summer at Praia da Mourisca (location: 4.224176°N,—8.771932°W), NW of Spain, by Torres et al. (2021). The fresh seaweed was washed using tap water, and milled with an A320 chopper (Moulinex, Spain), until obtaining a particle size of 1.0 cm. Hermetic plastic bags were used to store the wet samples at − 18 °C in darkness until further use.

Nasturtium officinale (No) was used as a model plant of the Brassicaceae family for the biostimulation assays.

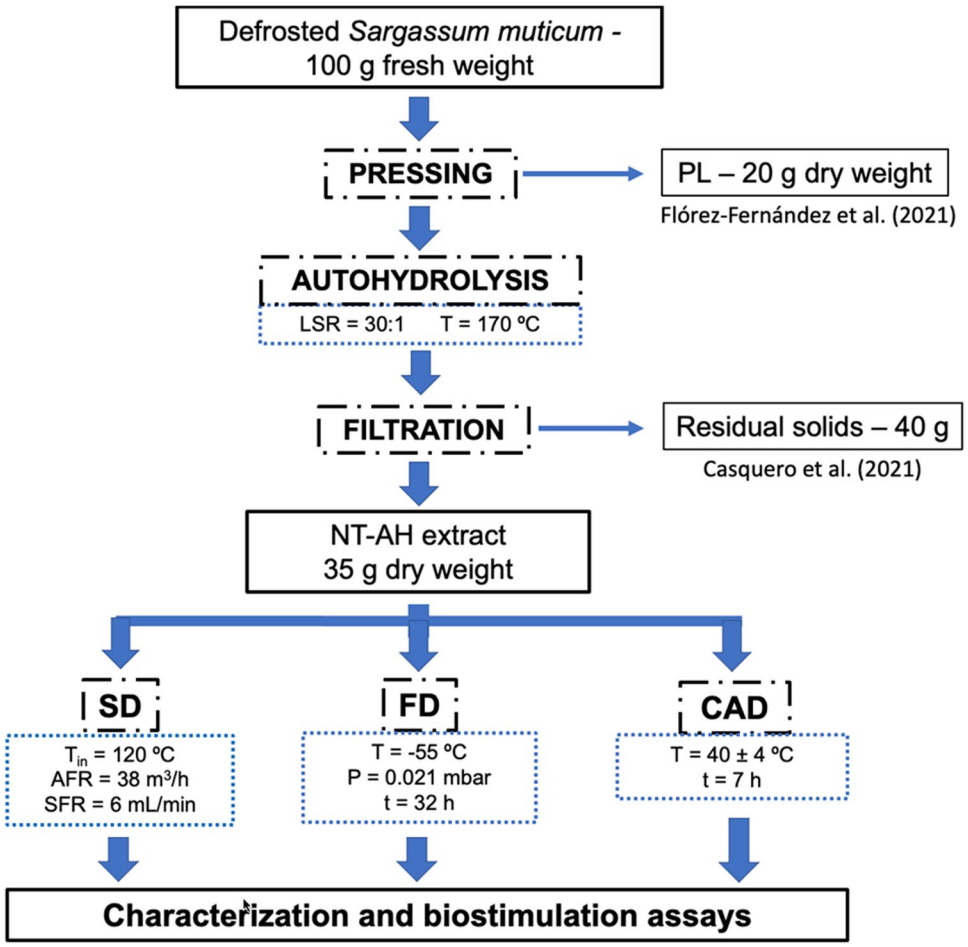

Fig. 1 Flow diagram of the processed raw material. PL: Pressed liquor (raw); LSR: Liquid–solid ratio; T: temperature (°C); NT-AH: Non-treated autohydrolysis extract; FD: Freeze-dried; SD: Spray-dried; CAD: Convective air dried; t: time (min); Tin: Inlet temperature (°C); AFR: air flow rate ($m^3\ h^{-1}$); SFR: feed solution flow rate ($mL\ min^{-1}$)

Extraction process

Algae were dried at room temperature (60 g), then mixed with water at a liquid:solid ratio of 30:1 (w/w). A pressurized reactor equipped with a stirred vessel of 3.7 L (Parr Instruments series 4848, USA) was used to heat the mixture to final temperatures in the range of 120–220 °C. After cooling, liquid and solid phases were separated through filtration. Before further analysis, liquid extracts were filtered through 0.45 µm membranes.

Dehydration processing

Three drying treatments were tried for the soluble extracts obtained after hydrothermal treatment of *S. muticum* to determine their influence on the biostimulant potential. Conventional dehydration was performed on a convective air oven (P-Selecta, Barcelona, Spain) at 40 °C. The corresponding dried samples were labelled as CAD. Freeze-drying was conducted on a freeze-dryer (Alpha 2–5 LD plus, Martin Christ, Germany) operating at -55 °C and 0.021 mbar, being the dehydrated samples labelled as FD. Spray-drying was carried out using a spray-dryer (BÜCHI B-290, Switzerland) equipped with a standard cyclone (1.5 mm nozzle). The operating conditions were optimized trying different settings following preliminary works reported in the recent literature (Baltrusch et al. 2022). The inlet temperature (T_{in}) was set at 120 °C, with a 6 mL min^{-1} feed solution flow rate and a 38 $m^3\ h^{-1}$ (100%) air flow rate. The outlet temperature was around 50 ± 1 °C. The dried samples were labelled as SD. For all dehydration treatments, the dehydration kinetics were monitored until constant weight. In all cases, dehydrated samples were placed into a desiccator containing silica gel for 24 h to cool down at room temperature, homogenizing their moisture content. Then, the samples were stored in dark airtight plastic bags at room temperature until further measurements. All the experimental measurements were performed at least in triplicate.

The dehydration kinetics were assessed as moisture ratio (MR), in terms of $(M_t\text{-}M_e)/(M_0\text{-}M_e)$, *vs* time. M_e and M_0 represent the equilibrium and initial moisture content (kg water kg^{-1} dry basis, d.b.), whereas M_t is the moisture content at any drying time (Moreira et al. 2013). The monitored drying kinetics were fitted by means of the two-parameters Page model (Page 1949), $MR = e^{-kt^n}$, with n and k being model parameters.

An estimation of the energy consumption for the three studied drying treatments was determined as the multiplication of the necessary drying time and power (López-Hortas et al. 2018).

Liquid extracts physicochemical characterization

Total phenolic content, antioxidant capacity, carbohydrates content, sulfates, proteins, phytohormones, fatty acids, yield, conductivity, and pH were measured for the liquid phase obtained by autohydrolysis, pH of the liquid samples was measured under constant stirring and at room temperature using a Crison GLP-21 (Spain). Conductivity was measured in a HI 8633 Hanna electroconductivity meter (Spain). Further measurements were performed as explained bellow.

Mineral characterization

Some of our elemental and mineral data of *S. muticum* are based on Flórez-Fernández et al. (2021).

Macroelements were determined by atomic emission spectrophotometry (Na, K and P) and atomic absorption spectrophotometry (Ca, Mg) through a 220 Fast Sequential Spectrophotometer (Varian, USA). In order to perform these measurements, samples were previously subjected to a microwave assisted digestion (Marsxpress, CEM) of ash (0.3 g) with H_2O_2 (1 mL) and 8 mL of HNO_3. Operating conditions were set at 1600 W for 15 min, maintaining a static end point temperature of 200 °C for 15 min.

An elemental analyzer (Thermo Flash EA 1112, Germany) was used to determine the C content. Operation conditions were set at 130 mL min^{-1} He gas flow, 100 mL min^{-1} gas flow reference, 250 mL min^{-1} oxygen flow, at an oxidation furnace temperature of 900 °C and a reduction furnace temperature of 680 °C. A 2.0 m, 6 × 5 mm multiple analysis column (Cromlab, Spain) was used, with temperature set at 50 °C and a chromatogram time of 420 s. Aspartic acid (Sigma, USA) was used as reference.

The Kjeldahl method was used to measure total N, results were converted to protein using the specific factor for brown seaweeds 5.38 ± 0.50 (Lourenço et al. 2002).

Analysis of phenolic compounds through phloroglucinol quantification

Phlorotannins (oligomers of phloroglucinol) were measured through a spectrophotometric method as performed by Koivikko et al. (2005). It is based on the Folin-Ciocalteu method (Singleton and Rossi 1965) with slight modifications. Firstly, a standard curve was set using 0.030 to 0.005 g L^{-1} of phloroglucinol. Afterwards, samples were mixed with 1 mL of Folin-Ciocalteu reagent, followed by 2 mL of a 20% (w/v) sodium carbonate solution. The mixture was then stirred in a vortex left in the dark during 45 min. Subsequently, absorbance was measured at λ = 730 nm. All measurements were performed at least in triplicate.

Trolox equivalent antioxidant capacity

Antioxidant capacity of seaweed extracts was measured by the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Re et al 1999). Liquid extract samples (10 µL) were placed in a test tube, followed by 1 mL of ABTS solution (absorbance 0.7 ± 0.1) and then incubated for 6 min at 30 °C in a water bath. Then, absorbance was measured at 734 nm and antioxidant capacity was calculated using a standard curve performed with Trolox (Sigma-Aldrich, Denmark). Measurements were performed at least in triplicate.

Carbohydrates and other derived groups content

High-Performance Liquid Chromatography (HPLC) was used to elucidate carbohydrate and other derived groups content. Oligosaccharide content was determined after dialysis of the studied extracts (Spectra/Por Float-A-Lyzer G2 Dialysis Membrane Tubing, MWCO 0.5 kDa, SpectrumLabs, USA), followed by a post-hydrolysis, performed with 4% H_2SO_4 (v/v), at 121 °C for 20 min. All samples were finally filtered through a 0.45 µm syringe filter, and then measured on a chromatograph. An Aminex HPX-87H column (300 × 7.8 mm, BioRad, USA) was used, operating at 60 °C, with a mobile phase of 0.003 M H_2SO_4 (w/w) at 0.6 mL min^{-1}. The HPLC equipment had a refractive index detector.

Soluble protein content

Soluble protein content of the obtained extracts was measured through the Bradford assay (Bradford 1976) with 1 to 10 µg mL^{-1} of bovine serum album (BSA, Sigma Aldrich, China) used for the standard curve. 0.5 mL of each sample was mixed in a test tube with 0.5 mL of Bradford Reagent (Sigma Aldrich, Germany). Afterwards, samples were incubated during 10 min and finally measured at 595 nm in a UV–vis spectrophotometer (Thermo Scientific Evolution 201, USA).

Soluble sulphate content

The gelatin-barium chloride method (Dodgson 1961) was used to determine the soluble sulphate content. Gelatin-$BaCl_2$ reagent was prepared as it follows: 0.5 g gelatin powder (Scharlau, Spain) was dissolved in 100 mL hot water (70 °C) and kept at 4 °C overnight. Afterwards, 0.5 g of $BaCl_2$ (Sigma-Aldrich, USA) was added and stirred until a cloudy solution was obtained. After 2–3 h, the reagent

was ready to use. 0.1 mL of extract samples or blank were mixed with 1.9 mL trichloroacetic acid solution (4%, v/v) and 0.5 mL of the mentioned reagent. The suspensions were mixed and then incubated at room temperature for 15 min. Absorbance was read at 500 nm.

Scanning electron microscope and particle size distribution

The morphology of the dried extracts was analyzed using a scanning electron microscopy (JEOL JSM6010LA, Japan) and the images were obtained at different magnifications. Samples were covered by a gold layer of 15 nm. Average particle size was calculated. SEM micrographs were analyzed using ImageJ software to obtain average particle diameters. Around 200 measurements were performed manually for each of the analyzed samples. In order to obtain the particle size distribution (PSD), results were depicted as frequency distributions of different ranges of diameter and adjusted to a Lorentzian curve using Prism GraphPad 6.0.

Nasturtium officinale seed germination and seedling growth bioassays

Experiments were performed under in vitro conditions. Germination and different growth parameters were assessed in order to evaluate the influence on the biostimulant effect of three drying techniques (FD: Freeze drying, SD: spray-drying, ACD: air convective drying) for the liquid extracts of *S. muticum* obtained by autohydrolysis. All assays were made by triplicate.

Four dilutions were prepared for each of the obtained extracts (PL, SD, CAD, FD), at different concentrations: 5 g L^{-1}; 0.5 g L^{-1}; 0.05 g L^{-1}; 0.005 g L^{-1} using distilled water as control.

Various Petri-dishes with perlite (beneath) and filter paper (above) were prepared for each treatment. Filter paper was then moistened with 50 mL of each extract. In each plate, 10 seeds were then sown and incubated at an angle of 70 to 80°. The germination percentage over the control was determined according to the European standard EN 16,086–1. The controlled environment growth cultures were incubated at 25 ± 5 °C in the dark for 72 h. Afterwards, different growth parameters were assessed:

GD Germination degree (%). Seeds were considered germinated once the radicle protruded more than 2 mm
RL average root length (mm)
SL average shoot length (mm)
R/S root:shoot ratio
RI Root Index (root development in relation to the control)
MLV Munoo-Liisa Vitality Index was used to compare the product of the germination degree by the average root length in the samples, with the control. This index was calculated according to Eq. (1).

$$MLV\ (\%) = \frac{GDs \times RLs}{GDc \times RLc} \times 100 \quad (1)$$

where GD_s = germination degree of each replica of a treatment, GD_c = average germination degree of the control, RL_s = average length of each replica of a treatment, and RL_c = average length of the three replicates of the control treatment.

Statistical analysis

Statistical analysis and graphics were performed using the GraphPad Prism 6.0 program. Significant differences between means were calculated through one-way or two-way ANOVA tests, and with a p value < 0.05.

Results

Schematic procedure

Figure 1 shows an overview of the extraction and further treatments. Similar autohydrolysis treatments have been already performed by González-López et al. (2012). Different drying procedures were analyzed to assess its influence on the potential use of the *S. muticum* AH extract as biostimulant (Fig. 2). It was observed that the drying kinetics for freeze drying treatment were 3.5 × longer than those obtained using conventional air-drying methodology. The lowest processing time was identified for spray-drying of *S. muticum* liquors (about 4 h).

Chemical characterization of the liquid extracts

Fundamental chemical characterization of the extracts and statistical analysis are summarized in Table 1. Means with no letter in common are significantly different (one-way ANOVA; α = 0.05). Data shown in the first row (pressed raw liquor, PL) contain results reported in previous studies (Pérez-Larrán et al. 2020; Flórez-Fernández et al. 2021). Sulfate content of spray-dried (SD) extracts was similar to non-treated (NT) extracts, while freeze-drying (FD) and convective-air-drying (CAD) treatments showed slight but significant decrease in sulfate content. Proteins showed a

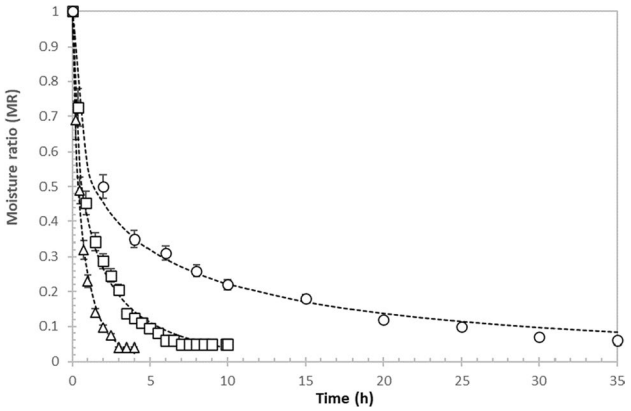

Fig. 2 Drying kinetics of tested extracts: (a) CAD: Convective air drying (squares); (b) FD: Freeze-drying (triangles) and (c) SD: Spray-drying (circles). Lines correspond to the Page model. Error bars smaller than symbols sizes

significant decrease in CAD treatment, compared to NT extract, but did not have huge variations.

Phloroglucinol content and TEAC values varied among different treatments similarly. PL had the lowest values; AH extracts showed higher levels of phloroglucinol and antioxidant capacity. When comparing the different drying treatments, SD appeared to conserve better antioxidant properties and phloroglucinol content. FD and specially CAD procedure caused a significant decrease of TEAC values and phloroglucinol content when compared to NT.

Macro-elements presented some variations among treatments. SD samples conserved significantly higher carbon, nitrogen and phosphorous content. CAD extracts showed significantly higher levels of sodium, magnesium and potassium.

Oligosaccharides showed small differences between the different drying techniques but slight (and not significantly) lower values, when compared to the NT extract. SD extracts conserved better a high oligosaccharide content. When compared to the PL, AH extracts had significantly lower O-Glucose and O-Gal + Xyl + Man content, except for O-Fucose content, which is significantly higher.

Electric conductivity (EC) and pH of the extracts used in the biostimulation assay were measured (Table 2). pH was generally higher in CAD samples, and lowest in NT and FD extracts. A decrease in pH was observed at higher extract concentrations (except in case of CAD extracts). EC increased strongly with increasing concentration and showed some differences among drying techniques. EC in the 100% (5 g L^{-1}) extracts was very high in all cases. Lowest EC values were obtained in SD extracts and highest in FD samples.

Table 1 Mineral and bioactive composition of the extracts (%, w/w) depending on different treatments

	Parameters	PL	AH-NT	AH-FD	AH-SD	AH-CAD
	Sulfate content (g (100 g)$^{-1}$)	–	3.54 ± 0.03a	3.38 ± 0.02b	3.51 ± 0.04a	3.19 ± 0.05c
	Protein content (g (100 g)$^{-1}$)	4.63*	4.75 ± 0.09a	4.68 ± 0.10ab	4.69 ± 0.08ab	4.49 ± 0.07b
	Phloroglucinol content (g (100 g)$^{-1}$)	2.47**	4.52 ± 0.06a	4.22 ± 0.05b	4.34 ± 0.07b	3.99 ± 0.08c
	TEAC (g Trolox (100 g)$^{-1}$)	0.75**	7.68 ± 0.05a	7.53 ± 0.07b	7.60 ± 0.02ab	7.36 ± 0.05c
Macroelements (%)	Carbon	18.90*	17.16 ± 0.32c	16.74 ± 0.06b	19.20 ± 0.08d	16.04 ± 0.20a
	N:P_2O_5:K_2O ratio	–	1.1: 1: 17	1: 1: 20.6	1.1: 1: 17.7	1: 1: 24
	Nitrogen	0.86*	0.98 ± 0.07b	0.78 ± 0.03a	1.02 ± 0.02b	0.75 ± 0.03a
	Phosphorous	–	0.39 ± 0.00b	0.33 ± 0.01a	0.39 ± 0.02b	0.32 ± 0.01a
	Potassium	11.65*	12.56 ± 0.11a	12.99 ± 0.41ab	13.30 ± 0.24b	14.59 ± 0.30c
	Calcium	0.57*	0.24 ± 0.05a	0.18 ± 0.01a	0.24 ± 0.07a	0.23 ± 0.05a
	Magnesium	1.37*	1.58 ± 0.02a	1.6 ± 0.02a	1.69 ± 0.06b	1.8 ± 0.04c
	Sodium	5.63*	5.66 ± 0.09a	5.55 ± 0.17a	5.75 ± 0.25a	6.57 ± 0.27b
Oligosaccharides and associated groups (%)	Total	33.15 ± 3.44a**	32.9 ± 3.7a	29.38 ± 2.08a	31.3 ± 2.7a	27.36 ± 2.10a
	O-Glucose	14.69 ± 0.61a**	2.12 ± 0.10b	2.01 ± 0.05b	2.06 ± 0.04b	1.78 ± 0.05b
	O-Fucose	4.29 ± 0.65a**	15.42 ± 0.21b	14.95 ± 0.10bc	15.33 ± 0.03b	14.31 ± 0.13c
	O-Gal + Xyl + Man	12.96 ± 1.49a**	7.31 ± 0.15b	6.56 ± 0.02c	7.01 ± 0.05c	6.04 ± 0.06bc
	Formic groups	–	3.21 ± 0.20a	2.79 ± 0.12bc	2.99 ± 0.16ab	2.45 ± 0.11c
	Acetyl groups	1.21 ± 0.69a**	1.32 ± 0.05a	0.98 ± 0.06a	1.02 ± 0.03a	0.95 ± 0.04a

PL raw pressed liquor, *AH* autohydrolysis extraction, *NT* non treated, *FD* freeze-dried, *SD* spray-dried, *CAD* convective air dried. Means with at least one common letter are not significantly different

* Data from Flórez-Fernández et al. (2021). ** Data fromPérez-Larrán et al. (2020)

Table 2 Electric conductivity (EC) and pH of the tested extracts, at different concentrations

	Concentration (%)	AH-NT	AH-FD	AH-SD	AH-CAD	σ
pH	0	6.1	6.1	6.1	6.1	0,00
	0.1	6.96	6.64	6.76	6.84	0.13
	1	6.38	6.25	6.29	6.35	0.06
	10	5.63	5.82	5.87	6.35	0.31
	100	5.2	5.6	5.77	6.63	0.60
EC (μS cm^{-1})	0	0.05	0.05	0.05	0.05	0.00
	0.1	12.64	22.57	6.96	10.62	6.68
	1	51.67	50.6	44	55.33	4.72
	10	501.7	480.7	446.7	500.7	25.72
	100	4730	5943	4790	5353	566.98

AH autohydrolysis extraction treatment, *NT* non treated, *FD* freeze-dryed, *SD* spray-dryed, *CAD* convective air dryed, *σ* standard deviations

In all cases standard deviations were lower than 5%

Morphological characterization of dried liquid extracts

Morphological characterization of the differently dried extracts is depicted in Fig. 3. Figure 3a–e shows the SEM results and Fig. 3f the particle size distribution of the spray-dried (SD) extracts.

SD extracts showed morphological differences within different operational settings. Particles in SD120 (T_{in} = 120 ºC, T_{out} = 50 ºC, feed rate = 20%) had an average diameter of 1.83 ± 0.62 μm, which was significantly smaller compared to SD150 (T_{in} = 150 ºC, T_{out} = 80 ºC, feed rate = 40%), with an average diameter of 2.13 ± 0.66 μm, when analyzed by unpaired t-test ($p < 0.05$).

Freeze dried (FD) extracts showed a more laminar structure, when compared to the granular appearance of SD samples. However, all the dried samples showed primarily an amorphous state. Additionally, crystal formation was observed in CAD, with T = 40 ºC, and SD120, which had an outlet temperature (T_{out}) of 50 ºC.

Biostimulant effects in *Nasturtium officinale*

The use of the different extracts had low influence on the germination rate (GR; Fig. 4a), this occurred due to the already high GR in the control treatment. Thus, only a decrease in the GR could be measured. A significant decrease of GR was observed in NT extracts at a concentration of 5 mg L^{-1}.

Munoo-Liisa vitality index showed greater variations (Fig. 4b). This assay evaluates root length and germination jointly, in relation to the control. FD treatment achieved the best results at a concentration of 1%, with a significantly higher index value.

Results showed slight increases in root and shoot growth when compared to the control in some cases, but differences were not statistically significant (Fig. 4c–d). However, shoot length tends to increase in all cases as the extract concentration increased, without finding a growth inhibitory effect even applying the highest dose. The treatments that have not been subjected to high temperatures (NT and FD) showed a similar evolution, with similar lengths. On the other hand, the treatments with a thermal drying process (CAD and SD) have higher growth values with a 100% (5 g L^{-1}) extract concentration.

Seeds germinated with the NT extracts showed a higher root:shoot ratio (Fig. 4e). In all cases this parameter decreased when the extract concentration increased, due to the higher growth observed in the aerial part.

Significant increases in root index values (root development in relation to the control; Fig. 4f) were obtained, at the concentration of 0.1% (5 mg L^{-1}) in SD and 1% (50 mg L^{-1}) in case of FD extracts, with an increase in root length of 17 and 24%, respectively. NT liquor needed a much higher dose (100%, 5 g L^{-1}) to show significant differences over the control, with a 17% increase in root length. The highest dose (100%, 5 g L^{-1}) of FD extract produced a phytotoxic effect leading to a significant reduction in root index value.

The drying process of the AH liquor seems to affect its efficiency as a biostimulant, being the SD extracts the most effective at various concentration, achieving the greatest increase in seed development using low doses of extract.

Discussion

Autohydrolysis and biostimulant products

The hydrothermal procedure of this study was performed following the optimized method of Flórez-Fernández et al. (2019) at 170 °C. The mentioned study already analyzed

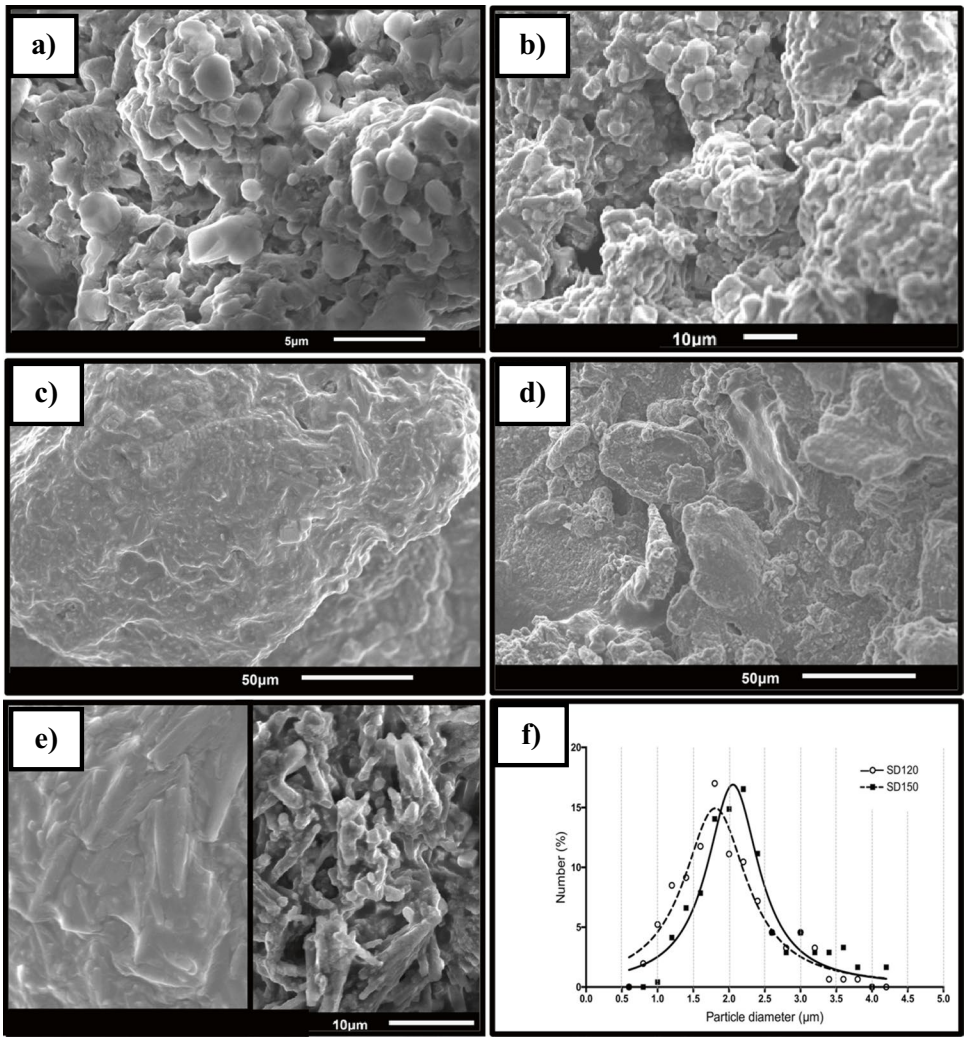

Fig. 3 Morphological characterization of extracts. **a**–**e**): Scanning electron microscopy micrographs showing *Sargassum muticum* extracts dried through different techniques: **a**) spray-drying at T_{in} = 120 °C and feed rate = 20% (SD120), **b**) spray-drying at T_{in} = 150 °C and feed rate = 40% (SD150), **c**) oven-drying and **d**) freeze-drying. **e**) Details of the crystals formed in spray-dried extract at T_{in} = 150 °C (left) and oven-dried extract (right). **g**) Particle size distribution of SD120 and SD150. Scale bars represent a) 5 µm, b) 10 µm, c) 50 µm, d) 50 µm, e) 10 µm

the biostimulant effect of the raw pressed liquor (sap), and solid residues of the autohydrolysis (AH) treatment were valorized by the incorporation to a seed coating mixture patented by Casquero Luelmo et al. (2021). Apart from this, no studies about the use of AH extracts for the development of biostimulant products were found in literature.

Physicochemical properties and biostimulant potential of the extracts

Until now, a deep analysis and valorization of the AH liquid extracts, from already pressed *S. muticum* solids, as potential plant biostimulants, has not been contemplated as in our study. In this paper we analyzed different liquor drying techniques and its impact on physicochemical properties, especially on the biostimulant potential of these extracts.

Phloroglucinol and TEAC values were notably higher in AH-extracts compared to the pressed liquor, probably due to a disruption of the algae cells and higher solubility of the cellular membrane caused by the increment of temperature, with the consequent release of these bioactive components. Phloroglucinol content and TEAC values significantly decreased with longer and/or more intense stress exposure during the drying process, phenolic compounds could undergo polymerization or oxidation (Leandro et al. 2020). In this sense, SD was the best technique to conserve these characteristics.

Seaweeds are rich in minerals. Due to their cell wall polysaccharides with negative surface charge, they easily retain cations or positively charged molecules from seawater (Alba and Kontogiorgos 2018). This, along with the natural presence of N, P and specially K, ensures a nutrient-rich composition which confers a potential value as fertilizer in agriculture (Silva et al. 2019), particularly in fruit crops which are very K demanding (Wang et al. 2022). High micronutrient content in seaweeds has also been related to an enhanced reactive oxygen scavenging response (i.e., oxidative stress tolerance) in plants, improving the response to environmental stress events (Bradáčová et al. 2016). The high content of arsenic in *S. muticum* has been pointed out by authors such

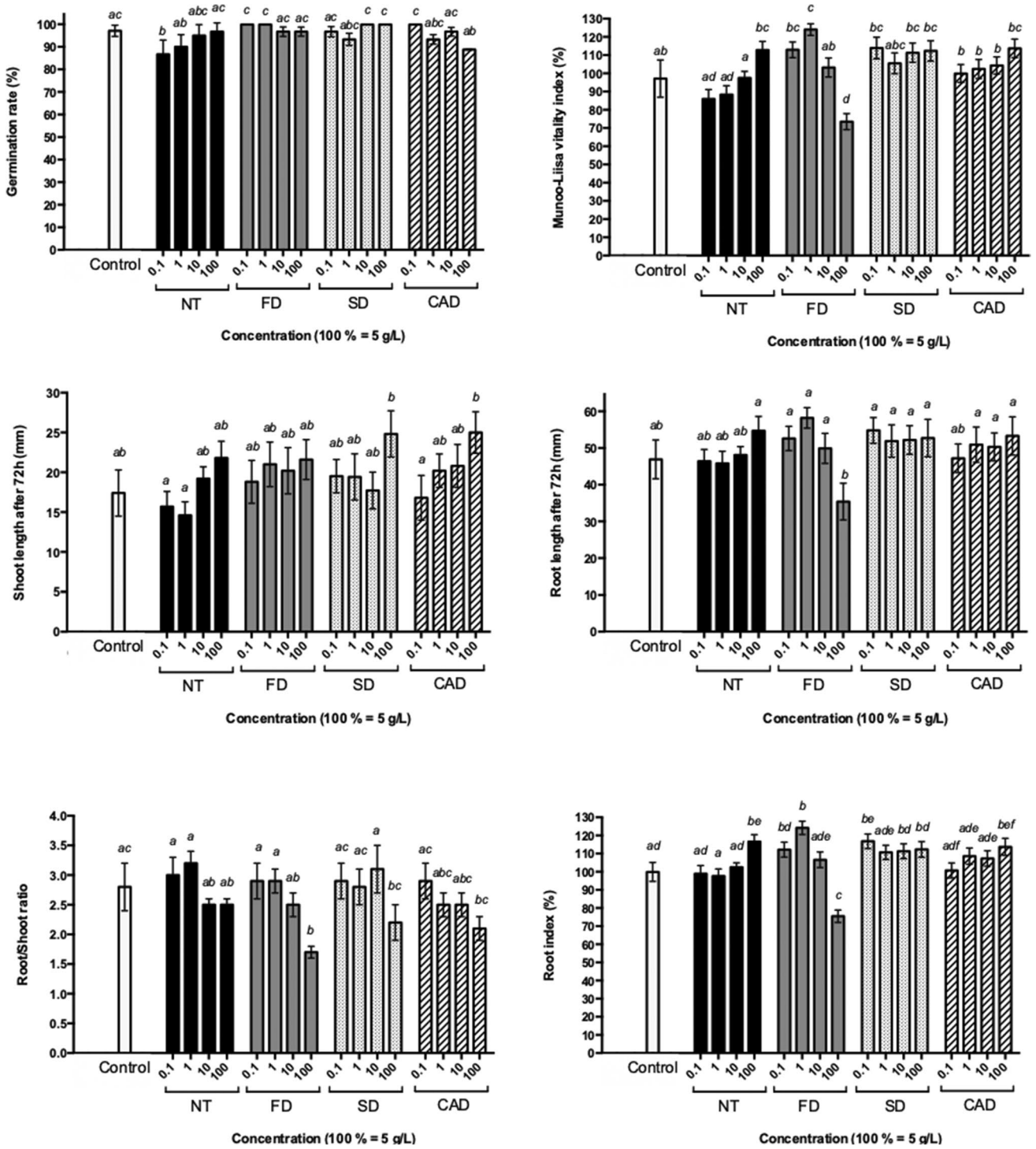

Fig. 4 Growth evaluation of *Nastrium officinale* after addition of different liquid extracts of *Sargassum muticum*. NT: Non-treated liquor, FD: Freeze-dried, SD: Spray-dried, CAD: Convective air dried. Control: No extract. Means with no letter in common are significantly different (two-way ANOVA; $\alpha = 0.05$; $n = 3$). Standard deviations are represented as error bars

as Devault et al. (2022). *Sargassum muticum* collected in our region (Galicia, NW of Spain) showed moderate to low levels of arsenic, ranging from 7.4 to 35.8 ppm (Balboa et al. 2016). EU Fertilizer Regulation (EC) No 2019/1009 sets the limit of arsenic concentration in organic biostimulants at 40 ppm. In our study, *S. muticum* extracts were used at maximum of 5 g L^{-1}. Even if we consider the complete transfer of the arsenic contained in the algae to the liquid extract, our

biostimulant product would have an arsenic concentration far below the European limit. In case of the oligosaccharide content, our results show that, in general, higher values correspond to lower stress exposure (i.e., less temperature and/or drying times).

In the context of an integral biorefinery system approach, it is notable that after AH extraction of an already pressed seaweed material, high mineral, oligosaccharide and phloroglucinol contents were still achieved when compared to similar studies with no previous pressing and same AH procedure (Álvarez-Viñas et al. 2019) or different extraction techniques (Flórez-Fernández et al. 2017). Thus, industry could benefit not only from further extractions steps as noted in this study, but also drying techniques which allow the conservation of sensible properties such as antioxidant capacity and phenolic content, increasing the shelf-life of the whole product.

Neutral or slightly acidic pH about 6.5 is preferable for biostimulants to increase plasma membrane permeability. However, the effective range can be up to pH 8.5 (Arthur et al. 2013). An acid medium can be detrimental for optimal root development, low soil pH favors the uptake of anions, inhibiting the uptake of other ions. EC of irrigation water is a limiting factor for seed germination and plays a crucial role in seed establishment and development (Khajeh-Hosseini et al. 2003). However, brown and green algal extracts contain several betaines and betaine-like compounds (Blunden et al. 1992) which can alleviate salinity-induced osmotic stress (Khan et al. 2009). This could help explain the high germination rates observed despite very high EC, compensating the high salinity of more concentrated extracts.

SEM and particle size analysis

Morphological differences among SD extracts could be explained due to a 20% lower feed rate in SD120, which caused a better pulverization and dispersion of the droplets compared to SD150, resulting different particle diameters. Thus, it is possible to increase the feed rate of the spray-drying process with higher temperatures (due to faster drying), but it may cause higher particle sizes, as in case of SD150.

Mainly amorphous states were observed within the dried extracts, with occasional crystal formation (Fig. 3). The transition from an amorphous to a crystalline state is determined by the glass transition temperature (T_g) which depends on moisture content and chemical composition, in this case, specifically on the assemblage of different polymer chain lengths (Shrivastava 2018). Thus, our results can be explained by the high carbohydrate content in the extracts, which influences the sticky nature of the dried powders due to its low T_g and hygroscopic nature. In addition, low residence times of the spray and vacuum drying techniques contribute to the observed caking phenomenon. Moisture content of the powders was found to be very low immediately after drying (Fig. 2), but fast rehydration can happen easily in contact with air humidity. Water acts as a plasticizer, decreasing drastically the viscosity and the T_g of the matrix by increasing the moisture content and water activity. This causes, again, interparticle fusion and stickiness, which can already happen at room temperature if a moisture content of 5–10% is achieved (Jaya et al. 2013). Stickiness could be a constraint for some applications but also an advantage regarding to potential uses as biostimulant on plant material (e.g., foliar spray).

Additionally, ensuring an amorphous molecular state of the matrix (*i.e.,* avoid crystallization) is important to guarantee product stability (e.g., inhibition of enzymatic reactions, protective effect on proteins, lipid encapsulation). Thus, the T_g of the studied system is an important factor to consider. Nevertheless, the heterogeneous nature of biomass materials hinders the definition of a direct correlation between a general physical feature, such as T_g, and the chemical reaction kinetics (Buera et al. 2005). For that reason, local heterogeneities probably allowed the formation of occasional crystals in SD120 and more commonly in CAD (probably due to longer drying time). It is assumed that FD and SD150 samples are less modified and more stable.

Biostimulant potential

Pressed raw liquor of *S. muticum* is obtained at very low yields of about 0.1 L kg^{-1}. Additionally, sap only contains 8.5% of dry weight. Thus, using this material as fertilizer, for instance in potato cultivation, would demand 800 kg of *S. muticum* (Xu et al. 2022) to cover the K needs to harvest a tonne of potatoes. In contrast, biostimulants require very low dosages, turning seaweed extracts more suitable for this kind of agricultural use.

Several studies have demonstrated biostimulant effects of seaweeds, as increasing germination percentage, root length and root:shoot ratio (Sivritepe and Özkan Sivritepe 2008; Khan et al. 2009), there are also studies conducted with biostimulants specifically prepared with *Sargassum* sp. (Di Filippo-Herrera et al. 2019; Noli et al. 2021; Pacheco et al. 2021). In contrast, there are also studies pointing out negative effects as reduced germination.

Negative effects on root development could be due to an excess of the algal biostimulant substances, as these promote growth at low concentrations, but can show inhibitory effects at high concentrations (Battacharyya et al. 2015).

The temperature reached and the time employed in the drying process may influence the loss of growth hormones or elicitor compounds, which trigger hormone-like responses. Auxins and cytokinins are involved in root development and their balance is of special importance. The different thermo-sensitivity of both hormones and

elicitor compounds can have a direct influence on root development (Stirk et al. 2004).

The presence of several growth-promoting phytohormones in seaweeds is well-documented (Battacharyya et al. 2015; du Jardin 2015). However, its mechanism of action is still under debate. Some studies showed that seaweed extracts elicit endogenous hormone-like activity (Khan et al. 2011), downplaying the importance of the effect of exogenous phytohormones. Further, the increased phytohormone-like activity has been reported to occur due to upregulation of biosynthetic genes (Rayirath et al. 2009; Wally et al. 2013). Thus, the biostimulant effect should be also evaluated attending to the biostimulant-derived organic compounds, which interact with receptors of the cell membrane triggering a signal-transduction pathway, rather than quantifying phytohormones, which may be below the threshold of action.

Higher root:stem ratios allow plants to be more effective in extracting nutrients from deeper soil layers and influence the maturity of the crop as a whole (Hernández-Herrera et al. 2014).

In conclusion, low stress drying of *Sargassum muticum* autohydrolysis extracts allowed a better conservation of the plant growth biostimulant properties. These extracts were non phytotoxic in assays with *Nasturtium officinale* (Watercress) and significant increases in root development at a concentration of 5 mg L^{-1} of spray-dried extracts and 50 mg L^{-1} in case of freeze-dried extracts. Optimal Munoo-Liisa vitality index were found with 50 mg L^{-1} of freeze-dried extracts.

Authors' contributions K.B., N.F.F., M.I. and M.D.T. performed the experimental tasks, K.B., M.I. and M.D.T. wrote a draft of the manuscript; N.F.F., M.D.T., M.E.L.M. and H.D. followed the experimental tasks, analyzed the results and corrected the manuscript. All authors reviewed the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests Authors declare there is no conflict of interest.

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