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Effect of several commercial seaweed extracts in the mitigation of iron chlorosis of tomato plants (*Solanum lycopersicum* L.)

Sandra Carrasco-Gil[1] · **Lourdes Hernandez-Apaolaza**[1] · **Juan José Lucena**[1]

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Abstract
Commercial seaweed extracts (SWEs) have been applied in agriculture for ameliorating biotic and abiotic stress in plants. However, the mechanisms of action of these extracts are only partially known. Most of the research work with SWEs has focused on abiotic stresses such as drought, salinity or high temperatures, but little is known about SWE effects on plants with nutrient imbalances. Therefore, the goal of this study was to investigate the effects of several commercial SWEs (based on *Ascophyllum nodosum* and *Durvillea potatorum*) in the mitigation of iron chlorosis of stressed tomato plants. Tomato plants were grown in a hydroponic system initially with Fe, and then Fe was removed from the nutrient solution. SWEs were applied twice, first during the growth period (+Fe) and second at the beginning of Fe deficiency (−Fe), following the recommended doses of manufacturers. Some of SWE treatments activated the antioxidant system in Fe-deficient tomato plants increasing SOD and CAT activity. However, SWEs application did not produce positive effects on biomass, chlorophyll content, activation of Fe acquisition strategies and Fe uptake with respect to the untreated control.

Keywords Seaweed extract · Tomato · Iron-deficiency · Antioxidant activity

Introduction

Seaweed extracts (SWEs) are widely used in agriculture for their beneficial effects on plant growth and tolerance enhancement to biotic and abiotic stresses (Sangha et al. 2014; Arioli et al. 2015; Battacharyya et al. 2015). While most of the beneficial effects of SWE application are described for abiotic stresses such as salinity, extreme temperatures and drought (Ibrahim et al. 2014; Mancuso et al. 2006; Shukla et al. 2018), little is known about the effect of SWEs on nutrient deficiencies. However, several studies revealed that SWE application may stimulate nutrient uptake and translocation in plants. Application of commercial SWE products by foliar spray, increased N, P, K, Ca, Zn and Fe concentration in tomato fruits (Dobromilska et al. 2008), Zn in grapevine leaves (Sabir et al. 2014), and K in almond leaves (Saa et al. 2015) in comparison to untreated plants. Also SWE applications in the nutrient solution stimulated N and S uptake in root and shoot of rapeseed (Jannin et al. 2013).

Brown seaweeds (Phaeophyceae) are the most commonly raw material used for commercial manufacture of extracts for applications in agriculture due to their abundance and distribution. Amongst the brown seaweeds, *Ascophyllum nodosum, Ecklonia maxima, Macrocystis pyrifera* and *Durvillea potatorum* are the most frequently commercially used by industries (Khan et al. 2009). Commercial SWE are available as liquid extracts or in a soluble powder form and may be applied near the root of the plant such as fertigation or by foliar spray in leaves. Commercial SWEs are not homogeneous products since their composition depend on the type of seaweed used, the season of harvest, the location and the extraction process in the manufacturing (Khan et al. 2009; Connan et al. 2004; Rayirath et al. 2009). Commercial biostimulant manufactured from similar sources are usually marketed as equivalent products, but may differ considerably in composition and thereby in efficiency (Lötze and Hoffman 2015). Many manufacturers do not reveal the technology of biostimulant production and formulation. Its different physicochemical composition may influence on the biologic activity of these extracts.

✉ Sandra Carrasco-Gil
sandra.carrasco@uam.es

[1] Department of Agricultural Chemistry and Food Science, Universidad Autónoma de Madrid, Av. Francisco Tomás y Valiente 7, 28049 Madrid, Spain

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The promoting effects on root growth stimulation, improved leaf development, as well as enhanced flowering and fruit set have been ascribed to the many types of biologically active molecules of plant growth regulators present in seaweeds. These metabolites comprise auxin, cytokinins, gibberellins, abscisic acid and ethylene as well as more recently discovered brassinosteroids, jasmonates, salicylic acid and strigolactones (Stirk and van Staden 2014). However, the phytohormone-like activity of SWEs might also be caused by chemical components in the extract other than phytohormones themselves (Rayorath et al. 2008; Wally et al. 2013). Besides, the pH and temperature at which the extraction processes are performed, may affect the stability of biological active compounds of the extract. At present, the modes of action of active compounds are only partially known, but it is plausible that these components exhibit synergistic activity (Fornes et al. 2002; Vernieri et al. 2005). This fact together with the high variability that these products present in their composition, may hinder its acceptance within the agricultural market. Moreover, several studies on the chemical composition of a variety of seaweed extracts showed that the plant nutrients content (usually macronutrients including N, P, K but also micronutrients) was insufficient to elicit physiological responses in plants at the concentrations that the SWEs were applied in the field (Blunden 1971, 1991; Khan et al. 2009). Nutrient additions of chelated trace elements, as well as the macro elements N, P and K to the raw SWE are often used (Verkleij 1992; Craigie 2011).

Iron (Fe) is an essential nutrient for plant development and it is involved in chlorophyll (Chl) synthesis, photosynthetic electron transport processes and protection against radical oxygen species (ROS) production (Morales et al. 1998; Broadley et al. 2012). A low Fe availability, especially in calcareous soils with alkaline pH, results in a reduction of plant productivity and quality (Marschner 1995). To cope with Fe deficiency, plants have developed two different strategies to facilitate the availability of Fe: strategy I (dicots and non-graminaceous plants) and strategy II (graminaceous plants) (Römheld and Marschner 1986). The first step in strategy I is the acidification of the rhizosphere. Subsequently, the root surface-localized iron chelate reductase (FCR) reduces Fe(III) to soluble Fe(II), which is then taken up into epidermal cells by the Fe-regulated transporter 1 (IRT1). In strategy II, plants release PhytoSiderophores (PS) by root, which would form stable Fe-PS chelates. These chelates are taken up by a plasma membrane-localized oligopeptide transporter, yellow-strip 1 (YS1) (Curie et al. 2001).

Adverse growing conditions such as Fe deficiency may be alleviated by the use of SWE products, enhancing the defense mechanism to reduce the oxidative stress and the chlorosis. The addition of SWEs may promote the root development and the photosynthesis, improving the nutrient uptake by FCR activation. Therefore, the goal of this study was to investigate the effects of several commercial SWEs in the mitigation of iron chlorosis of Fe deficient stressed tomato plants. These products were also evaluated with respect to the effectiveness in plant growth-promoting activity under Fe deficiency, as these products can be considered and used as biostimulant in agriculture.

Materials and methods

Commercial seaweed extracts characterization

Two commercially available liquid seaweed extracts (AN1 and AN2) of *A. nodosum* with an acid and basic pH respectively, one solid raw material of *A. nodosum* (AN3) used as ingredient for the manufacture of commercial extracts, and one commercially available liquid seaweed extracts of *D. potatorum* (DP) with acid pH were selected as biostimulant treatments. According to the labelling of the commercial products, AN1 was obtained by an exclusive and patented cold extraction process containing algae *A. nodosum* (> 98%), ethyl paraben (0.2–0.3%), potassium sorbate (0.2–0.3%); AN2 did not show the extraction method used, but containing algae *A. nodosum* (100%), polysaccharides of polyuronic structure, and potassium alginate; AN3 was obtained by alkaline hydrolysis extraction followed by a gentle drying on hot rotating cylinders, containing algae *A. nodosum* (100%); and DP was obtained by a process of enzymatic digestion at low temperatures containing algae *D. potatorum* (100%), trace elements, vitamins, amino acids, growth hormones and enzymes. All of them used different extraction processes (acidic, alkaline or enzymatic) and were classified as 100% SWE without any additive or enrichment substance.

The pH was analysed in the concentrated liquid SWEs and in a 5% (w/v) solution of the solid seaweed raw material, using a pH-meter (Orion dual star, Thermo scientific, MA, USA). Liquid SWE samples were freeze-dried and weighed and dry matter (DM) was calculated as dry weight/fresh weight × 100 (%). Liquid seaweed extracts (1 ml) and solid seaweed raw material (0.4 g DW) were digested using a microwave (CEM Corporation MARS 240/50, Matthews, NC, USA) with 8 ml HNO_3 (65%) and 2 ml H_2O_2 (30%). The microwave digestion programme was completed in two steps with a slope of 15 min to 200 °C following by 40 min at a constant temperature of 200 °C. All reagents used were Suprapur® grade (Merk® KGaA, Darmstadt, Germany). Samples were then filtered through a 0.20-µm filter paper and made up to 25 ml with deionized water (type I reagent grade). Total iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn) concentrations were determined by atomic absorption spectrometry using a Perkin-Elmer Aanalyst TM 800 instrument (Perkin Elmer, Waltham, MA, USA). A total

of four analytical replicates of each seaweed extract were performed.

The concentration of the principal plant regulators such as cytokinins (*trans*-zeatin, *t*Z, zeatin riboside, ZR, and isopentenyl adenine, iP), gibberellins (GA_1, GA_3 and GA_4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to Albacete et al. (2008) with some modifications. Briefly, 0.05 g of dry raw seaweed material (AN3) homogenized in liquid nitrogen and 150 µl of liquid seaweed extracts (AN1, AN2 and DP) dropped in 0.5 ml of cold (− 20 °C) extraction mixture of methanol/water (80:20, v:v). Solids were separated by centrifugation (20,000×*g*, 15 min) at 4 °C and re-extracted for 30 min at 4 °C in additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus C_{18} cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of plant pigments and then evaporated at 40 °C under vacuum either to near dryness or until organic solvent was removed. The residue was dissolved in 1 ml methanol/water (20:80, v:v) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 µg l^{-1}) and corrected for 10 µg l^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%.

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L. Moneymaker) plants were grown in a growth chamber with a photosynthetic photon flux density at leaf height of 1000 µmol m^{-2} s^{-1} photosynthetically active radiation, 16-h, 25 °C, 40% humidity/8-h, 20 °C, 60% humidity day/night regime. Seeds were surface sterilized and germinated in vermiculite for 12 days in 1/20 diluted Hoagland nutrient solution in distilled water. Seedling were pre-adapted to hydroponic system in 10-l boxes (28 plants per box) in 1/5 diluted Hoagland nutrient solution with 20 µM Fe and pH 6.0 during 3 days. Plants were then transferred to 50 ml plastic pots (one plant per pot) and grown in full-strength Hoagland solution containing in mM: 7.5 NO_3^-, 1 HPO_4^{2-}, 1.05 SO_4^{2-}, 3.5 K^+, 2.5 Ca^{2+}, 1 Mg^{2+}; and in µM: 23.2 H_3BO_3, 4.6 Mn^{2+}, 1.2 Zn^{2+}, 0.185 Cu^{2+}, 0.06 MoO_4^{2-}, 4.6 Cl^-, 46 Na^+, with 20 µM Fe for 12 days. The source of Fe was Fe(III)-HBED (N,N′-bis(2-hydroxybenzyl)-ethylenediamine-N,N′-diacetic acid). The pH was fixed at 7.5 ± 0 by the addition of 0.1 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) and 0.1 g L^{-1} $CaCO_3$ to simulate calcareous conditions. After that, the deficiency of Fe was induced with 1 µM Fe-HBED and plants were grown in Fe-deficient nutrient solution, pH 7.5, 0.1 mM HEPES, 0.1 g L^{-1} $CaCO_3$ during 1 week. A total of 16 pots per treatment was performed.

Seaweed extract treatments were applied twice during the experiment following the recommended doses (10 L ha^{-1} AN1 (1.1 ml l^{-1}), 2 L ha^{-1} AN2 (0.2 ml l^{-1}), 0.05 g L^{-1} AN3, 10 L ha^{-1} DP (1.1 ml l^{-1})) of manufacturers. First application was after 3 days pre-adapted hydroponic period and the second one was at the beginning of Fe-deficient period. The Fe content of the SWE itself was compensated with $FeCl_3$ up to 1 µM to avoid possible effects of Fe coming from SWEs. A control treatment without SWE and 1 µM Fe-HBED was also performed.

Plant analysis

Plants were collected 8 days after Fe deficiency induction (25 days-old plants). The plant material was divided in three sets, one for oxidative stress parameters analysis, one for micronutrient concentration determination and other for the iron-chelate reductase (FCR) activity measurement. The dry weight (DW) of root and leaves were also measured.

Oxidative stress parameters

Enzymes were extracted from 0.1 g of intact frozen roots and leaves with 1 ml extraction solution, freshly prepared containing 50 mM potassium phosphate buffer at pH 7.8, 2 mM Na_2-EDTA (ethylene diamine tetraacetic acid), 10 mM DTT (1,4-dithiothreitol), 20 mM ascorbic acid, 0.6% PVPP (polyvinyl polypyrrolidone) and 50 µl protease inhibitors cocktail. The extracts were centrifuged at 14,000×*g* for 15 min at 4 °C, and the supernatants were used for the enzymatic assays. Total superoxide dismutase activity (SOD; EC 1.15.1.1) was assayed according to Giannopolitis and Ries (1977) with some modifications. Briefly, 300 µl reaction mixture containing 50 mM potassium phosphate buffer pH 7.8, 0.1 mM Na_2-EDTA, 13 mM methionine, 2 mM riboflavine and 75 mM NBT (nitroblue tetrazolium) were added to 10 µl of crude extract in a microplate. The reaction was started by exposing the mixture to cool white fluorescent light and absorbance at 560 nm was measured at 0, 15 and 30 min using a spectrophotometer (Spectro start nano, BMG Labtech, Germany). One unit of SOD activity was defined as the amount of enzyme that causes 50% NBT reduction by superoxide radicals, and the specific activity

was expressed as units mg^{-1} of protein. Catalase activity (CAT, EC 1.11.1.6) was determined according to Aebi (1984) with some modifications. CAT activity was assayed in a 3 ml reaction volume at 25 °C by adding 0.1 ml diluted extract to a solution containing 50 mM phosphate buffer pH 7.0 and 10 mM H_2O_2. The activity was measured by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption using a spectrophotometer (Spectro start nano, BMG Labtech, Germany). Activity was expressed as units (mmol of H_2O_2 decomposed per minute) per mg of protein. Lipid peroxidation was determined by the formation of malondialdehide (MDA), a by-product of lipid peroxidation that reacts with thiobarbituric acid. The resulting chromophore absorbs at 535 nm, and the concentration was calculated directly from the extinction coefficient of 1.56 M cm^{-1} following the procedure described by Carrasco-Gil et al. (2012).

Micronutrient analysis

Prior to mineral analysis, roots and leaves were washed with 0.3% HCl (v/v) and 0.1% Tween 80 and rinsed twice with distilled water. All plant tissues were placed in an oven at 60 °C for 72 h until constant weight. Plant samples (0.3 g dry weight (DW) of tissue) were digested with 8 ml HNO_3 (8%, Suprapur® Sigma-Aldrich, Madrid, Spain) after dry mineralization at 480 °C for 2 h. A total of four biologic replicates were used. Iron, Mn, Zn and Cu concentration were determined in root and leaf of tomato plants by atomic absorption spectrometry using a Perkin-Elmer AAnalyst TM 800 instrument (Perkin Elmer, Waltham, MA, USA). The translocation rate of micronutrients was calculated (concentration in leaf/concentration in root) to evaluate the micronutrients transport to aerial part.

Assessment of chlorophyll content during Fe deficient period

Leaf chlorophyll index was assessed at the beginning and at the end of Fe deficient period using a SPAD 502 apparatus (Minolta Co., Osaka, Japan). The SPAD data was expressed as the decrease (%) of SPAD values during the Fe deficient period. Data was the average of four measurements of new developed leaf levels during the Fe-deficient period in a total of eight plants per treatment.

Iron-chelate reductase activity (FCR)

Iron-chelate reductase activity was assayed according to Escudero et al. (2012) with some modification. Briefly, 300 ml reaction containers covered with aluminium foil to avoid light exposure were placed in the growth chamber. Each beaker contained 250 ml of reduction assay solution consisting of macronutrient solution used in the growth period, 2 mM MES to buffer the pH at 6.0 and 300 µM Na_2BPDS (bathophenanthroline disulfonic acid) as Fe(II) trapping and colorimetric reagent. Each solution was continuously aerated. Roots of 25 day old plants were washed three times in macronutrient solution containing 37.5 µM Na_2BPDS and transferred to the reaction container. The reaction was started by adding 100 µM Fe-EDTA as substrate of the enzyme. Aliquots of 5 ml were withdrawn at 10, 30 and 60 min for absorbance measurement. Eight plant replicates per treatment and one blank without plant were analysed. Fe(II)-$(BPDS)_3$ concentration was calculated after the determination of absorbance at 535 nm. The slope of the plots of Fe(II) (µmol g^{-1} fresh root) produced versus time was used as the Fe(II) reduction rate for each plant.

Statistical analyses

Statistical analysis was carried out with SPSS for Windows (v. 21.0), using a Levene test for checking homogeneity of variances and ANOVA or Welch's tests ($p < 0.05$ or 0.10) were performed. Post hoc multiple comparisons of means were carried out using Duncan's or Games-Howell's test ($p < 0.05$ or 0.10) as appropriate.

Results

Commercial SWEs characterization

The pH values were acid for AN1 and DP and basic for AN2. The solid raw material AN3 showed a neutral pH after its dissolution in distilled water (Table 1). All liquid commercial SWEs presented a low dry matter (DM) content (< 16%). In general, the micronutrients concentration were not homogenous between SWEs with significant differences specifically in Fe and Mn concentration. The AN1 presented

Table 1 Determination of micronutrients (Fe, Mn, Cu and Zn) concentration, pH and dry matter (DM) in seaweed extracts (AN1, AN2, AN3 and DP)

	AN1 (µg ml^{-1})	AN2 (µg ml^{-1})	AN3 (µg g^{-1})	DP (µg ml^{-1})
pH	4.2 ± 0.2 c	14.1 ± 0.1 a	6.3 ± 0.1*b	4.1 ± 0.2 c
DM (%)	15.3 ± 0.1 a	5.4 ± 0.5 c	–	9.5 ± 0.1 b
Fe	53.1 ± 1.3 a	39.9 ± 2.0 a	24.6 ± 2.1 b	3.5 ± 0.9 c
Mn	11.9 ± 0.2 b	20.9 ± 0.1 a	2.9 ± 0.5 c	1.1 ± 0.2 d
Cu	1.5 ± 0.1 b	3.0 ± 0.6 a	2.7 ± 0.6 a	0.6 ± 0.2 c
Zn	12.1 ± 3.7 a	4.1 ± 0.1 b	11.3 ± 0.9 a	5.3 ± 0.4 b

Data are means ± SE (n = 4). Significant differences between treatments ($P < 0.05$) within the same line are indicated by different letters

*5% (w/v) solution of the solid seaweed raw material

the highest Fe concentration following by AN2 > AN3 > DP and AN2 presented the highest Mn concentration following by AN1 > AN3 > DP. The AN2 and AN3 showed the highest Cu concentration and the AN1 and AN3 showed the highest Zn concentration.

The presence of plant regulators and its concentration significantly varied among the SWE products (Table 2). The ethylene precursor ACC was found only in DP. The *t*Z cytokinin was found only in AN1, and the iP cytokinin was found in all SWEs but at different concentrations ranging from 0.64 to 366 ng g^{-1}. The gibberellins GA_1, GA_3 and GA_4 were presented in AN2 and AN3. In AN1 was found GA_1 and GA_3 and in DP was found only GA_4. The highest gibberellins GA_1, GA_3 and GA_4 concentrations were detected in AN3. The IAA was only found in AN3. The ABA was presented in all SWE except in DP, with the highest concentration in AN3. The SA was found in AN2, AN3 and DP but not in AN1. The highest concentration of SA was found in AN2 with an increase of 2258-/134-fold with respect to AN3 and DP extracts. The JA was presented in all SWEs, and the highest concentration was found in AN1 with an increase of 2.5×10^4-/1.6×10^4-/227×10^4-fold with respect to AN2, AN3 and DP extracts.

Plant experiment

The application of SWEs significantly increased SOD activity in root and shoot with AN1 and AN2 treatments compared to control plants (Fig. 1). The CAT activity significantly increased in roots after the application of AN1 and DP, but no differences were observed in leaves compared to the control treatment. The MDA concentration did not decreased either in roots or leaves after the Fe-deficient period compared to the control, but the application of DP extract significantly increased the MDA concentration in leaves with respect to the commercial extracts made of *A. nodosum* (AN1, AN2, AN3).

The application of SWEs did not increase the Fe concentration neither in root nor in leaf tissue compared to the untreated control (Table 3). However, other micronutrients (Mn, Cu and Zn) were significantly increased with some of the treatments. AN1 significantly increased the Mn and Zn concentration in leaf, AN2 significantly increased the Mn, Cu and Zn concentration in root and leaf, and AN3 significantly increased the Cu concentration in root compared to untreated control. Furthermore, the ratio shoot/root micronutrient concentration was calculated in tomato plants to evaluate the effect of SWEs on Fe, Mn, Cu and Zn translocation to the shoot (Table 3). Neither of the SWE treatments significantly increased Fe translocation to shoot. However, AN1 significantly enhanced the Mn and Zn translocation to shoot (by 1.9- and 1.4-fold, respectively), AN2 significantly decreased the Cu and Zn translocation to shoot (by 2.0- and 1.7-fold, respectively), AN3 significantly decreased (by 2.8-fold) the Cu translocation to shoot and significantly increased (by 1.4-fold) the Zn translocation to shoot, and DP significantly decreased (by 2.0-fold) the Cu translocation to shoot compared to untreated control.

The application of SWEs did not increase root DW and leaf DW compared to the untreated control after 7 days of Fe-deficient period (Fig. 2), except in the case of AN2 extract that significantly increased the root DW with respect to the control (1.3-fold) and other SWEs (AN1, AN3 and DP; 1.2, 1.4 and 1.5-fold respectively). By contrast, AN2 significantly reduced the leaf DW compared to the untreated control and the rest of SWE (1.5-fold).

The first application of SWEs under Fe sufficiency (day 0 in Fig. 3) did not increase SPAD values in the tomato leaves after 12 days of growth. The second application of SWEs at the beginning of Fe deficiency, did not reduce the

Table 2 Plant regulators concentration (ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), Cytokinins (*trans*-zeatin, *t*Z, zeatin riboside, ZR and isopentenyl adenine, iP), gibberellins (GA_1, GA_3 and GA_4), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), and the jasmonic acid (JA)) in SWEs (AN1, AN2, AN3 and DP) and their contribution in nutrient solution for tomato plants after every individual application of the seaweed extracts

	In seaweed extract product				In nutrient solution for plant			
	AN1 ng g^{-1}	AN2 ng g^{-1}	AN3 ng g^{-1}	DP ng g^{-1}	AN1 pg ml^{-1}	AN2 pg ml^{-1}	AN3 pg ml^{-1}	DP pg ml^{-1}
ACC	nf	nf	nf	0.40				0.046
tZ	434	nf	nf	nf	87.3			
ZR	nf	nf	nf	nf				
iP	1.42	366	121	0.64	0.29	4.56	6.04	0.075
GA_1	0.95	0.23	1.43	nf	0.19	2.9×10^{-3}	0.07	
GA_3	1.55	0.24	13.0	nf	0.31	2.9×10^{-3}	0.65	0.009
GA_4	nf	3.83	9.2	0.08		4.8×10^{-2}	0.46	
IAA	nf	nf	92.4	nf			4.62	
ABA	16.44	1.11	61.3	nf	3.31	1.4×10^{-2}	3.07	
SA	nf	1.8×10^{-3}	13.5	0.80		22.4	0.67	0.093
JA	9.78×10^5	38.7	60.5	0.43	1.97×10^5	0.48	3.03	0.050

nf not found

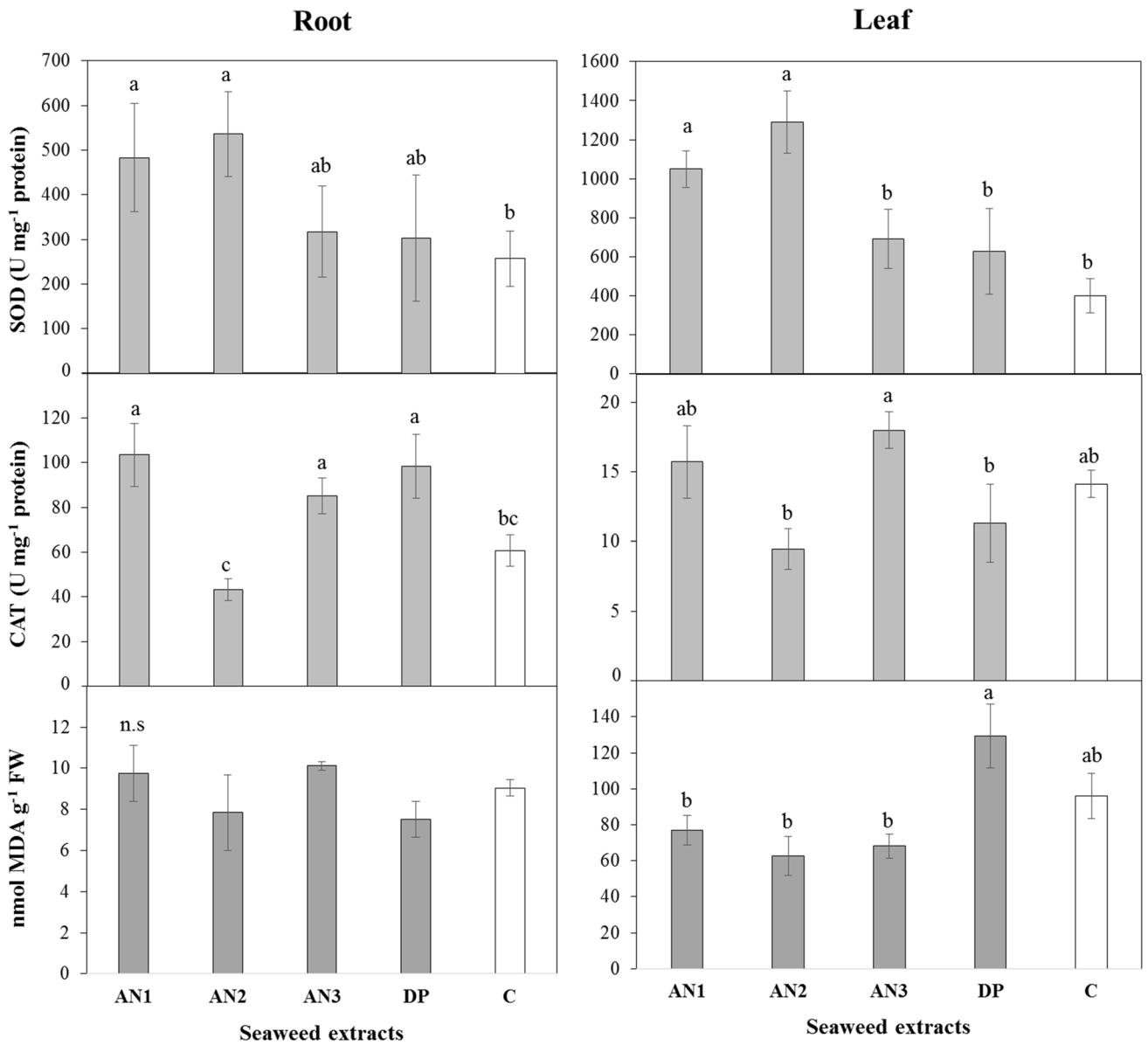

Fig. 1 Oxidative stress indexes (SOD and CAT activity, and MDA concentration) of roots and leaves after 7 days of Fe deficiency and treated with commercial SWEs (AN1, AN2, AN3, DP). A control (C) treatment without SWE application was performed. Data are means ± SE (n = 4). Significant differences between treatments ($P < 0.10$) are indicated by different letters. Not significant (n.s)

iron chlorosis after 7 days compared to the untreated control (Fig. 3). After 7 days of Fe deficiency, tomato plants treated with AN1 and DP had the same SPAD decrease as the untreated control (7%). However, AN2 and AN3 treatments showed a higher SPAD decrease (27% and 13% respectively) in tomato leaves compared to the untreated control.

None of SWE treatments increased the root FCR activity with respect to the untreated control (Fig. 4). However, AN2 treatment decreased the root FCR activity below the untreated control and the rest of the SWEs, but not significantly.

Discussion

Analysis of the composition of most SWE commercial products would be a useful first step to better hypothesize and or depict a cause-effect relationship of their mechanism of action. The components of SWE products depend on the type of seaweed used for extraction and also how the seaweed was handled after harvest and how it was processed (Battacharyya et al. 2015). All of SWEs applied in the experiment used different extraction process that affected the pH of the extract (acid or basic) and therefore the active compounds present in the products. Another factor that

Table 3 Micronutrients concentration (Fe, Mn, Cu and Zn; μg g^{-1} DW) in root and leaf and translocation rate (TR; leaf metal concentration/root metal concentration) of tomato plants treated with commercial SWEs (AN1, AN2, AN3, DP) after 7 days of Fe deficiency

	AN1	AN2	AN3	DP	C
Root					
Fe	200 ± 17 n.s	263 ± 32	202 ± 14	314 ± 56	215 ± 37
Mn	43 ± 2 c	86 ± 12 a	59 ± 4 bc	78 ± 10 ab	62 ± 4 bc
Cu	12.4 ± 1.8 bc	24.1 ± 4.1 a	17.2 ± 2.9 ab	12.4 ± 1.2 bc	8.2 ± 2.5 c
Zn	102 ± 7 b	241 ± 25 a	77 ± 24 b	114 ± 3 b	127 ± 9 b
Leaf					
Fe	28.4 ± 1.6 n.s	30.9 ± 1.7	28.2 ± 1.1	28.4 ± 1.7	31.9 ± 2.2
Mn	64 ± 2 b	73 ± 5 a	60 ± 2 bc	55 ± 1 c	52 ± 1 c
Cu	9.7 ± 0.9 b	14.1 ± 0.5 a	8.2 ± 0.4 b	8.1 ± 0.7 b	9.6 ± 0.7 b
Zn	76 ± 5 ab	80 ± 4 a	66 ± 2 bc	63 ± 3 c	63 ± 2 c
Translocation rate					
Fe	0.14 ± 0.01 n.s	0.11 ± 0.01	0.14 ± 0.01	0.09 ± 0.01	0.14 ± 0.03
Mn	1.50 ± 0.05 a	0.84 ± 0.18 b	1.01 ± 0.26 b	0.71 ± 0.13 b	0.83 ± 0.03 b
Cu	0.78 ± 0.22 ab	0.58 ± 0.13 b	0.47 ± 0.05 b	0.65 ± 0.11 b	1.27 ± 0.31 a
Zn	0.74 ± 0.05 a	0.33 ± 0.03 d	0.85 ± 0.05 ab	0.55 ± 0.01 bc	0.49 ± 0.02 c

A control (C) treatment without SWE application was performed. Data are means ± SE (n = 4). Significant differences between treatments ($P < 0.05$) within the same line are indicated by different letters

n.s not significant

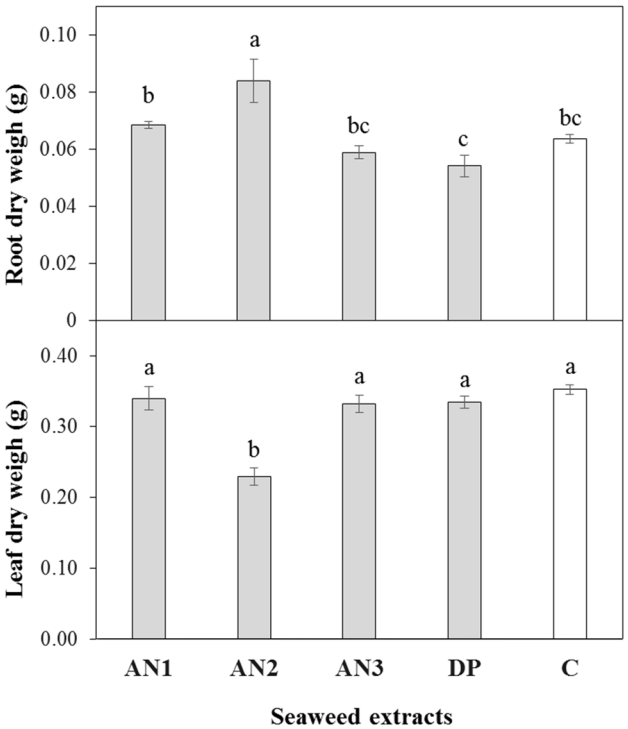

Fig. 2 Dry weight (DW; in g $plant^{-1}$) of root and leaf tomato plants after 7 days of Fe deficiency and treated with commercial SWEs (AN1, AN2, AN3, DP). A control (C) treatment without SWE application was performed. Data are means ± SE (n = 4). Significant differences between treatments ($P < 0.05$) are indicated by different letters

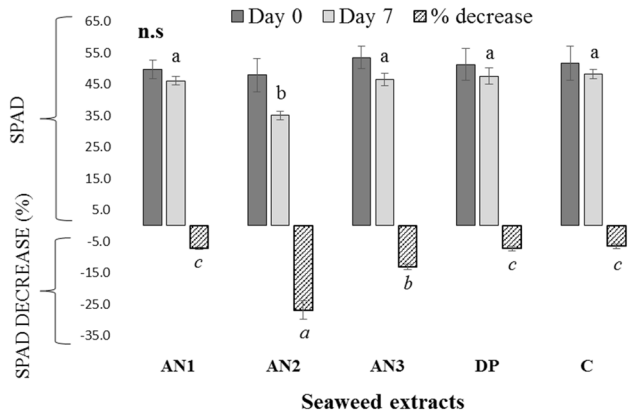

Fig. 3 Soil-plant analysis development (SPAD) values of tomato leaves at day 0 and 7 of Fe deficiency, and SPAD decrease (in %) of tomato leaves after 7 days of Fe deficiency and treated with commercial SWEs (AN1, AN2, AN3, DP). A control (C) treatment without SWE application was performed. Data are means ± SE (n = 8). Significant differences between treatments ($P < 0.05$) are indicated by different letters (in bold for SPAD values at day 0 without Fe deficiency, in regular for SPAD values at day 7 after Fe deficiency, in italics for SPAD decrease after 7 days of Fe deficiency). Not significant (n.s)

contributes to variation of composition in SWE biostimulants is the nutrients concentration. The SWEs applied in this experiment presented significant differences in micronutrients concentration (Fe, Mn, Cu and Zn), despite AN1, AN2 and AN3 being made of *A. nodosum*. Similar results were found in the comparison of micronutrients concentration of two commercial seaweed products (Maxicrop and Algifert),

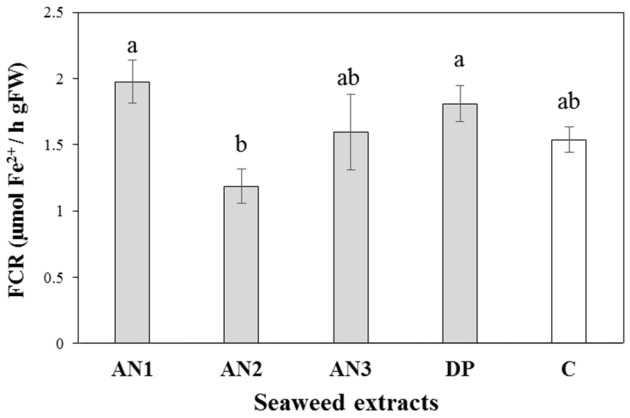

Fig. 4 Iron-chelate reductase (FCR) activity of tomato roots after 7 days of Fe deficiency treated with commercial SWEs (AN1, AN2, AN3, DP). A control (C) treatment without SWE application was performed. Data are means ± SE (n = 8). Significant differences between treatments ($P < 0.05$) are indicated by different letters

manufactured from *A. nodosum*, and three liquid seaweed products (Afrikelp, Basfoliar Kelp and Kelpak) manufactured from *E. maxima*, where Fe, Mn, Cu, Zn and B concentration significantly varied, (Verkleij 1992; Lötze and Hoffman 2015). For instance, Fe concentration in Maxicrop was 2200 mg kg^{-1} and in Algifert was 60 mg kg^{-1} DW. In the case of *E. maxima* products, all of them were harvested on the same location (South African coastline) but in different years. Moreover, the presence of plant regulators and their concentrations varied significantly among the SWE products (Table 2). The ethylene precursor ACC was only detected in DP extract manufactured from *E. maxima*. It has been reported that brown seaweeds (Phaeophyceae) produced low rates of ethylene (Broadgate et al. 2004). Ethylene elicits physiological responses in plants such as fruit ripening, senescence of flowers and leaves and inhibition of shoot and root elongation. It is a stress hormone, being generated in response to various biotic and abiotic stresses (Bleecker and Kende 2000). The *t*Z cytokinin was detected only in AN1, and the iP cytokinin was found in all SWE but at different concentrations. Cytokinins are essential for cell division as well as influencing many developmental processes in vascular plants such as controlling the root/shoot ratio, delaying senescence, breaking bud dormancy, influencing nutrient mobilisation, apical dominance, floral development and seed germination (Kieber 2002; Sakakibara 2006). The cytokinin-like activity of brown seaweeds has been extensively studied using bioassays such as the *Amaranthus* betacyanin and soybean callus bioassays. The results reported changes in cytokinin-like activity suggesting that cytokinin levels and profiles are linked to growth phases and environmental conditions of seaweeds (Stirk and van Staden 2014). The auxin IAA was only detected in AN3 extract from *A. nodosum* but also other authors identified IAA in *E. maxima*. Auxins are key regulators for almost every aspect of plant growth and development, being involved in cell division, elongation and differentiation as well as in tropic responses, promoting root growth, apical dominance, vascular development, flowering and senescence (Mano and Nemoto 2012; Murphy 2002; Woodward and Bartel 2005). The SWEs AN1 and AN2 made of same algae (*A. nodosum*) showed significant differences regarding plant regulators content probably due to the extraction process (acid and basic pH respectively). The plant regulators concentrations in the nutrient solution coming from SWE application (following the manufacturer recommendation rates) were low ($2.9 \times 10^{-3} - 87.3$ pg ml^{-1}). However, JA concentration in the nutrient solution coming from AN1 extract was high (1.97×10^{5} pg ml^{-1}). Wally et al. (2013) quantified phytohormones in SWEs and determined that the levels of phytohormones present in the various commercial SWEs were insufficient to alter plant phenotypes in plants, suggesting that other SWE components were altering endogenous and biosynthetic pathways of plants.

Reactive oxygen species (ROS) production is a common factor in many abiotic stresses such as salinity, ozone exposure, UV irradiation, temperature extremes, drought and nutrient deficiency. Plants have an enzymatic antioxidant system to protect cells from oxidative damage caused by ROS (Mittler 2002). Superoxide dismutase (SOD), and catalase (CAT) are the first enzymes in the detoxification pathway and contain Fe, either in heme (CAT) or non-heme (Fe-SODs) form. The lack of Fe in plants reduced CAT activity and increased total SOD activity (decreasing Fe-SOD and increasing CuZn-SOD and Mn-SOD) (Molassiotis et al. 2006; Sun et al. 2007; M'sehli et al. 2014). In the present experiment, the application of AN1 and AN2 extracts increased total SOD activity in root and shoot after Fe deficient period compared to untreated plants. The increase of total SOD activity may be due to Fe deficiency (in AN2; see CAT activity), but also due to SWE application (in AN1; see CAT activity). Several studies reported an increase of SOD activity in leaves after the application of an *A. nodosum* extract in unstressed turf grasses (Fike et al. 2001) and drought stressed tall fescue (Zhang 1997). Also, the application of a *Ulva rigida* and *Fucus spiralis* extracts by spray at 25% concentration in unstressed or moderate water stressed beans respectively, increased SOD activity in leaves (Mansori et al. 2014). The activation of antioxidant enzymes after the application of SWE is also supported by the increase of CAT activity observed in tomato roots treated with AN1 and DP extract compared to the CAT activity of untreated Fe deficient tomato roots (Fig. 1). Other authors, also reported an increase of CAT activity in leaves of moderate water stressed beans treated with 25% of *F. spiralis* and 50% of *U. rigida* extracts (Mansori et al. 2014) and unstressed and salt stressed barley, soaked previous germination with different concentration of *S. latifolium* extract when compared

to the control (Sofy et al. 2017). Besides, the accumulation of ROS due to Fe deficiency, can damage membrane lipids increasing lipid peroxidation and the concentration of MDA (Mohamed and Aly 2004; Sperotto et al. 2008). The application of SWEs (*U. rigida* and *F. spiralis*) decreased the MDA level in leaves of water-stressed bean plants (Mansori et al. 2014) and in unstressed and salt stressed barley whose seeds were previously soaked with different concentration of *Sargassum latifolium* extract compared to the control (Sofy et al. 2017).

At present, the chemical components of SWEs that may be responsible for abiotic stress tolerance in treated plants is unknown. It has been suggested that positive anti-stress effects of SWEs may be related to cytokinin activity (Fike et al. 2001; Ervin et al. 2004; Zhang and Ervin 2008), but also jasmonic acid (JA) activated plant defense responses to environmental stresses including drought, salinity, and low temperature (Du et al. 2013). The highest contribution of cytokinin (tZ) and JA to the nutrient solution came from AN1 application, which increased the activity of both antioxidant enzymes (SOD and CAT) compared to untreated plants. However this positive response of antioxidant enzymes to Fe deficiency was also observed with AN3 application, whose contribution of cytokinin and JA to the nutrient solution was very low.

Regarding SWE application effect on biomass, AN1, AN3 and DP extracts did not increase root DW and leaf DW compared to the untreated control during 7 days of the Fe deficiency (Fig. 2). However, extensive literature review reported the beneficial effect of SWEs on plant growth development (Khan et al. 2009; Craigie 2011; Battacharyya et al. 2015). Only AN2 extract application significantly increased the root DW, but simultaneously reduced the leaf DW compared to the untreated control and other SWE products. Therefore one or several active compounds presented in AN2 could elicit an imbalance in the shoot growth. Focusing on plant regulator presented in AN2, SA concentration in the nutrient solution (22.5 pg ml^{-1} or 1.6×10^{-7} µM) was higher than in other SWE products, but very low for plant growth inhibition. Kovácik et al. (2009) reported an inhibitory effect on plant growth in chamomile after the application of higher concentrations of SA (250 µM). However, growth-stimulating effects of SA have been reported in root soybean (Gutiérrez-Coronado et al. 1998) and root and leaf chamomile (Kovácik et al. 2009) with a minimum of SA concentration of 10 nM and 50 µM respectively.

The SWE application did not increase the SPAD values in tomato leaves during the growth period, and did not reduce the iron chlorosis after 7 days of Fe deficiency compared to the untreated control (Fig. 3). Contrary to our results, several studies reported that the application of SWEs increased the chlorophyll content mainly due to an increase in the biogenesis of chloroplasts, a reduction in chlorophyll degradation and a delay in senescence (Jannin et al. 2013; Rayorath et al. 2008; Nair et al. 2012; Blunden et al. 1997). It could be possible that under nutrient sufficiency, unstressed plants were not influenced by SWE application (with doses applied), and when Fe deficiency appears, SWE application should be increased for attenuating chlorosis. Iron deficiency induces FCR activity in roots in strategy I plant species in conjunction or not with other adaptive responses such as rhizosphere acidification and changes in root hair and transfer cell development to enhance Fe uptake in plant (Schmidt 1999). In our study, none of SWE treatments increased FCR activity with respect to the untreated control (Fig. 4) and also no differences were shown in Fe concentration plant tissue and Fe translocation rate from root to shoot after SWE supply (Table 3). However, Durand et al. (2003), focusing on N metabolism, reported that application of SWE on *A. thaliana* (by foliar spraying or addition in the growth medium) enhanced nitrate reductase (NR) activity in leaves, resulting in improved nitrogen assimilation.

As mentioned above, SWE treatments did not increase Fe concentration in root and in leaf tissue compared to the untreated control (Table 3). The low concentration of Fe (1 µM) in the nutrient solution, impedes an increase of Fe in the plant, but also there was no difference in Fe distribution. However Mn and Zn concentration in plant were significantly increased with AN1 and AN2 treatments, and Cu concentration was significantly increased with AN2 and AN3 treatments, all of them made from *A. nodosum*. It can not be dismissed that the increase of Mn was due to the profusely described Fe/Mn antagonism, but also Cu and Zn may share membrane transporters with Fe, increasing their concentration under Fe deficiency (Socha and Guerinot 2014). Moreover, AN2 extract increased micronutrients concentration in both root and shoot, possible due to a significant higher root development (Fig. 2a) increasing the absorption area and a lower shoot development producing a concentration effect, in comparison with the rest of treatments. DP treatment made from *D. potatorum* did not increase the micronutrients uptake in tomato plants. The contribution of SWE products to the micronutrients supply of nutrient solution was evaluated. The contribution of Mn, Cu and Zn to the nutrient solution from all SWE products was less than 7%, except Cu and Zn from AN1, which contribution was 14% and 17% respectively to the nutrient solution (Table 3). These values do not justify the variation observed in the plant content. Several studies reported that SWE supply improved nutrient uptake by roots. The application of a commercial product from *A. nodosum* in rapeseed increased nitrogen uptake (21% in shoot and 115% in roots; Jannin et al. 2013). The elemental nutrient composition of that SWE, did not show significant N content (data not shown). As mentioned before, the application of a commercial product from *A. nodosum* by foliar spray, increased N, P, K, Ca, Zn and Fe in tomato

fruits (Drobomilska et al. 2008), or Zn in grapevine leaves (Sabir et al. 2014) in comparison to untreated plants. However the elemental nutrient composition of the commercial extracts applied in those experiments, were not previously analyzed by the authors, so it can not be ruled out that the increase of the mineral content in tomato fruit or grapevine leaves comes from the extra-contribution of the commercial extracts, that many times are enriched with chelated trace elements and macro elements N, P and K (Verkleij 1992; Craigie 2011), and not from the stimuli nutrient uptake.

In summary, the application of commercial SWEs (AN1, AN2, AN3 and DP) on Fe-deficient tomato plants activated the antioxidant system increasing SOD and CAT activity. However these commercial SWEs did not produce positive effects in biomass, chlorophyll content, activation of Fe acquisition strategies and Fe uptake with respect to the untreated control, following recommended doses of manufacturers. Also, DP extract (based on *D. potatorum*) exerted less protection against lipid peroxidation than AN1, AN2 and AN3 extracts (base on *A. nodosum*). It seems that the potential benefits of SWEs application on micronutrient deficient plants, are in the area of antioxidant activities.

Many of commercial SWEs ensure enhance nutritional quality of the plant, but in many cases those products are enriched with nutrients that works as fertilizers not as biostimulants. It should be taken into account that different authors working with commercial extracts obtained from the same seaweed (as example *A. nodosum*) showed variation in the results probably due to (1) their composition which depend on the extraction process in the manufacturing, and (2) doses, frequency and time of application. Research in the extraction method and application rates is needed to preserve the maximum active compounds and ensure the effectiveness of the product. These advances will allow designing commercial seaweed extracts of quality, which guarantee to farmers the benefits indicated on the package.

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Compliance with ethical standards

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

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