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Research article

Ascophyllum nodosum extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants

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ABSTRACT

Global changes in climate are leading to increased occurrence and duration of drought episodes with concurrent reduction in crop yields. Expansion of the irrigated land area does not appear to be a viable solution in many regions to deliver crop productivity. The development of crop drought tolerance traits by either genetic modification or plant breeding represent the principal approaches to meeting this challenge to date. Biostimulants are an emerging category of crop management products which can enhance crop productivity under abiotic stress conditions. The ability of some biostimulant products such as *Ascophyllum nodosum* extracts (ANE) to enhance the tolerance of crops to drought stress has been observed by growers. The objective of this study was to investigate if different commercial ANE biostimulants provided the same tolerance to tomato plants (cv. Moneymaker) subjected to a defined drought period. A compositional characterisation of the key macromolecules of ANEs was performed. In addition, the role of ANE biostimulants in inducing changes of chlorophyll and osmolytes levels, MDA production, dehydrin isoform pattern and dehydrin gene expression levels was assessed. The three ANE biostimulants evaluated were found to provide different levels of tolerance to drought stressed tomato plants. The level of drought tolerance provided was related to changes in the concentration of osmolytes and expression of *tas14* dehydrin gene. Taken together, our results highlight that despite the fact all ANE biostimulants were manufactured from the same raw material, their ability to maintain crop productivity during and after drought stress was not the same.

1. Introduction

Drought is a normal, recurring feature of climate which occurs in virtually all climatic regimes. Even in more humid climatic zones, drought is often a common feature. Agriculture is one of the key sectors affected by drought. The impact of drought on crop productivity has led to major consequences for food security and the economy of different world regions. World regions most impacted by drought include South-Central Asia, Southeast of South America, Central Europe and Southeast of the United States (Carrão et al., 2016). The occurrence of agricultural drought depends on the crop evapotranspiration demand and the soil moisture availability to meet this demand (Wilhite, 2011). Globally, rain fed agriculture is practised in 80% of the total agricultural area (Monneveux et al., 2013). Irrigation is the first line solution for agricultural drought but this is not without cost and problems (e.g. sustainability and salinity).

Strategies beyond irrigation for providing crop drought tolerance include speciality crop inputs, traditional plant breeding and genetic modification strategies to reduce drought stress. A key factor to successful implementation of these strategies is a better understanding of drought tolerance, which includes a series of protective mechanisms which function at the morphological and physiological levels. Typical mechanisms include development of vigorous root system, formation of epidermal wax, shedding of older leaves, regulation of stomatal closure to reduce dehydration, modulation of photosynthetic performance, repression of cell growth or induction of senescence (Wilkinson and Davies, 2010; Fang and Xiong, 2015). Reducing transpiration presents an opportunity to alleviate the adverse effects of water deficit and improve crop productivity under drought conditions (Prakash and Ramachandran, 2000). Speciality crop inputs promoted to reduce crop

Abbreviations: 2-ME, 2-mercapthoethanol; ABA, abscisic acid; ANE, *Ascophyllum nodosum* extract; DW, dry weight; EBIC, European Biostimulant Industry Consortium; FW, fresh weight; GM, genetically modified; HPAEC-PAD, High performance anion exchange chromatography with pulsed amperometric detection; LEA, late-embryogenesis-abundant; MDA, malondialdehyde; PVPP, polyvinylpolypyrrolidone; qRT-PCR, quantitative real time polymerase chain reaction; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; RH, relative humidity; ROS, reactive oxygen species; RWC, relative water content; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBARS, thiobarbituric acid reactive substance; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TW, turgid weight

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drought stress include antitranspirants which can be categorised into three major groups based on their mode of action, namely films, reflecting and physiological antitranspirants (Del Amor et al., 2010).

Plants also respond and adapt to drought stress at biochemical, cellular and molecular levels. For instance, by the mobilization of stress-related hormones, production of osmolytes, elimination of reactive oxygen species (ROS) and accumulation of stress protective proteins such as LEA (Late Embryogenesis Abundance) proteins (Olvera-Carrillo et al., 2011; Fang and Xiong, 2015). Each mechanism depends on the expression and regulation of an assortment of genes with diverse functions (Nakashima et al., 2014). Conventional breeding for adaptation to drought stress is far more complicated than breeding for other traits (Fita et al., 2015). Another way to increasing yield under water stress is based on the generation of genetically modified (GM) crops with tolerance to drought (Reguera et al., 2012). The first drought tolerant GM crop was commercially launched in 2012 with market approval in USA and Canada. Monsanto, in collaboration with BASF, developed a genetically modified maize variety with improved resistance to water stress by expression of bacterial genes encoding RNA chaperones. DroughtGard™ maize remains the only drought tolerant GM crop with multi-region approval in 2017 and planting increased 15-fold from 50,000 hectares in 2013 to 810,000 hectares in 2015 reflecting high farmer acceptance (James, 2015).

Biostimulants are an emerging class of crop management products that target the modulation of crop stress to increase productivity. A number of definitions of a biostimulant have been proposed and reviewed (Yakhin et al., 2017). The European Biostimulant Industry Consortium (EBIC) are leading the international marketplace in defining and seeking regulation for biostimulant products in Europe. EBIC has defined biostimulants as "containing substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality". du Jardin (2015) assigned biostimulants into 8 categories: (i) humic substances, (ii) complex organic materials, (iii) beneficial chemical elements, (iv) inorganic salts, (v) seaweed extracts, (vi) chitin and chitosan derivatives, (vii) antitranspirants and (viii) free amino acids and other N-containing substances with microorganisms a potential ninth category.

Seaweed extracts are prominent in the biostimulant market, representing the fastest growing biostimulant product category (Watkins, 2015). The effects of seaweed extracts on plants have been reviewed (Craigie, 2011; Sangha et al., 2014) with a range of biostimulant effects reported, including drought tolerance. It is important to recognise that seaweed extract biostimulants are not a homogenous category of products. Seaweed extract biostimulants vary depending on the seaweed specie used for manufacture (e.g. brown, green or red), the spatio-temporal source of the seaweed raw material and the process used for manufacture/extraction (Khan et al., 2009; Sharma et al., 2013). Most of the commercial seaweed extracts with biostimulant effects are manufactured with brown algal species, with *Ascophyllum nodosum* Le Jol the dominant specie due to its long history of positive results in enhancing crop productivity (Craigie, 2011). *Ascophyllum nodosum* extract (ANE) biostimulants have previously been reported to increase drought stress tolerance of grasses and crops (Spann and Little, 2011; Elansary et al., 2016, 2017; Martynenko et al., 2016; Santaniello et al., 2017). Additionally, a recent transcriptome analysis of the model plant *Arabidopsis thaliana* reported the dysregulation of abiotic stress genes important for drought tolerance after the application of ANE biostimulants (Goñi et al., 2016).

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crop plants around the world and is particularly sensitive to a number of environmental stresses, including drought. Responses of tomato to drought stress depend on several factors including duration and severity of the drought period as well as its inherent tolerance mechanisms (Iovieno et al., 2016; Patanè et al., 2016). Due to the agronomic and economic relevance of tomato, different approaches to reduce the impact of drought on fruit yield and quality have been proposed, including the application of a biostimulant enriched in betaines (Petrozza et al., 2014). GM tomato plants have been shown to have increased drought stress tolerance without affecting plant growth under non-drought conditions. Successful gene modifications include the introduction of genes encoding dehydrins or enzymes involved in the synthesis of osmoprotectants (Gerszberg and Hnatuszko-Konka, 2017).

This study focused on generating data to support answers to the following questions: Do ANE biostimulants have a role in maintaining crop productivity during periods of drought; Are all ANE biostimulants the same in terms of their ability to induce drought tolerance in tomato; What are the effects of biostimulants on some of the molecular players involved in mediating drought tolerance in tomato.

2. Material and methods

2.1. Plant material and growth conditions and drought stress treatment

Tomato seeds (*Lycopersicon esculentum*, cv. Moneymaker) were purchased from Liscahane Nurseries, Tralee. Seeds were surface sterilised with sodium hypochlorite for 1 min before being thoroughly rinsed with distilled water. Seeds were set in plug trays using growth medium of compost: vermiculite: perlite (5: 1: 1). On day 21, seedlings were then transferred to 2 litre pots (same growth medium as previous) and 2g of slow releaser fertilizer containing $N/P_2O_5/K_2O$ (7/7/7, w/w/w) was applied to each pot. The resultant plants were raised in a growth room at a temperature of 27/22 ± 2 °C (day/night; 16/8 h) and 70 ± 5% relative humidity (RH) under a light intensity of 120 μmol $m^{-2} \cdot s^{-1}$ in a complete randomised block design. Plants were irrigated with 125 mL water every other day in order to create equal soil moisture conditions in all the pots. Temperature and relative moisture content were recorded regularly with a portable USB data logger (Log32TH, Dostmann electronic GmbH).

2.2. Treatment application and drought stress conditions

Three commercially available liquid seaweed extracts of *A. nodosum* (ANE A, ANE B and ANE C) manufactured using different methods were applied to plants as biostimulant treatments. ANE A was manufactured using a proprietary process at high temperatures and neutral pH. ANE B and ANE C were manufactured using a proprietary process at high temperatures and alkaline pH. Prior to imposition of severe drought, ANE biostimulants and control treatments were applied by foliar spray at a dilution of 0.33% (v/v) on 35-day-old tomato plants. Distilled water was applied as a control. After 24 h, drought stress was induced by withholding water for 7 days. To minimize the influence of any positional effect on drought stress responses, the relative position of the pots in the growth room was changed every other day. After the drought treatment, plants were re-watered, and 24 h later ANE treatments were applied again as foliar spray at 0.33% (v/v). Control plants were sprayed with equal volume of distilled water. Recovery stage after water withdrawal was maintained for 2 weeks under conditions described at section 2.1 to obtain 56-day-old plants. This experimental protocol is evaluating the drought tolerance stage (until T1) and growth promotion effects after stress (from T1 to T3). The 2 applications programme before and after stress period is based on current farmer practice for the use of ANE biostimulants. Leaf tissue was sampled before first ANE biostimulant application (T0), at 7 days after subjecting plants to drought stress (T1), at 48 h after the second ANE treatment in the 3rd day of the recovery stage (T2) and at the end of the recovery stage (T3). The samples were snap-frozen in liquid nitrogen, ground and kept in −80 °C until further analysis. Similar tomato plants were selected and grown under unstressed conditions for 56 days. ANE biostimulants and control treatments were applied by foliar spray as described above to evaluate growth promoting effects on non-drought

stressed tomato plants. Sampling points for unstressed plants corresponded to 42-day-old (T1), 45-day old (T2) and 56-day old tomato plants (T3).

2.3. Chemical compositional analysis of ANEs

Total solids from ANE liquid formulations were determined after drying in a convection oven for 18 h at 105 °C. These same samples were then used to determine ash by placing in a furnace for 6 h at 550 °C. Sulphate content linked to carbohydrate molecules was determined quantitatively after hydrolysing the samples with 2 M trifluoroacetic acid (TFA) for 5 h at 100 °C. Sulphate ion was precipitated in a strongly acid medium with barium chloride-gelatine (Lloyd et al., 1961). The resulting turbidity was measured spectrophotometrically at 420 nm and compared with an appropriate calibration standard curve using Na_2SO_4 (0–10 mM). L-fucose, total uronic acids, laminarin and total polyphenol content were determined spectrophotometrically following the method of Goñi et al. (2016). A quantitative analysis of soluble mannitol from ANEs was carried out by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) according to Goñi et al. (2016).

2.4. Growth parameters, relative water content and chlorophyll determination

Tomato plants were harvested at the end of the recovery stage and measurement of fresh weight (FW) of the plants (leaf + stem) was obtained. Plant dry weight (DW) was determined by drying frozen ground samples in a convection oven for 18 h at 105 °C. DW was measured and recorded before every subsequent metabolite analysis at all stated sampling times. The chlorophyll content was determined using an extraction method with an ammonia/acetone mixture (1/9, v/v). 100 mg of frozen ground leaf samples from the four different sampling times during the experiment (T0, T1, T2 and T3) were macerated and incubated in 0.5 mL extraction solution. After incubation for 2 h at 4 °C, samples were centrifuged at 20,000 *x g* for 10 min at 4 °C. The supernatants were collected and diluted with acetone 80% (v/v) before measuring the absorbance at 646.6 and 663.5 nm in a microtiter plate using a Varioskan Flash instrument (Fisher Scientific). Equation for the determination of total chlorophyll (chlorophyll a + chlorophyll b) in buffered aqueous 80% acetone was used (Porra, 2002). The results are expressed on a dry weight basis ($mg \cdot g^{-1}$ DW).

2.5. Relative water content

Measurements of relative water content (RWC) were performed on leaves collected at three sampling times (T0, T1 and T2). FW of the leaves selected was immediately measured after cutting. In order to obtain the turgid weight (TW), the leaves were immersed in distilled water in a closed petri dish and incubated under normal room temperature and dim light for 18 h. At the end of the imbibition period, the leaves were taken out, properly wiped to remove the water on the surface and weighed. Afterwards, the leaves were put in a convection oven for 24 h at 80 °C to obtain DW. RWC was calculated according the equation:

$$(\text{RWC in }\%) = [(\text{FW-DW})/(\text{TW-DW})]*100.$$

2.6. MDA content

The content of malondialdehyde (MDA) was measured following a modified thiobarbituric acid reactive substance (TBARS) assay. Briefly, 50 mg of frozen ground leaf material from three sampling times (T0, T1 and T2) was homogenized in 0.5 mL of ethanol 80% (v/v) and incubated for 60 min at 4 °C. After centrifugation at 20,000 x g for 10 min at 4 °C, the supernatant was recovered and divided in two aliquots of equal volume. Each aliquot was mixed with either (1) a solution of 20% (w/v) trichloroacetic acid (TCA), or (2) a solution of 20% TCA and 0.5% (w/v) thiobarbituric acid (TBA). Both mixtures were then incubated for 40 min at 95 °C. After that, samples were cooled in an ice bath and centrifuged at 2000 x g for 5 min at 4 °C. The absorbance at 440, 532 and 660 of the supernatant was read against a blank in a microtiter plate using a Varioskan Flash instrument (Fisher Scientific). MDA equivalents were calculated according the equations established by Hodges et al. (1999). The results were expressed as nmol $MDA \cdot g^{-1}$ DW.

2.7. Proline content

50 mg frozen ground leaf material from three sampling times (T0, T1 and T2) were homogenized in 0.5 mL of ethanol 70% (v/v) and incubated overnight at 4 °C under dark. The homogenates were centrifuged at 20,000 *x g* for 10 min at 4 °C. The supernatants were used for the estimation of proline content in leaf tissues. 200 μL of supernatant was mixed with 400 μL of reaction mixture [1% (w/v) ninhydrin in acetic acid/water/ethanol (60/20/20, v/v/v)] and incubated for 20 min at 95 °C. After cooling at room temperature, the absorbance was measured at 520 nm in a microtiter plate using a Varioskan Flash instrument (Fisher Scientific). After preparing a calibration standard curve with L-proline (Sigma-Aldrich), proline content was expressed as $mg \cdot g^{-1}$ DW.

2.8. Chromatographic determination of soluble sugars

Soluble sugars were extracted from 15 mg of frozen ground leaf material from three sampling times (T0, T1 and T2) with 0.5 mL of aqueous 2% (w/v) PVPP and incubated for 25 min at 90 °C. Then, leaf extracts were sonicated for 5 min and centrifuged at 20,000 *x g* for 20 min at 4 °C. The levels of glucose, fructose and sucrose were determined by HPAEC-PAD using a Carbopac PA-100 column (see section 2.3). An isocratic gradient of 50 mM NaOH (degassed by bubbling with helium) at 1 $mL\,min^{-1}$ was applied. Compounds were identified by comparison of retention time to that of commercial standards (Sigma-Aldrich) and sugars were quantified by peak integration. Glucose, fructose and sucrose content was expressed as $mg \cdot g^{-1}$ DW.

2.9. Isolation of tomato leaf heat-stable protein fraction

Frozen ground leaf material from three sampling times (T0, T1 and T2) was solubilized in the extraction buffer (100 mM Tris–HCl (pH 7.5), 2% (w/v) PVPP, 2 mM PMSF, 2 mM EDTA), followed by centrifugation at 21,000 x *g* for 20 min at 4 °C. The crude protein extracts were heated for 20 min at 85 °C, cooled down on ice for 20 min and centrifuged at 21,000 x *g* for 20 min at 4 °C to remove coagulated proteins. The protein concentration of the heat-stable protein fractions was determined by the Bradford method using the Bio-Rad protein assay as described by the manufacturer. 250 μg of protein per sample were trichloroacetic acid (TCA) precipitated (20%, v/v), washed three times with cold acetone and dried. Protein pellets were resuspended in 1 × Laemmli sample buffer with and without 5% (v/v) 2-mercapthoethanol and heated for 20 min at 80 °C for further SDS-PAGE and immuno-analysis under reducing and non-reducing conditions.

2.10. SDS-PAGE and immuno-analysis (western blot) of dehydrin isoforms

4 μg of heat-stable protein extracts were resolved on a 14% SDS-PAGE using a Mini-Protean II Cell (Bio-Rad). Duplicate gels were prepared, one to transfer onto 0.2 μm nitrocellulose membrane (Whatman) with a Mini Trans-Blot Cell (Bio-Rad) and the other for staining with Coomassie Brilliant Blue R-250. Electrotransferred nitrocellulose membranes were blocked with phosphate-buffer saline containing 0.1%

(v/v) Tween-20 and 5% (w/v) non-fat dry milk powder. The membranes were probed with polyclonal anti-dehydrin affinity purified serum (dilution 1/2000) raised against the conserved K-segment for dehydrin C-terminal (AS07-206A, Agrisera), which were detected with rabbit antiserum against IgG horseradish peroxidase conjugate diluted 5000-fold (NA934VS, GE-Healthcare). The immuno-complexes were visualized using the Pierce ECL chemiluminescence detection system (Thermo-Scientific). Immunoreactive bands were quantified by densitometry of scanned autoradiographs using the software ImageJ (NIH) and the results were expressed as the relative fold-change with respect to the levels of accumulation of control plants. The molecular mass of the separated polypeptides was estimated in comparison to the mobility of pre-stained electrophoresis marker (ColorBurst, Sigma-Aldrich). Experiments were carried out independently at least three times.

The phosphorylation state of dehydrin isoforms was determined by alkaline phosphatase treatment. Aliquots of heat-stable protein extracts (100 μg) were precipitated overnight with absolute ethanol (1:9, v/v). Protein pellets were then solubilized in 1xCutSmart Buffer (NEB) and the solubilized proteins were incubated with 10 units of calf intestine alkaline phosphatase (M0290S, NEB) for 6 h at 37 °C. The reaction was stopped by precipitating the proteins with absolute ethanol (1:9, v/v). Protein pellets were resuspended then in 1 × Laemmli sample buffer and heated for 20 min at 80 °C. These protein samples were separated by SDS-PAGE and analysed by western blot technique as described above.

2.11. RNA extraction and relative gene expression by qRT-PCR of the tas14 transcript

Total RNA was isolated from about 50 mg of frozen ground leaf material from three sampling times (T0, T1 and T2) by RNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. RNA was treated with RNase-free DNase Set (Qiagen, UK) in order to remove efficiently genomic DNA contamination. RNA concentration and purity was measured in a μDrop™ Plate RNA using a Varioskan Flash instrument (Fisher Scientific). RNA integrity was checked on a 1.2% agarose gel with SYBR™ Gold staining (Thermo Fisher Scientific, Ireland). Expression analysis of *tas14* dehydrin gene (*Solyc02g084850.2*) was performed by real time-PCR using a Roche LightCycler® 96 System (Roche, UK). Quantitative PCR was performed using about 300 ng of total purified RNA and the LightCycler® RNA Master SYBR Green I one-step kit (Roche, UK) according to the manufacturer's instructions. The expression level of the tomato *actin* (*Solyc01g104770.2*) gene was used as the reference gene. $2^{-\Delta\Delta CT}$ was used to quantify normalized gene expression (Schmittgen and Livak, 2008). The primers sequences used were as follows: *tas14*, forward 5′-TCATCACCATGAGGGGCAAC-3′ and reverse 5′-ACCTTCATGTTGTCCAGGCA-3'; Actin, forward 5′-TCTTGA AGCGTTTTAAAAGATGGC-3′ and reverse 5′- TCACCAGCAAATCCAGC CTT-3'. Amplicon specificity was confirmed by electrophoresis on a 2% agarose gel stained with SYBR™ Gold staining (Thermo Fisher Scientific, Ireland).

2.12. Statistical analysis

Growing of plants, ANEs treatments, non-drought/drought stress trials and sampling of plants were done in three independent experiments (biological replicates) with at least six plants per treatment (N = 18). For phenotypic assessment, a minimum of six plants were evaluated for each experiment and treatment. For RWC determination, two leaves (central and top position) were collected from each of the six plants per treatment. For every subsequent metabolic, proteomic and molecular analysis, three leaves (central and top position) were harvested from each of the six plants per treatment. The leaves were pooled and represent a single sample out of the three biological replicates. Chemical compositional analysis of ANEs was performed in triplicates as three independent assays. For biochemical and molecular tomato leaf analysis, 3 extractions of each treatment were performed and the experiment was repeated three times. Statistics were evaluated with the Statgraphics Plus v 5.1 software (Statistical Graphics Corp., USA) and SigmaPlot 12.0 for Windows. Phenotypic differences between non-drought and drought untreated tomato plants were analysed with the unpaired *t*-test at $p \leq 0.05$. ANEs compositional analysis data and the effect of ANEs treatment on plants was analysed with a one-way analysis of variance (ANOVA). The significance level was set at $p \leq 0.05$ and performed by Tukey-HSD's test.

Table 1
Compositional analysis of three *A. nodosum* biostimulants (ANE A, ANE B, ANE C) currently used in agricultural practice.

Component[1]	Treatment		
	ANE A[3]	ANE B	ANE C
Solids % (w/v) extract	29.50 ± 0.29 a	39.10 ± 0.83 b	19.47 ± 0.32 c
Ash (except sulphate) % (w/w)[2]	23.48 ± 0.12 a	39.71 ± 0.08 b	39.95 ± 0.11 c
Sulphate % (w/w)	15.98 ± 0.34 c	10.29 ± 0.22 b	8.02 ± 0.18 a
Uronic acid % (w/w)	13.05 ± 1.10 c	10.82 ± 0.36 b	8.49 ± 0.41 a
Fucose % (w/w)	15.12 ± 0.60 c	8.10 ± 0.20 b	6.90 ± 0.60 a
Polyphenol % (w/w)	3.60 ± 0.13 a	12.10 ± 0.1 c	7.30 ± 0.14 b
Laminarin % (w/w)	2.30 ± 0.04 b	3.57 ± 0.17 a	n.d [4] c
Mannitol % (w/w)	8.02 ± 0.19 c	6.94 ± 0.22 b	1.93 ± 0.03 a
Other % (w/w)	18.45 ± 0.37 b	8.47 ± 0.25 a	27.41 ± 0.19 c

[1] Data are the means ± SD (n = 9).
[2] ANEs chemical compositional analysis is expressed with respect to their dry content.
[3] Different small letter within the same row indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).
[4] Not detected: value below limit of detection (< 0.05% w/v sample).

3. Results

3.1. Compositional analysis of ANEs

The liquid ANE formulations used in this study showed statistically significant differences in their concentration of solids and chemical composition (Table 1). ANE B showed the highest concentration of solids while ANE C displayed the lowest value, 1.5 and 2-fold more dilute than ANE A and ANE B formulations, respectively. On the whole, ANE A and ANE B formulations were primarily composed of uronic acids (representing mainly alginate), fucose, mannitol, laminarin, polyphenols and ash. The level of change of some of these components was not always proportional with the changes in total solids. For example, the concentration of mannitol, uronic acids or fucose in ANE A was found to be 15 to 86% higher than ANE B. On the other hand, our analysis showed that the amount of laminarin in ANE A was 1.5 fold lower than ANE B. The product composition pattern of ANE C showed similarities with ANE A and ANE B. However, this ANE contained lower amounts of carbohydrates such as fucose, uronic acids or mannitol while the presence of laminarin was not detected. All ANE biostimulants contained a high proportion of sulphate from the total ash content. Fucoidan is a sulphated, fucose rich, heteropolysaccharide that may be extracted from the cell wall of *A. nodosum*. Interestingly, the highest sulphate to fucose ratio was observed for ANE B (1.27) while ANE A showed the lowest value (1.06). The analysis of polyphenols, determined as phloroglucinol equivalents, indicated that ANE C and ANE B contained 2 to 3.4-fold higher amounts of this component on a dry weight basis than ANE A. The amount of unknown compounds for ANE A and ANE B was lower than 20% (w/w) while ANE C had the highest percentage of unidentified organic components (Table 1).

3.2. Effects of ANEs on tomato growth and chlorophyll content under drought stress

To evaluate the capacity for drought stress tolerance induced by

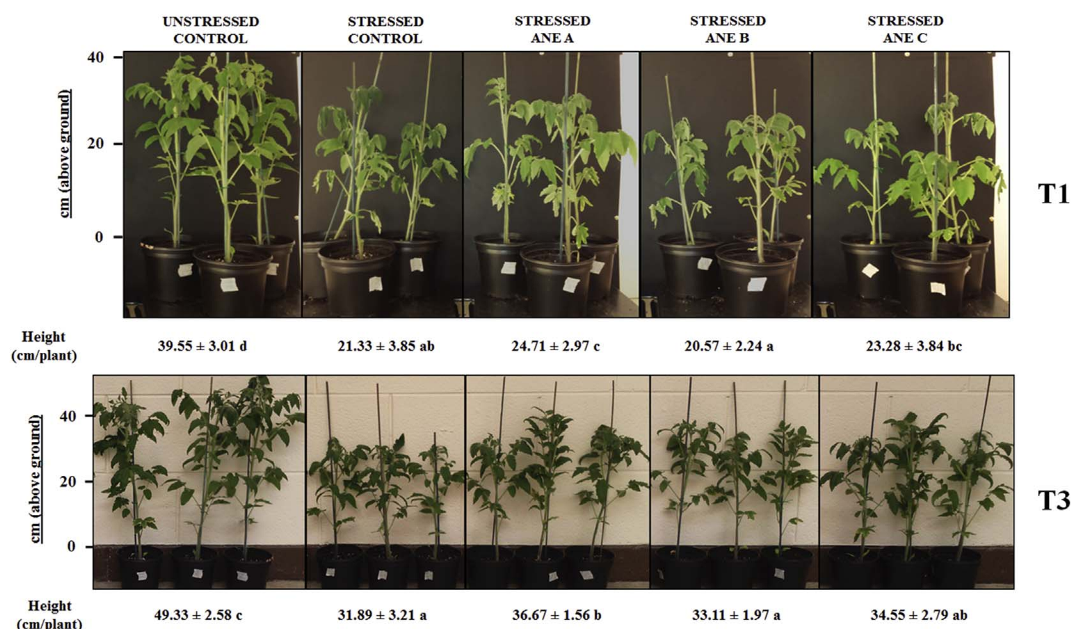

Fig. 1. Drought stress tolerance of ANEs-treated tomato plants. ANEs biostimulants and control (distilled water) treatments were applied to 35-old-day tomato plants (cv. Moneymaker) before subjecting them to drought stress by withholding water for 7 days. The stressed plants were rehydrated to allow recovery for 2 weeks. A second ANE biostimulant application was applied 24 h after re-watering the plants. Similar tomato plants were selected and grown under unstressed conditions for 56 days. Control treatments were applied by foliar spray as described above. A scale length above the ground is indicated on the left of the image to evaluate plant growth. Means followed by different small letter within the same row indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

Table 2
Effects of drought stress and ANE A on RWC in leaves of tomato plants (cv. Moneymaker).

Sampling time	Treatment		
	Unstressed Control[1]	Stressed Control	Stressed ANE A
T0	76.24 ± 0.70 a[2]	76.43 ± 1.16 a	76.48 ± 1.26 a
T1	76.58 ± 0.39 c	65.48 ± 0.14 a	73.05 ± 0.74 b
T2	76.54 ± 1.19 a	75.43 ± 1.16 a	76.00 ± 1.58 a

[1] Data are the means ± SD ($n = 9$).
[2] Different small letter within the same row indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

Table 3
Effects of drought stress and ANEs on growth parameters in 56-old-day tomato plants (cv. Moneymaker).

Treatment	Biomass	
	FW (g/plant)[2]	DW (g/plant)
Unstressed Control	29.71 ± 3.43 c[3]	4.07 ± 0.26 c
Stressed Control[1]	15.77 ± 1.21 a	1.80 ± 0.14 a
Stressed ANE A	19.98 ± 2.21 b	2.37 ± 0.26 b
Stressed ANE B	15.31 ± 1.65 a	1.70 ± 0.18 a
Stressed ANE C	20.27 ± 2.06 b	2.44 ± 0.25 b

[1] Drought stress was applied by withholding water for 7 days. The stressed plants were rehydrated to allow recovery for 2 weeks later.
[2] Data are the means ± SD ($n = 18$).
[3] Different small letter within the same column indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

ANE biostimulants, 35-old day tomato plants (cv. Moneymaker) were treated with three different commercial ANEs while control plants were sprayed with distilled water. After 7 days without watering, severe drought stress was evident in untreated plants compared to non-drought control plants (Fig. 1). Compared to unstressed control, the RWC (%) of drought untreated plants was decreased by 14.49% (Table 2). The difference in drought tolerance between the untreated and ANE A-treated drought plants was also remarkable (Fig. 1). While control plants showed severe wilting of all the leaves and plant growth inhibition, we found that ANE A-treated plants had less noticeable visual stress symptoms on leaves and significant higher plant height. On the contrary, plants treated with ANE B and ANE C displayed generalized wilting and a similar plant growth pattern compared to control. The RWC of ANE A-treated plants was also significantly higher under drought stress in comparison with control (Table 2). However, tomato plants treated with ANE B and ANE C were only able to maintain hydration level around 70% of RWC throughout the dehydration period. These phenotypical and physiological differences clearly illustrate the higher tolerance to severe water deficit induced by ANE A application compared to the other two commercial ANEs.

At the end of the recovery stage, after re-watering and applying the second ANE foliar application, an enhanced plant growth and greater foliar density was observed in both ANE A and ANE C-treated plants compared to untreated plants. Both growth parameters, above ground plant FW and DW, were significantly increased over control by between 25 and 30%. On the other hand, growth and biomass of ANE B-treated plants was almost identical to untreated drought plants and approximately 50% lower than unstressed control plants (Table 3). The effects of ANE treatments on unstressed tomato plants over the same growth period was also tested. Interestingly, a similar but not statistically significant increase of plant FW, ranging between 8.10 and 8.88%, was observed for the three commercial ANE biostimulants used in this study (Fig. S1).

Chlorophyll is one of the major chloroplast components for photosynthesis and the concentration of chlorophyll in leaves can be used as reliable indicator of metabolic and energetic imbalance in tomato plants under drought stress. The total foliar chlorophyll content was expressed with respect to dry weight. Since tomato leaves have a high composition of water, using fresh weight as a basis for expressing compositional results was considered less accurate because it was significantly affected by the dehydration and further rehydration stages of this experiment. Growth time markedly increased the total chlorophyll content in tomato leaves from T0 to T1 sampling points (Fig. 2). However, well-watered untreated plants exhibited significantly higher

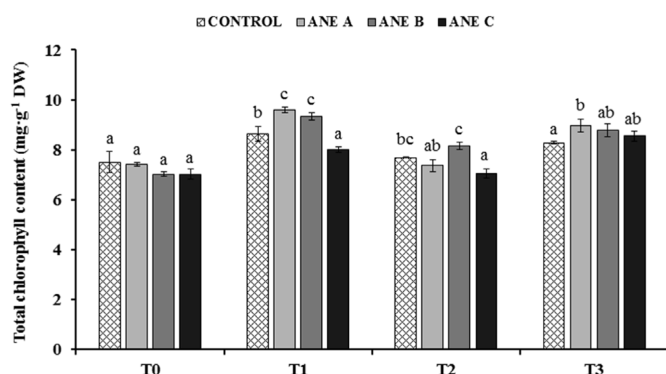

Fig. 2. Effect of drought stress and ANEs biostimulants on the total foliar chlorophyll content in tomato plants (cv. Moneymaker). T0: before first ANE biostimulant application; T1: 7 days of drought stress; T2: 2 days after the second ANE treatment on the 3rd day of the recovery stage; T3: end of the recovery stage. Means followed by different small letter indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

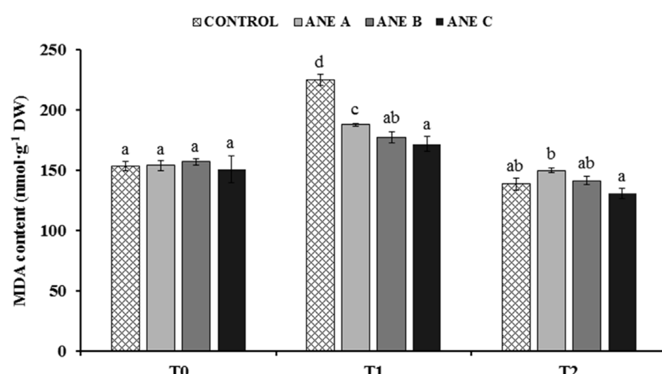

Fig. 3. Effect of drought stress and ANEs biostimulants on the MDA content in leaves of tomato plants (cv. Moneymaker). T0: before first ANE biostimulant application; T1: 7 days of drought stress; T2: 2 days after the second ANE treatment on the 3rd day of the recovery stage. Means followed by different small letter indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

levels than those observed in drought control plants after 7 days without watering (Table S1). Besides the water stress effect, the total chlorophyll content of tomato leaves also changed due to application of ANEs at the end of the drought period (Fig. 2). Both ANE A and ANE B-treated plants showed significantly higher chlorophyll content than the untreated drought plants (11 and 8%, respectively), while this parameter decreased up to 7.5% in plants treated with ANE C. After 2 days of the second ANE application and 3 days of re-watering to allow plant recovery, the total chlorophyll content was lowered to similar values observed at the start of the experiment for all the plants except for those treated with ANE B. The application of this biostimulant resulted in a significant chlorophyll content increase, exhibiting values that were between 10% and 14% higher with respect to tomato plants treated with ANE A and ANE C. The chlorophyll content did not mirror exactly the growth parameters chosen to test the effects of ANE treatments at the end of the recovery stage. Only the total chlorophyll content of ANE A-treated plants was significantly higher than in untreated drought plants by 9%. In contrast, no statistically significant changes in total chlorophyll content were detected for plants treated with ANE B and ANE C compared to drought control at T3 (Fig. 2).

3.3. Effects of drought stress and ANEs on lipid peroxidation in tomato leaves

Lipid peroxidation was measured in terms of MDA content. Accumulation of MDA is a typical symptom of membrane lipid damage under drought stress conditions and a noticeable increase of this parameter (up to 35%) was confirmed in untreated drought plants with respect to well-watered control (Table S1). MDA accumulation in ANE-treated plants under water stress was significantly decreased in all the treatments tested compared to control (Fig. 3). However, lowest values were found under the effect of ANE B and ANE C, representing a decrease of 30%. The formation of MDA equivalents reflected a decreasing kinetic after re-watering the plants. At the start of the recovery stage, all plants showed similar MDA content values and we did not observe statistically significant differences with respect to control. However, it was noted that MDA accumulation of plants treated with ANE C was significantly decreased by 15% compared to ANE A-treated plants (Fig. 3).

3.4. Effects of drought stress and ANEs on proline and soluble sugars content in tomato leaves

The osmoprotectant accumulation achieved under drought stress was measured by determining the variations in endogenous concentrations of proline and soluble sugars. After withholding water for 7 days, the leaf proline content in untreated plants accumulated 6.3-fold compared to control plants grown under unstressed conditions (Table S1). Each ANE treatment stimulated a significant increase of proline levels with respect to control under drought stress conditions (Fig. 4). However, while the proline concentration in tomato plants treated with ANE B and ANE C were slightly higher, showing values that were 13% and 20% of control values, ANE A-treated plants increased their proline levels by 2-fold. After re-watering and applying a second ANE foliar treatment, the concentration of proline decreased ostensibly in both control and ANE-treated plants. Interestingly, the abundance of this compatible osmolyte in plants treated with ANE A and ANE C increased by 2.1-fold and 1.3-fold in comparison to the ANE B-treated plants or corresponding controls, respectively (Fig. 4).

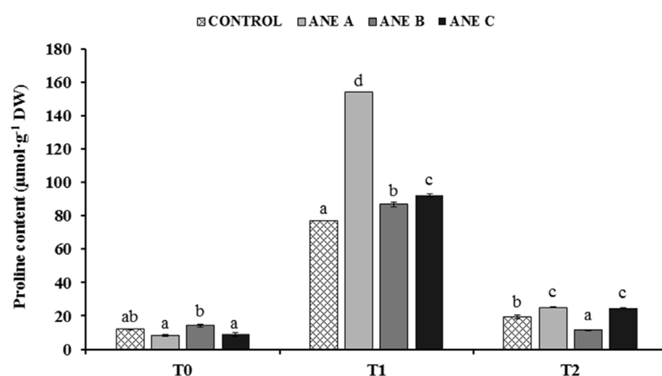

Fig. 4. Effect of drought stress and ANEs biostimulants on the proline content in leaves of tomato plants (cv. Moneymaker). T0: before first ANE biostimulant application; T1: 7 days of drought stress; T2: 2 days after the second ANE treatment on the 3rd day of the recovery stage. Means followed by different small letter indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

The soluble sugar content of tomato leaves was quantified by HPAEC-PAD after detecting glucose, fructose and sucrose as the main chromatographic peaks of plant extracts (Fig. S2). The results revealed that the total soluble sugar content, calculated as the sum of glucose, fructose and sucrose, ranged between 4.97 and 7.93 mg g^{-1} DW in plants growing under unstressed conditions before applying the first ANE treatment (Table 4). It was observed that although untreated plants induced a noticeable accumulation of soluble sugars after 7 days without watering; this accumulation was significantly higher in ANE A-treated plants by 1.3-fold. In response to drought stress, these treated plants increased foliar sucrose, glucose and fructose concentrations by 40%, 28% and 15% with respect to untreated plants, respectively. However, ANE B or ANE C treatment did not have any significant effect on the accumulation of total soluble sugars in plants affected by

Table 4
Effects of drought stress and ANEs on soluble sugars content in tomato leaves.

Sugar[1]	Sampling time	Treatment			
		Control[2]	ANE A	ANE B	ANE C
Glucose (mg·g^{-1} DW)	T0	1.35 ± 0.01 c	1.35 ± 0.02 c	1.19 ± 0.08 b	0.87 ± 0.02 a
	T1	6.68 ± 0.17 ab	8.53 ± 0.44 c	5.79 ± 0.28 a	6.72 ± 0.44 b
	T2	1.46 ± 0.01 c	1.03 ± 0.03 a	1.12 ± 0.01 b	1.68 ± 0.02 d
Fructose (mg·g^{-1} DW)	T0	3.15 ± 0.06 d	2.30 ± 0.05 c	2.11 ± 0.04 b	1.26 ± 0.01 a
	T1	11.33 ± 0.44 bc	13.02 ± 0.78 c	8.22 ± 0.60 a	10.65 ± 0.77 b
	T2	1.89 ± 0.06 b	2.52 ± 0.01 c	1.42 ± 0.03 a	3.09 ± 0.01 d
Sucrose (mg·g^{-1} DW)	T0	3.43 ± 0.06 c	3.39 ± 0.02 a	2.20 ± 0.10 b	2.84 ± 0.09 c
	T1	9.66 ± 0.35 ab	13.50 ± 1.03 c	10.18 ± 0.33 b	8.96 ± 0.28 a
	T2	3.59 ± 0.10 c	4.22 ± 0.02 d	2.81 ± 0.02 b	2.05 ± 0.02 a

[1] Data are the means ± SD (n = 9).
[2] Different small letter within the same row indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

drought stress, showing slightly lower values compared to untreated plants (24.19 and 26.33 mg g^{-1} DW, respectively, versus 27.66 mg g^{-1} DW). It was also noted that there was a significant decrease of glucose and fructose levels in plants treated with ANE B with respect to drought control. In line with the observed variations in endogenous proline levels, plant re-watering resulted in a pronounced decrease in the concentration of soluble sugars. This effect was more pronounced in plants treated with ANE B, showing 22–25% lower glucose, fructose and sucrose values than untreated plants. On the other hand, total soluble sugars or sucrose content of ANE A-treated plants was 12 and 18% higher compared to control after 2 days of the second ANE application and 3 days of re-watering, respectively.

3.5. Analysis of dehydrin isoform pattern in tomato leaves treated with ANEs under drought stress

Using a commercial polyclonal antiserum raised against the consensus K-segment of plant dehydrins, eight polypeptide bands were recognized in heat-stable protein extracts from leaves of tomato plants (cv. Moneymaker). These bands showed a molecular mass range determined by SDS-PAGE from 15 to 38 kDa (Fig. 5). The analysis of the thermostable fractions revealed that 3 low molecular weight polypeptides (15, 18, 25 kDa) and one 32 kDa molecular specie slightly accumulated in untreated plants during drought stress. The re-watering of these control plants resulted in an evident intensity reduction of most of the bands, although the dehydrin-like proteins of 34 and 38 kDa were present throughout the time of experiment and their relative band

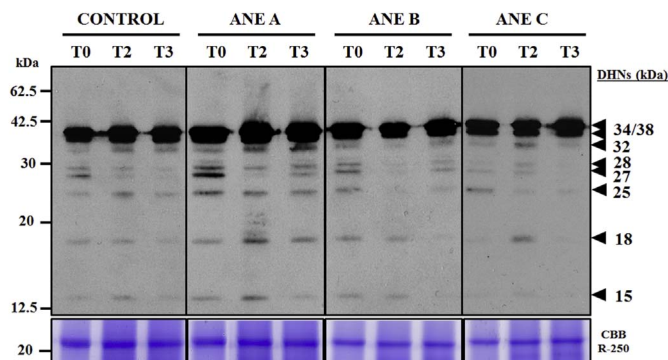

Fig. 5. Effect of drought stress and ANEs biostimulants on the dehydrin-like protein pattern in leaves of tomato plants (cv. Moneymaker). Boiling resistant protein extracts (4 µg of protein) were separated by SDS–PAGE (14% polyacrylamide) and transferred to nitrocellulose membranes probed with antisera raised against K-segment of dehydrins. The results shown are representative of three biological replicates. A duplicated gel stained with Coomassie Brilliant Blue R-250 (CBB R-250) was used as protein loading control. T0: before first ANE biostimulant application; T1: 7 days of drought stress; T2: 2 days after the second ANE treatment on the 3rd day of the recovery stage.

intensity remained relatively unchanged with respect to the different ANE treatments (Fig. S3A). In tomato plants treated with ANE A, prolonged drought stress conditions produced significant increases in the relative accumulation of the detected bands of 32, 18 and 15 kDa (Fig. S3B, S4B-C). The two lowest molecular weight dehydrin-like proteins reached maximum values, being 3.9 and 5.3-fold higher than those of untreated plants. Interestingly, we also detected the presence of three faint bands above the 18 kDa band. The presence of these additional bands were confirmed when we analysed the phosphorylation status of heat-stable fractions from tomato leaves (Fig. S4A). After re-watering and applying a second foliar treatment of ANE A, the abundance of the bands of 32, 28 and 27 kDa increased significantly between 1.7 and 2.4 fold with respect to untreated plants (Figs. S3B–D). Although the 18 and 15 kDa dehydrin like-proteins abundance decreased with respect to the water deprivation period, they maintained a significantly higher level of relative abundance compared to the control (Figs. S4B–C). As we can observe in Fig. 5, protein extracts from plants treated with ANE B exhibited a related accumulation pattern when compared to untreated plants. Whereas the bands of 32, 18 and 15 kDa maintained a similar level of relative abundance throughout the experiment (Fig. S3B, S4B-C), the dehydrin-like isoforms of 28, 27 and 25 kDa decreased sharply under drought stress (Fig. S3C-D, S4A). At the recovery stage, the relative abundance of these proteins of 28 and 27 kDa increased significantly with respect to untreated plants (Figs. S3C–D). The application of ANE C treatment also substantially affected the accumulation of the 18 kDa dehydrin-like protein, which relative abundance increased by 2-fold of that in untreated plants under drought stress (Fig. S4B). As also observed in ANE-B-treated plants, the detected bands of 28, 27 and 25 kDa decreased significantly their relative abundance levels (Fig. S3C-D, S4A). Re-watered plants treated with ANE C maintained similar accumulation levels of most of the detected polypeptides compared to control.

The presence of post-translational phosphorylation was estimated by evaluating the gel mobility shift of immunoreactive bands after alkaline phosphatase treatment of these heat stable fractions (Fig. S5A). The immunoblot performed on SDS-PAGE from samples of ANE A-treated plants under drought stress showed that most of the detected dehydrin-like protein appear to be phosphorylated. For example, the bands detected around 18 kDa ran at slightly lower mass when the protein extract was treated with alkaline phosphatase. This difference of molecular mass can be due to the phosphorylation of these dehydrin-like proteins. On the other hand, the relative amount of the other detected proteins between 34 and 25 kDa decreased significantly while a new polypeptide was detected at 55 kDa. Analyses performed under reducing conditions and SDS-PAGE electrophoresis, followed by Western blotting, did not show the presence of gel mobility shifts from the dehydrin-like proteins (Fig. S5B).

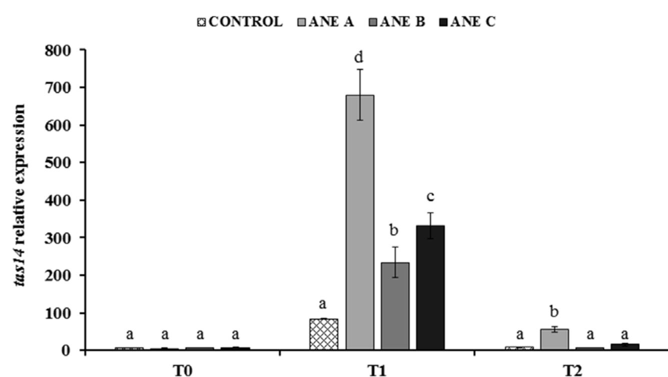

Fig. 6. Effect of drought stress and ANEs biostimulants on the *tas14* dehydrin gene expression in leaves of tomato plants (cv. Moneymaker). Results were expressed as the relative fold-change with respect to the *actin* gene expression levels. The results shown are representative of three biological replicates. T0: before first ANE biostimulant application; T1: 7 days of drought stress; T2: 2 days after the second ANE treatment on the 3rd day of the recovery stage. Means followed by different small letter indicate significant differences between treatments based on Tukey-HSD test ($p \leq 0.05$).

3.6. Analysis of tas14 dehydrin gene expression in tomato leaves treated with ANEs under drought stress

In order to examine whether drought stress and different ANE biostimulants affected the regulation of *tas14* dehydrin isoform at the transcriptional level in tomato leaves, relative changes in gene expression were analysed by qRT-PCR using specific primers for this transcript (Fig. 6). At the start of the experiment, the relative gene expression of *tas14* with respect to the reference gene *actin* was found at low constitutive levels and no statistically significant differences were observed between plants. Even though *tas14* gene expression was clearly induced in all plants subjected to drought stress, ANEs induced a significant up-regulation compared to untreated plants. Overall, the *tas14* transcript level of ANE A-treated plants reached the highest level (8-fold of control levels), showing values that were 290% and 205% of expression levels in plants treated with ANE B and ANE C, respectively. Although *tas14* mRNA levels were quickly down-regulated after re-hydration for 48 h, a noticeable accumulation of this transcript was observed in plants treated with ANE A twice, showing values that were between 3.5 and 7.7 times higher than those observed in the rest of the treatments.

4. Discussion

The impact of drought on agricultural production in an era of increasing CO_2 levels with concurrent global climate change is difficult to predict, plan for and reduce, due to the numerous factors involved. The utilisation of speciality crop inputs such as biostimulants in crop husbandry practice, which are sustainable and environmentally friendly, are likely to prove popular in meeting this challenge. Developing knowledge of the mode of action and robustness of biostimulants for meeting challenges, such as drought, is important to build credibility and acceptance in agricultural practice. In this study we observed significant differences in the ability of three ANE biostimulants of varying composition to induce tomato plant tolerance to an acute drought stress. Reduction in plants growth rate under soil water deficit is common to many plant species and our drought stress conditions confirmed a plant biomass decrease of approximately 50% between stressed and unstressed untreated plants. However, two of the three ANE biostimulants (ANE A and C) significantly enhanced plant fresh and dry weight at the end of the recovery period. In addition, ANE A reduced water loss levels by 11.6% when compared to the drought control and maintained better plant growth without wilting damage on leaves after 7 days without watering. A study on soybean plants highlighted the ability of ANE biostimulants to provide drought tolerance (Martynenko et al., 2016). The ANE was shown to regulate leaf temperature and was suggested to do this through stomatal control. An additional study by Spann and Little (2011) on Hamlin sweet orange further supported the role of ANE biostimulants in reducing the effects of drought stress in greenhouse grown citrus trees. This study found that a 50% deficient irrigation reduced the shoot growth of container-grown citrus nursery trees by approximately 30% compared to fully irrigated control trees, but ANE biostimulant treatments prevented most of this growth reduction, and maintained tree growth at levels similar to the fully irrigated trees (Spann and Little, 2011). The reported level of growth enhancement under drought conditions in these studies is similar to that reported here.

The results of the ANE biostimulant compositional analysis and drought stressed plant phenotype data were not found to be related. A study by Santaniello et al. (2017) also reported on an ANE biostimulant providing tolerance to drought in *Arabidopsis thaliana*, with increased plant survival the measured phenotype. However, it is difficult to make a comparison of the compositions between the 2 studies as different components have been analysed. There is very little data published linking chemical composition with biostimulant activity and changes at the molecular level within the plant. The European Union REACH registration for seaweed extract has identified alginate, fucoidan, laminaran, mannitol and polyphenols as key components within seaweed extracts (Authority, 2012). However, the relationship between the concentration and their physicochemical characteristics to biostimulant activity is poorly understood. There are some reports relating ANE biostimulant components such as alginate oligosaccharides (Liu et al., 2013) and mannitol (Gerszberg and Hnatuszko-Konka, 2017) to drought tolerance in different crop species. The compositional variation of the three different ANE biostimulants used in this study clearly demonstrates the level of heterogeneity that exists within this category of biostimulant.

It is generally accepted that leaf yellowing is the first visual symptom of drought induced leaf senescence in different plant species. Leaf yellowing is the result of chlorophyll degradation in senescing leaves. The effect of drought stress on chlorophyll metabolism has been the subject of controversy and conflicting results have been reported depending on the plant material, and the experimental procedures used for investigations (Cornic and Massacci, 1996). Compared to unstressed tomato plants at the same developmental stage, the drought protocol implemented in this study negatively impacted the total chlorophyll content per gram of dry plant tissue. The most significant differences in chlorophyll levels between water stressed plants were observed at T1, during drought stress, and T3, the recovery period post the stress event. Above all, ANE A-treated plants had the highest chlorophyll levels over both these periods. Other ANE biostimulants have previously been shown to induce photoprotective defence systems under short-term periods of severe drought stress (Santaniello et al., 2017) or enhance leaf chlorophyll content of plants from different economic crops (Blunden et al., 1996). The chlorophyll content of wheat plants treated with short-chain alginate oligosaccharides, one of the main carbohydrates observed in ANEs, was also significantly increased during a water deficit period (Liu et al., 2013). Many studies indicate the “stay-green” trait (higher chlorophyll content) is associated with improved yield and transpiration efficiency under water-limited conditions in sorghum and wheat (Borrell et al., 2000; Verma et al., 2004). Increasing the chlorophyll levels after any water deficit may be positive for the re-establishment of the photosynthetic capacities of the leaves, and in combination with other metabolic processes, lead growth recovery.

The 3-carbon dialdehyde MDA is one of the fragmentation products from the oxidation of polyunsaturated fatty acids in cell membranes by ROS. This metabolite is considered harmful to the plant cells and it has been well established that most MDA in leaf tissue originates in mitochondria and chloroplasts membranes, organelles with highly oxidative metabolism and high percentages of polyunsaturated fatty acids (Mano, 2012). Plant cells counter the cascades of uncontrolled lipid

peroxidation under drought stress with multiple protection mechanisms such as low molecular mass antioxidants or the activation of antioxidant enzymes (Gill and Tuteja, 2010). The consistent decrease of MDA accumulation in all ANE-treated tomato leaves under drought stress reflected that these biostimulants products can provide a further layer of protection. Previous studies have indicated the effectiveness of ANE biostimulants in enhancing the antioxidant system and/or reducing lipid peroxidation incidence in some crops subjected to drought stress as compared to control plants (Spann and Little, 2011; Elansary et al., 2016, 2017; Santaniello et al., 2017). The incomplete chemical composition of these ANEs reported in these studies did not allow us to establish commonalities with the three ANE biostimulants used in this work, but it is very likely that a pool of common bioactive molecules extracted from *A. nodosum* can provide an unspecific and basal enhancement of the antioxidant machinery of treated plants. This hypothesis is supported by other studies in unstressed plants treated with ANEs where the expression levels of genes encoding antioxidant enzymes were differentially upregulated (Goñi et al., 2016) and enhanced accumulation of antioxidant phytochemicals (Lola-Luz et al., 2014) was observed. Interestingly, Liu et al. (2013) also demonstrated that the exogenous application of alginate oligosaccharides promoted antioxidant enzymes activities and decreased content of MDA under drought stress. However, the ability of all ANE treatments to counteract the lipid peroxidation surge did not translate to enhanced accumulation of biomass in all cases when subjected to drought stress.

Accumulation of proline under stress in many plant species has been correlated with stress tolerance, and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants. However, proline accumulation cannot be regarded as a specific marker for drought tolerance, as its accumulation represents a general response to various abiotic stresses in plants (Hayat et al., 2012). In the present study, free proline content in the leaf tissues of tomato plants cv. Moneymaker treated with ANE A under drought stress was significantly increased when compared with untreated plants. ANE A induced the highest levels of proline and was significantly higher than ANE B and C. High levels of proline enable plants to maintain low water potentials, allowing additional water to be taken up from the environment, thus buffering the immediate effect of water shortages (Szabados and Savouré, 2010). Patanè et al. (2016) reported that tomato genotypes more sensitive to soil water deficit responded to drought stress through less proline in leaves. Although one recent investigation attests to positive effects of ANE treatment as a foliar spray on endogenous accumulation of proline in turfgrasses in response to drought and salinity stress (Elansary et al., 2017), another report suggests lack of such positive effects on drought stressed medicinal plants treated with the same commercial ANE as a soil drench (Elansary et al., 2016). Hence, significant proline accumulation cannot be considered as a common response to ANE application in crops subjected to drought stress, and the extent of its accumulation may depend on the biostimulant used, application type or crop class. In the post drought stress period (T2), ANE A and C-treated plants had significantly higher levels of endogenous proline than the control and ANE B, which correlates with the biomass accumulation at the end of the protocol. These positive results may play an essential role for tomato plant recovery from drought stress. Availability of total soluble sugars has been also used as a physiological measure of drought stress tolerance, because carbohydrates provide energy and solutes for osmoprotectant accumulation. Only ANE A was found to significantly increase the concentration of glucose and sucrose during the drought period (T1) of the protocol. Sucrose and glucose either act as substrates for cellular respiration or as osmolytes to maintain cell turgor, while fructose is not related to osmoprotection and seems related to secondary metabolite synthesis (Rosa et al., 2009). Therefore, sucrose and glucose enrichment in ANE A treated tomato plants may play an important role in the osmoprotectant accumulation at a cellular level when plants are under acute drought conditions. One recent investigation also found the positive effects of ANE on endogenous accumulation of total nonstructural carbohydrates in turfgrass subjected to drought stress, however the carbohydrate compositional analysis was not specified (Elansary et al., 2017).

Besides the accumulation of proline and soluble sugars, which is one of the most commonly found metabolic responses of higher plants to water deficit, plants are also able to respond and adapt to drought stress through the synthesis of specific stress-induced proteins as part of that stress tolerance mechanism (Gerszberg and Hnatuszko-Konka, 2017). LEA proteins constitute a superfamily of proteins that are very hydrophilic, heat stable and markedly induced during water and cold stress or by exogenous ABA, suggesting a protective role during water limitation. LEA proteins are involved in many functions, including prevention of membrane leakage, membrane and protein stabilisation, protection of cytosolic structures, and maintenance of water balance and ion sequestration. Among these proteins, dehydrins (group II LEA) have been extensively studied in relation to drought and cold stresses (Olvera-Carrillo et al., 2011). Recently, a comprehensive comparative genomic analysis have identified a total of 27 LEA family members in tomato dividing them into 7 groups based on sequence similarities. Among these LEA genes, 6 of them had the dehydrin domain constituted for 3 specific motifs and showed predicted molecular weights rangeing between 8.87 and 25.37 kDa (Cao and Li, 2015). Our characterization of the stress-induced accumulation of dehydrins in tomato leaf using a commercial antibody against the conserved dehydrin K-segment (EKKGIMDKIKEKLPG or similar) revealed the differential accumulation of eight dehydrin-like isoforms with apparent molecular weights between 15 and 38 kDa. Considering that the specificity of the antibody recognition was previously demonstrated with a synthetic consensus K peptide (Navarro et al., 2015) and the dehydrin immunodetection was performed in heat stable protein extracts, the presence of polypeptides with higher apparent molecular weights may suggest the presence of post-translational modifications or the formation of oligomeric structures that slow their gel migration. Several dephosphorylation states have been reported in dehydrin and dehydrin-like proteins expressed in tomato and table grapes (Godoy et al., 1994; Navarro et al., 2015), demonstrating that phosphorylation strongly affects their gel mobility and the different isoforms are resolved as discrete bands. In this study, comparisons of protein migration before and after alkaline phosphatase treatments for detected dehydrin-like proteins showed that 18, 25 and 34 kDa polypeptides were likely phosphorylated. Different multimeric complexes of LEA proteins have also been experimentally detected (Hernández-Sánchez et al., 2014). Our reducing electrophoresis analysis was carried out in the presence of SDS-buffer and 2-ME but none of the bands detected migrated differentially. Although it is reasonable to hypothesize the absence of strong intermolecular and/or intramolecular disulphide bonds which leads to the formation of oligomers; reducing conditions are not always enough to disrupt dimers into monomers at high concentrations of dehydrin proteins (Still et al., 1994).

The expression of the eight dehydrin-like proteins constitutively expressed in leaves of tomato plants were highly regulated by both the drought stress and the ANE biostimulant applied. Whereas the prolonged exposure to water deficit determined an increase in the accumulation levels of 15, 18, 25 and 32 kDa polypeptides, showing that these isoforms are drought responsive, a noticeable intensity decrease of these bands was also observed in the post-stress drought period. The results obtained also revealed that the dehydrin-like profiles of ANE-treated tomato plants differed appreciably from one another. Only ANE A treatment altered considerably the dehydrin response in drought stressed tomato plants. With the exception of the 25 kDa band, this biostimulant maintained significantly higher levels of the drought responsive dehydrin-like proteins in treated tomato plants. Moreover, the enhanced accumulation of these dehydrin-like proteins detected at recovery stage of the ANE A-treated plants was most likely as a result of a second application at one day after re-watering. Despite the putative role of the tomato dehydrin genes, which typically accumulate and up-

regulate during drought stress (Godoy et al., 1994; Cao and Li, 2015; Iovieno et al., 2016), a direct correlation between the accumulation of these LEA genes and enhancement of drought stress tolerance has only been demonstrated for the *tas14* gene (Muñoz-Mayor et al., 2012). Plants overexpressing *tas14* gene achieved improved long-term drought tolerance on the basis of shoot biomass and fruit yield without affecting plant growth in unstressed conditions. As previously described for the dehydrin isoform pattern, the transcription of *tas14* was highly up-regulated by water withholding and pretreatment with ANE A compared to control, the effect of ANE B treatment was significantly lower. However, the effect of ANE C on the expression of this dehydrin gene can be considered intermediate between these two previous ANE biostimulants. Tomato *tas14* gene encodes a set of distinct polypeptides of molecular masses between 19 and 22 kDa due to their different phosphorylated forms (Godoy et al., 1994). According to its apparent molecular weight, phosphorylation status and accumulation pattern observed in the immuno-analysis in control and ANE-treated plants, the polypeptide of 18 kDa could be identified as TAS14 dehydrin. Thus, the differential expression of this stress protective protein in the ANE-treated tomato plants highlights the fact that these plants are indeed experiencing different degrees of drought stress tolerance, as a consequence of the ANE treatment itself.

5. Conclusions

Clear phenotypic differences were observed between ANE formulations at the end of the drought period with ANE A maintaining better plant growth without symptoms of drought stress. Physiological measurement of osmolytes support a metabolic/physiological basis to the effect. Gene transcription and proteomic analysis of stress protective proteins support a potential mode of action in providing this tolerance. Although there are similarities between 2 of the ANEs in terms of their impact on the measured markers, the intensity of the tolerance appears to be different with ANE A providing stronger tolerance than ANE C. Taken together, our results highlight that despite the ANE biostimulants being manufactured from the same raw material, their ability to maintain crop productivity during drought stress was not the same.

Contributions

OG, PQ and SOC designed the experiment; OG and PQ performed experiments; OG, PQ and SOC contributed in validating, writing, and approving the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.02.024.

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