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The potential use of *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* as biostimulants on maize plants

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ABSTRACT

Nitrogen deficiency and drought stress are among the major stresses faced by plants with negative consequence on crop production. The use of plant biostimulants is a very promising application in agriculture to improve crop yield, but especially to prevent the effect of abiotic stresses. Algae-derived biostimulants represent an efficient tool to stimulate the root development: while macroalgae have already been widely adopted as a source of biostimulants to improve plants growth and resilience, far less information is available for microalgae. The objective of this work is to investigate the stimulant ability on maize roots of two green algae species, *Chlamydomonas reinhardtii* and *Chlorella sorokiniana*, being respectively the model organism for *Chlorophyta* and one of the most promising species for microalgae cultivation at industrial scale. The results obtained demonstrate that both *C. reinhardtii* and *C. sorokiniana* cells promoted the development of maize root system compared to the untreated negative control. *C. sorokiniana* specifically increased the number of secondary roots, while improved micro-nutrients accumulation on roots and shoots was measured in the case of *C. reinhardtii* treated plants. When these microalgae-derived biostimulants were applied on plants grown in stress conditions as nitrogen deficiency, improved development of the root system was measured in the case of plants treated with *C. sorokiniana* biomass. Microalgae cultivation for biostimulant production can thus be considered as a bio-based process providing solutions for improving plant resilience toward stress conditions.

1. Introduction

Intensive agriculture, together with other anthropic activities, can contribute to the damaging of soil fertility and increased pollution of the environment, especially air and water. Meeting human needs without compromising Earth system resilience poses the challenge to redesign agricultural technologies and practices. In the last decade, the interest for the positive effects on the plant growth and production mediated by the use of biostimulants has increased [1–4]. Biostimulants were described by du Jardin [5] as: "substances or materials, with the exception of nutrients and pesticides, which, when applied to plants, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes in plants in a way that provides potential benefits to growth, development, or stress response." Furthermore, the recent EU regulation 2019/1009 states that: "plant biostimulant" means a product stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: (a) nutrient use efficiency; (b) tolerance to abiotic stress; (c) quality traits; (d) availability of confined nutrients in soil or rhizosphere. In the scientific literature, four major groups of biostimulants have been described: 1) humic substances, 2) protein hydrolysates and amino acid formulates, 3) seaweed extracts and 4) plant-growth-promoting microorganisms [6]. The use of macro-algae (seaweed) extracts as biostimulants has been reported up from early human civilization [2,7]. Macro-algae improve seedling development, flowering and the resistance to several abiotic stresses, since their extracts are composed by vitamins, phytohormones, polysaccharides, fatty acids and phenolic compounds [2,8]. Brown macroalgae have been widely used to produce different commercially available products [9]. However, these macroalgae species are harvested from coastal regions or cultivated directly on the sea, being their biomass and chemical properties subjected to alterations due to the environmental conditions; moreover, the contamination of waters has reduced the suitable areas for their cultivation [10].

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The need to reduce the cost and to have a more controlled and sustainable system of production of macroalgae led to focus on microalgae [10,11]. Microalgae can be easily grown using open-pound systems or using more controlled and secure photobioreactor systems exploiting the autotrophic growth [10,12]. Industrially, several species of these micro-organisms have been widely used as food additives [13], for the production of lipids and antioxidant [14,15] and also as organisms involved in the bioremediation of wastewaters [16]. Co-cultivation of microalgae and plants in hydroponics systems resulted in improved productivity due to the onset of positive metabolic interactions [17]. In agriculture, microalgae-derived extracts showed several biostimulants activities on plants, improving their germination [18] and nutrient uptake [19,20], affecting the biomass production [20–24], inducing the expression of root traits [11,19,25] and increasing the abiotic stresses resistance [25–27] on different plant species such as lettuce [21], tomato [18,20,22,23,25,27], sugar beet [11] and wheat [19,24,26]. Different microalgae species were investigated for their biostimulant properties, from cyanobacteria [20,26,28], to eukaryotic species as the green algae *Dunaliella* [27,29], *Chlorella ellipsoidea* [20,26], *Chlorella vulgaris* [11,19,21], *Chlorella sorokiniana* [20,24], *Scenedesmus quadricauda* [11], *Acutodesmus Dimorphus* [18], *Nannochloris* [25] or diatoms as *Phaeodactylum tricornutum* [29]. Both marine and freshwater microalgae species can be considered as alternative source of biostimulants. However, in order to conceive a possible production of microalgae-based biostimulants locally near to the cultivated fields, the use of freshwater strains appear more suitable, potentially using the same water source adopted for crops irrigation for the preparation of the growth medium.

In this work two freshwater microalgae species, *Chlamydomonas reinhardtii* (CR) and *Chlorella sorokiniana* (CS), were tested for their biostimulant actions on hydroponically grown maize seedlings. CS was chosen among other species because it represents one of the most promising strain for industrial cultivation of microalgae [30–33]. In the case of CR, this species was chosen being the model organism for green algae, recently emerging as a sustainable platform for production of biocommodities [17,34–38]: investigation of its possible use to produce biostimulants could pave the way toward sustainable biorefinery process for the valorization of the biomass produced. Differently from previous work reporting the possible biostimulant activity of microalgal biomass, here we focused on the effects of microalgae-based treatments in abiotic stress conditions, as nitrogen (N) and water deficiency, to evaluate their ability to enhance the maize plants resistance to these abiotic stresses, that can affect the crop production. Biostimulant activity of microalgae have been usually associated with release of active peptides, polysaccharides or phytohormones. Indeed, cell breakage is one of the key but also costly point for the overall process in biostimulant production: different methods have been reported to weaken or remove the cell wall in microalgae through physical, chemical or enzymatic treatment [10]. The biostimulant properties of *Chlamydomonas reinhardtii* (CR) and *Chlorella sorokiniana* (CS) extracts obtained by acid hydrolysis were recently reported on tomato plants [20]. Here, we tested the biostimulant properties of lyophilized powders deriving from CR and CS intact cells in comparison with cells treated by physical methods to partially disrupt their cell wall.

2. Material and methods

2.1. Preparation of algae fresh cultures and their treatments

Chlamydomonas reinhardtii 4a + (CR) and *Chlorella sorokiniana* UTEX 1230 (CS) were obtained from the Culture Collection of Algae at Goettingen University (Germany, http://sagdb.uni-goettingen.de/). Microalgal cells were grown in 1 L flasks in a climatic chamber at a 16 h light/8 h dark regime at 22 °C/18 °C and light intensity (120 μmol m^{-2} s^{-1} PPFD) using the TAP (Tris Acetate Phosphate) culture medium [39,40]. When they reached the concentration of 1 * 10^7 cell/mL, the fresh culture was centrifuged, washed three times with water to remove the dissolved salts present in the growth medium and about half of the biomass was freeze-dried (LIO5P 4K, 5Pascal, Italy), obtaining the powders of the fresh cultures of CR and CS, referred as CRW and CSW, respectively. The remaining biomass of the fresh culture was treated to disrupt the cellular wall and membrane. CR was blended for 10 min for three times, following the indications of McMillan et al. [41], obtaining the CR + B preparation. The fresh culture of CS was placed in 2 mL tubes together with glass beads, centrifuged for 1.5 min twice and freeze-dried, obtaining the preparation referred as CS + B. The efficiency of the cell disruption step was analyzed by chlorophyll extraction in acetone 80% for CR [34,42] and DMSO for CS [43] by comparing the absorption spectra of pigments extracted before or after a centrifugation step at 1000 ×*g* for 3 min where the pellet was discarded. This centrifugation step essentially removed intact cells from the CS + B and CR + B samples. Protein quantification of all the lyophilized preparations was performed using the Micro BCA™ Protein Assay Kit (Thermo Fischer Scientific), after resuspension in water. All the preparations were normalized to the same amount of chlorophyll. Total N and C was determined using a CHN analyzer (CHN IRMS Isoprime 100 Stable Isotope Ratio Mass Spectrometer, Elementar, Como, Italy).

2.2. Plant growth conditions

2.2.1. Microalgae treatments

The maize seeds (*Zea mays* P0943, Pioneer Hi-Bred Italia Sementi S. R.L.) were germinated for 72 h at 26 °C and 100% relative humidity in the dark after soaking in water for 24 h. The seedlings were then transferred to a climatic chamber at 16-h light/8-h dark regime at 24 °C/18 °C, 40–50% relative humidity and light intensity (200 μmol m^{-2} s^{-1} PPFD). The seedlings (six per 2-L pot) were put in 0.05 mM $CaSO_4$ solution (1.8 L) for 24 h; after that the different treatments were performed for 5 days using a nutrient solution containing 100 μM $MgSO_4$, 400 μM $CaSO_4$, 200 μM K_2SO_4, 5 μM KCl, 175 μM KH_2PO_4, 25 μM $NH_4H_2PO_4$, 0.2 μM $MnSO_4$, 2.5 μM H_3BO_3, 0.2 μM $ZnSO_4$, 0.05 μM $CuSO_4$, 0.05 μM $NaMoO_4$ and 2 μM Fe-EDTA [44,45], supplemented with the different algae fresh cultures and preparations applied at the same rate equal to 2 mg C_{org} L^{-1} [11]. Considering the N content in microalgae biomass (Supplementary Fig. S1), plant treatments with CR or CS caused ad addition of ~33 μM of total N to nutrient solution. Maize seedlings were also treated with a commercial extract derived from the macroalgae *Ascophyllum nodosum* (MC EXTRA, Valagro, Italy), referred as MA. According to manufacturer specification MA was composed of 20% organic C and 1% organic N: MA was thus applied at the same rate of 2 mg C_{org} L^{-1}. An addition of 7.1 μM of total N to nutrient solution was caused by MA treatment. Negative controls (C) were obtained growing maize seedlings in the same nutrient solution and conditions without any algae treatment. The experiment was repeated three independent times (N = 3, biological replicates).

2.2.2. Low and high N tolerance experiment

The maize seeds were germinated, and seedlings were grown for 24 h in 0.05 mM $CaSO_4$ as described above. Then, seedlings were grown for 7 days using a nutrient solution containing 100 μM $MgSO_4$, 200 μM K_2SO_4, 5 μM KCl, 175 μM KH_2PO_4, 25 μM $NH_4H_2PO_4$, 0.2 μM $MnSO_4$, 2.5 μM H_3BO_3, 0.2 μM $ZnSO_4$, 0.05 μM $CuSO_4$, 0.05 μM $NaMoO_4$ and 2 μM Fe-EDTA. $Ca(NO_3)_2$ was added to the nutrient solution at the final concentration equal to 0.1 mM for low N condition and equal to 10 mM for the high N one. The treatments with CSW, CS + B and MA were carried out adding a quantity of each product in order to use the same dose of C_{org} equal to 2 mg C_{org} L^{-1} [11]. For each treatment (CSW, CS + B and MA) a control (CSW C; CS + B C and MA C) was prepared treating maize seedlings grown in nutrient solution with the same composition and balancing the extra amount of N supplied with the algae treatment with an equal amount of $NH_4H_2PO_4$. This quantity was calculated based on total N of each algae product (MA, CSW and CS + B). The experiment

was repeated three independent times (N = 3, biological replicates).

2.2.3. Drought tolerance experiment

The maize seeds were germinated and grown as before described for microalgae treatment experiments. The nutrient solution was supplemented with 10% of polyethylene glycol (PEG, MM 6000, Sigma-Aldrich) in order to induce an osmotic stress [46]. The algae treatment was carried out supplying the CSW, CS + B and MA at the same rate equal to 2 mg C_{org} L^{-1} [11]. In addition, some maize seedlings were treated only with PEG (PEG) and other grown without PEG and algae treatments (C). The experiment was repeated three independent times (N = 3, biological replicates).

2.3. Phenotypic analysis of maize seedlings

The length, surface area, volume and secondary roots number of the total root system (primary, seminal and lateral roots) were analyzed using the WinRHIZO™ scanner and software [47]. The shoot height, the fresh and the dry weight of both roots and shoots were measured for six plants for each experiment. Photosynthetic parameters like relative chlorophyll, Fv/Fm, differential leaf temperature, non-photochemical quenching and the quantum yield of photosystem II were analyzed using the MultispeQ V2.0 instrument and software [48].

2.4. Analysis of macro- and micronutrients

The content of macro- (Mg, P, K, Ca) and micronutrients (Mn, Fe, Cu, Zn) in roots and shoots were determined using an inductively coupled plasma-mass spectrometry (ICP-MS). Maize roots and shoots were rinsed with deionized water, blot-dried and air-dried for 72 h in a hot air oven at 60 °C. Plant tissue samples were weighted (about 10 mg) and digested with 350 μL of 69% HNO_3 in a 3-mL TFM microsampling insert (Milestone SRL). Three inserts were placed in a 100-mL vessel containing 10 mL of deionized water and 1 mL of 30% H_2O_2. The digestion was carried out at 180 °C for 20 min with a microwave oven (StartD microwave, Milestone SRL). The NIST 1515 (apple leaves) was used as standard reference material. The samples were diluted to 2% HNO_3 with sterile deionized water and analyzed using the Agilent 7500cx ICP-MS (Agilent). A custom multi-element standard solution (Romil LTD) was used to quantify each element.

2.5. Statistical analysis

Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test using the GraphPad Prism 7 (GraphPad Software). Statistically significant variations with a p value <0.05 are marked with letters.

3. Results

3.1. Microalgae preparations effects on the maize root system

CS and CR microalgae species were grown under autotrophic conditions in closed photobioreactors to produce biomass to be used as potential biostimulant product. Biostimulant properties of CS and CR were tested on maize seedling applying untreated cells or samples upon physical treatment to partially disrupt the cell wall. Microalgal cells were harvested at the end of the growth curve by centrifugation: part of the culture was freeze dried and used for plant treatments herein called CRW and CSW. Alternatively, harvested cells were physically treated to break the cell wall to improve the release of the cellular content and then: CR, which has a very weak cell wall, was treated by blending [41], whereas CS, which has a cell wall much stronger and resistant than CR, was treated by using glass beads as detailed in the Methods section. The efficiency of the cell disruption was analyzed measuring the chlorophyll content in intact cells compared to the chlorophyll content in the sample obtained after cell treatment and removal of unbroken cells (Supplementary Fig. S1). According to the results obtained, respectively 64.5% and 80.6% of broken cells were obtained for CR and CS by physical treatments (Supplementary Fig. S1A, B). Samples composed of disrupted cells where thus freeze dried and used for plant treatments, herein called CR + B and CS + B.

All the microalgae preparations (CRW, CSW,CR + B and CS + B) were analyzed to determine the level of C and N (Supplementary Fig. S1D): these values were then considered in order to formulate plants treatments at the same organic carbon concentration (2 mg of C_{org} L^{-1}) as reported by Barone et al. [11]. Considering the N content in microalgae biomass (Supplementary Fig. S1), plant treatments with CR or CS caused ad addition of ~33 μM of total N: it is thus possible to consider the biofertilizer effect of microalgae-based treatment as negligible. Biostimulant activity of macro- or microalgae based products has been usually associated to peptides released by the algal cells [10,49,50]. The amount of proteins of the supernatant after resuspension in water of the freeze dried products obtained (CRW, CSW, CR + B and CS + B), was analyzed as reported in Supplementary Fig. S1C. Samples composed of partially broken cells (CR + B and CS + B) were characterized by an higher protein release compared to intact cells (114,3% and 137,2% respectively), as a result of the partial degradation of the cell wall. As in the case of chlorophyll extraction, also in this case CS was characterized by an higher relative increase of proteins in the supernatant, confirming that the glass beads method lead to a better cells disruption compared to the blending.

Microalgal preparations were thus applied on maize seedlings hydroponically grown for 5 days (Fig. 1A). Moreover, a commercial biostimulant product, composed of macroalgae (MA) extract was applied for comparison at the same organic carbon concentration. Control condition (C) consisted in the growth of seedlings in the nutrient solution (N content was 25 μM $NH_4H_2PO_4$) without any algae treatment.

Fresh (FW) and dry (DW) weights of both roots and shoots were generally increased upon treatments with macroalgae extract (MA) or microalgae-based biomass (CRW, CR + B, CSW, CS + B) compared to the control case, even if the observed differences were not statistically significant (Supplementary Fig. S2). We thus evaluated the effects of the different treatments on roots apparatus measuring the number of secondary roots (<0.5 cm), the total root area, length, and volume (Fig. 1B–E). The commercial macroalgae-based biostimulant product herein tested (MA) improved root area and root volume compared to control (C), while secondary roots number and root length were not affected. Plants treated with CRW and CR + B showed intermediate values between those of control (C) and MA treatment in the case of root area and root volume (Fig. 1C–E). CSW and CS + B treatments showed the highest difference compared to the control in terms of roots parameters, improving not only root area and root volume, as in the case of MA, but also the total root length and, in the case of CS + B, the secondary roots number (Fig. 1B–E).

In order to characterize the possible physiologic effects of the different treatments applied, several photosynthetic parameters such as maximum Photosystem II quantum yield (Fv/Fm), relative chlorophyll content, photoprotective non-photochemical quenching (NPQ) and Phototsystem II quantum yield at the light intensity of growth (Phi2) were measured on mature plants (Fig. 2A–D). Moreover, stomatal conductance was estimated by measuring the differential leaf temperature as previously reported [51] (Fig. 2E). (Fig. 2A–E). No significant differences were observed in these parameters except for the leaf temperature differential, which was increased in absolute value in all algae-treated samples compared to the control (C), with the most evident effect in the case CS + B (Fig. 2E).

This value represents the ratio between the temperature of the leaf surface and the external environment, suggesting that all the algae treatments induced a more efficient heat dissipation compared to the negative control (Fig. 2D). Leaf temperature differential was previously reported to be directly related to stomatal conductance [51]: the

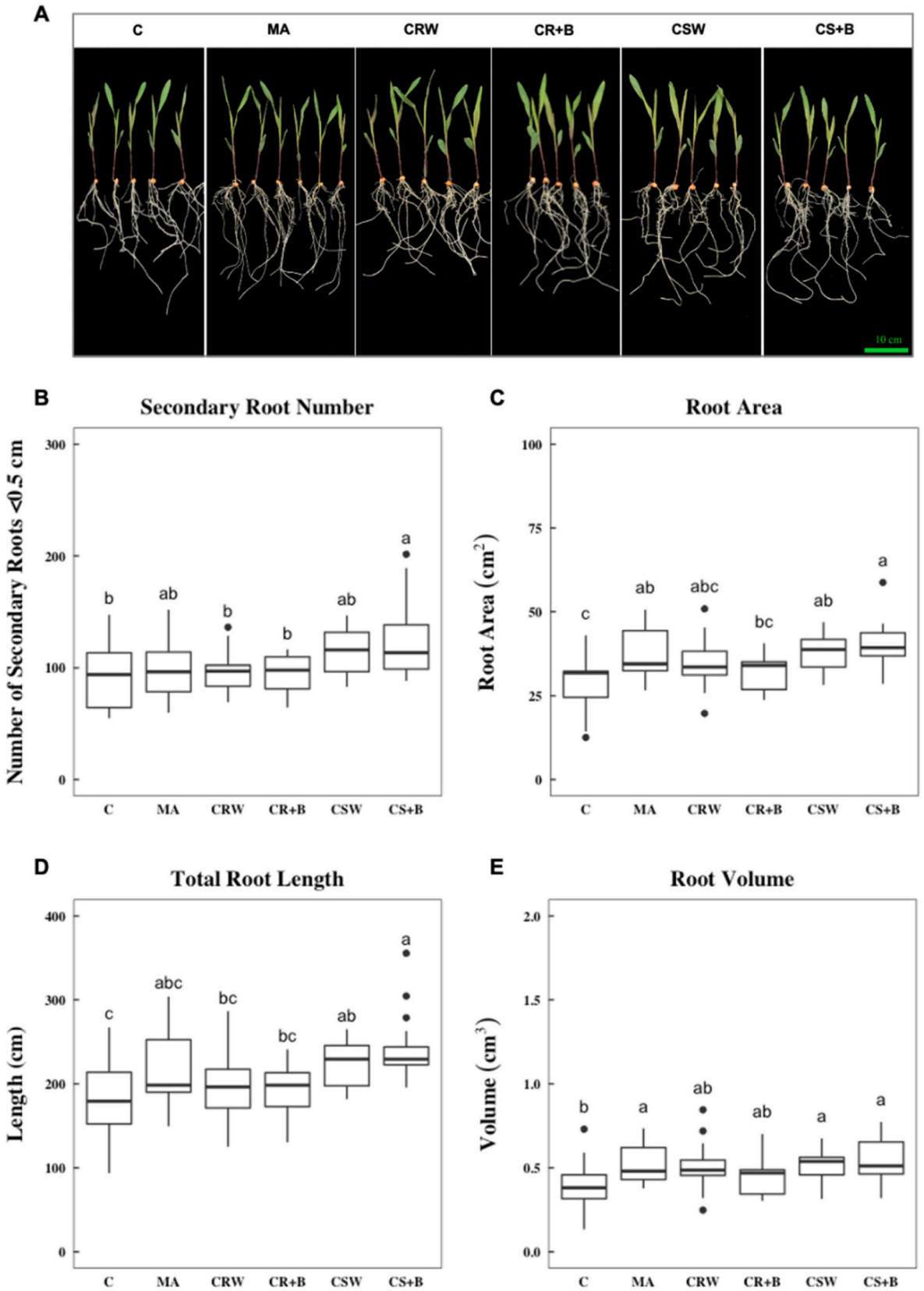

Fig. 1. Phenotypic characterization of hydroponically grown maize plants after 5 days of treatment: control (C), MA, CR whole culture (CRW), CR broken cells (CR + B), CS whole culture (CSW) and CS broken cells (CS + B). (A) Maize seedlings at the end of the experiment, scale bar refers to 10 cm. Total number of secondary roots <0.5 cm (B), surface area (C), root length (D) and volume (E) of primary seminal and lateral roots measured by the WinRHIZO™ software. The boxes represent the interquartile range (IQR) with the median line inside the boxes and the whiskers that represent 1.5 times the IQR ($n = 5$ plants, $N = 3$ independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

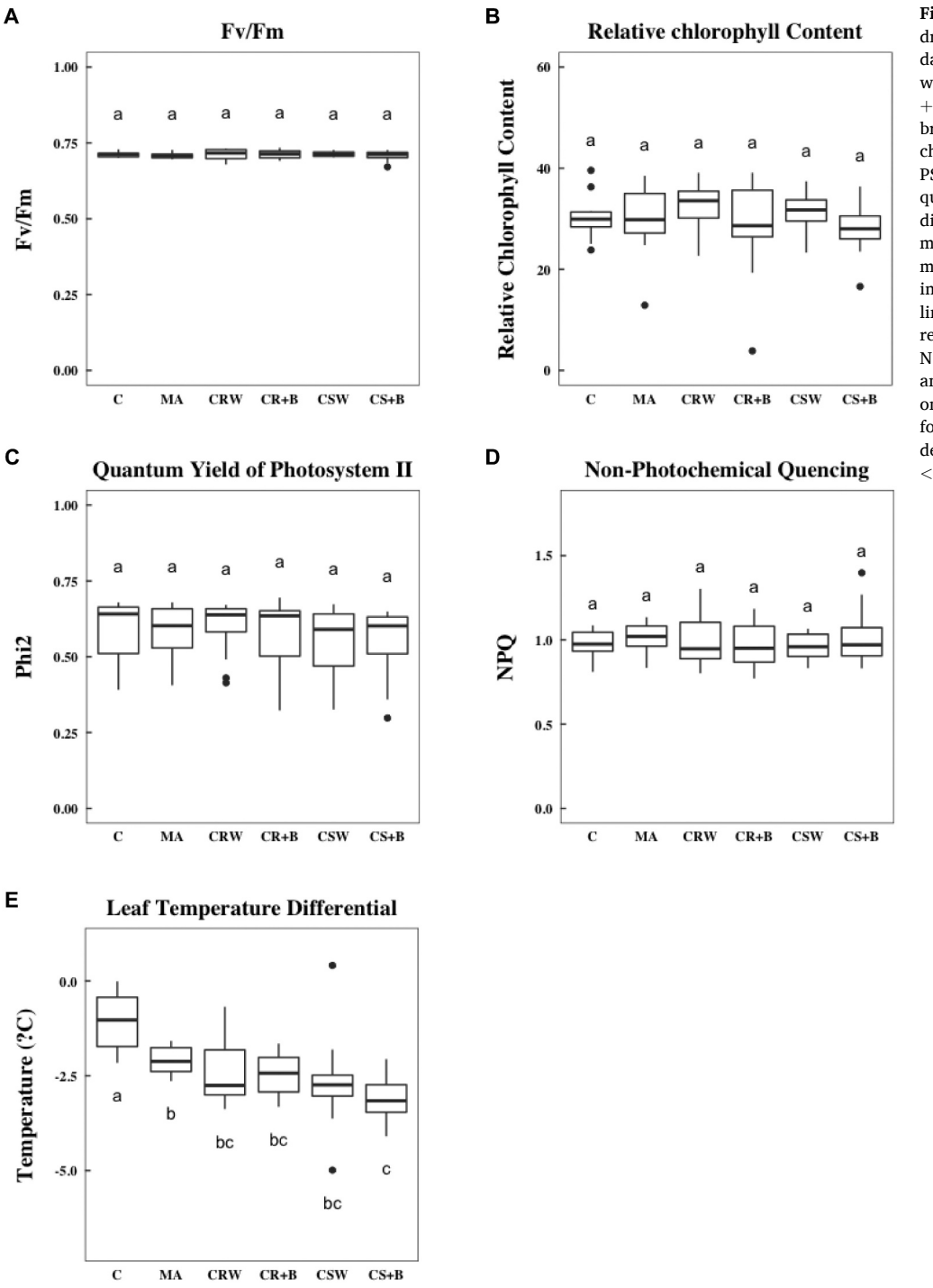

Fig. 2. Photosynthetic parameters of hydroponically grown maize plants after 5 days of treatment: control (C), MA, CR whole culture (CRW), CR broken cells (CR + B), CS whole culture (CSW) and CS broken cells (CS + B). Fv/Fm (A), relative chlorophyll content (B), quantum yield of PSII (Phi2) (C), non-photochemical quenching (NPQ) (D) and leaf temperature differential (E) of maize shoots were measured by the MultispeQ V2.0 instrument and software. The boxes represent the interquartile range (IQR) with the median line inside the boxes and the whiskers that represent 1.5 times the IQR (n = 5 plants, N = 3 independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

increase root formation in plants treated with MA or microalgae cultures is thus enabling an increased stomata opening and improved water transpiration.

3.2. Microalgae preparations induce the uptake of specific elements

The biostimulant effects of CR and CS preparations on maize seedlings were also tested on roots and shoots nutrient accumulation. Concerning Mg, P, K and Ca macronutrients no significant differences were observed for each treatment in both roots and shoots compared to the control or MA treatment (Table 1). Interestingly, CR preparations showed an improved tissue content of specific micronutrients (Table 1). The CR + B preparation induced an ~5-fold higher Cu level in the roots compared to the other treatments, whereas the maize seedlings treated with CRW showed an improved amount of Mn^{2+} in the shoots (Table 1). A positive effect on Cu^{2+} and Mn^{2+} concentration in hydroponically grown maize seedlings was previously observed in response to the treatment with animal derived protein hydrolysates [45]. The increased accumulation of Mn^{2+} in shoots in CRW treatment could be related to a biostimulant dependent trigger of Mn^{2+} assimilation and/or improved Mn^{2+} allocation in the different plant tissues, possibly because of increased Mn^{2+} demand. Mn^{2+} is indeed as cofactor of several enzymes involved in key metabolic process as nitrogen assimilation, chlorophyll biosynthesis, photosynthesis and ROS scavenging among others [52]. In

Table 1
Macro- and micro- nutrients amount in maize roots and shoots after 5 days of treatments with: CR, CS, MA preparations and extract, and the standard solution.

Treatment	Mg	P	K	Ca	Mn	Fe	Cu	Zn
	mg/g				μg/g			
Roots								
C	1056 ± 0,096 a	10,154 ± 1232 a	24,107 ± 4259 a	101,468 ± 0,589 a	19,844 ± 7039 a	101,468 ± 36,761 ab	8838 ± 1854 b	40,096 ± 7656 a
MA	1125 ± 0,217 a	9519 ± 1248 a	36,325 ± 28,232 a	117,573 ± 0,637 a	19,687 ± 5539 a	117,573 ± 27,107 a	7223 ± 1537 b	41,412 ± 2345 a
CRW	1052 ± 0,136 a	10,144 ± 2131 a	24,602 ± 5414 a	88,436 ± 0,404 a	22,988 ± 2939 a	88,436 ± 28,457 abc	7684 ± 2367 b	40,111 ± 6216 a
CR + B	1006 ± 0,078 a	9602 ± 1224 a	22,024 ± 2649 a	69,292 ± 0,509 a	15,937 ± 3537 a	69,292 ± 16,417 bc	39,205 ± 14,173 a	37,849 ± 4857 a
CSW	0,976 ± 0,121 a	9772 ± 0,681 a	23,491 ± 4817 a	83,572 ± 0,51 a	15,538 ± 2964 a	83,572 ± 25,501 abc	8371 ± 0,955 b	52,842 ± 28,814 a
CS + B	1064 ± 0,147 a	10,156 ± 1295 a	23,359 ± 3557 a	53,242 ± 0,628 a	15,393 ± 2429 a	53,242 ± 7764 c	8409 ± 1173 b	38,088 ± 2399 a
Shoots								
C	1564 ± 0,142a	6238 ± 0,692 a	59,881 ± 7409 a	1732 ± 0,147 a	14,006 ± 3127 b	48,311 ± 18,948 a	5808 ± 0,957 a	43,224 ± 5,7 ab
MA	1593 ± 0,118 a	6601 ± 0,533 a	72,573 ± 11,441 a	1,98 ± 0,385 a	15,968 ± 2159 ab	59,149 ± 31,842 a	5791 ± 0,415 a	44,296 ± 4799 ab
CRW	1729 ± 0,159 a	6125 ± 0,426a	66,487 ± 9137 a	2012 ± 0,158 a	19,851 ± 5195 a	66,119 ± 26,814 a	6771 ± 2439 a	48,489 ± 7178 a
CR + B	1536 ± 0,108 a	5845 ± 0,348 a	66,396 ± 6583 a	1761 ± 0,301 a	15,924 ± 2214 ab	52,942 ± 18,287 a	7478 ± 1498 a	39,216 ± 2445 b
CSW	1554 ± 0,081 a	6131 ± 0,494 a	62,836 ± 4364 a	2034 ± 0,483 a	15,206 ± 1374 ab	55,543 ± 18,109 a	5423 ± 0,246 a	36,555 ± 4471 b
CS + B	1636 ± 0,152 a	6756 ± 0,715 a	73,385 ± 8795 a	1735 ± 0,301 a	17,903 ± 1783 ab	47,384 ± 8056 a	6015 ± 0,423 a	42,642 ± 4609 ab

Macro- (Mg, P, K, Ca) and micronutrients (Mn, Fe, Cu, Zn) content in roots and shoots of hydroponically grown maize plants after 5 days of treatment: C (control), MA, CR whole cells (CRW), CR broken cells (CR + B), CS whole cells (CSW) and CS broken cells (CS + B). The average values are reported ± SD ($n = 2$ plants, $N = 3$ independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

addition, it was previously reported that the biostimulants based on animal protein hydrolysates stimulated the expression in maize roots of genes involved in metal micronutrients uptakes both in hydroponics and in soil [53,54] justifying the increase in the accumulation of these elements in roots in response to biostimulant application. Santi et al. [53] observed an up-regulation of transcripts involved in Fe uptake without a significant increase in the micronutrient content in roots unlike Cu, Mn and Zn. Similarly, in our experiment maize plants grown in presence of Fe-EDTA displayed in general similar Fe levels between control and microalgal preparations except for CS + B treatment, where Fe content was reduced in roots but not in shoots (Table 1).

3.3. Microalgae preparations further improved root formation in presence of PEG

Root formation has been previously reported to be stimulated in plants treated with polyethylene glycol (PEG) which partially reduces the availability of water for roots [55]. In order to evaluate the possible effect of microalgae-based biostimulants on roots formation in presence of PEG, dedicated experiments were performed as described in the following. According to the results reported in Figs. 1 and 2, only CS-derived preparations was evaluated, because they showed the most effective biostimulant activity on the maize root apparatus. Commercial biostimulant macroalgae-based product (MA) was also included in this experiment for comparison. Maize seedlings were thus hydroponically grown in a solution supplemented with 10% of polyethylene glycol in presence (MA, CSW and CS + B treatments) or absence (PEG treatment) of the different algae products. The standard nutrient solution without PEG and biostimulant preparations was used as control (C). PEG treatment caused only a slight increase in secondary root formation, which was much more evident in MA, CSW or CS + B treatments. Moreover, both CSW and CS + B treatments led to a significant improvement of the total root area and length compared to the C and PEG, whereas CS + B also induced an increment in the root volume (Fig. 3A–E). No significant differences were observed between CSW, CS + B and MA (Fig. 3B–E).

In our experiment, CS + B determined the highest root fresh weight compared to PEG treatment (Fig. 4A) despite no significant differences were observed for dry weight of the same tissue (Fig. 4C), suggesting an improved water acquisition. In addition, CSW and CS + B treatments showed the highest temperature differential in absolute value, confirming the higher stomatal conductance in presence of microalgae biostimulants (Supplementary Fig. S3), as in the case of the previous experiment in absence of PEG (Fig. 2). Moreover, in presence of PEG, higher Fv/Fm and lower NPQ values were measured for CSW treated plants compared to PEG and C conditions (Supplementary Fig. S3).

Thus, the biostimulant effect of the microalgae-based products had a biostimulant activity on PEG-treated plants improving root formation and stomatal conductance.

3.4. Microalgae preparations positively affect the root apparatus in response to low N

The increase of nitrogen use efficiency and/or the obtaining of sufficient production under N paucity is one of the major challenges of sustainable agriculture. Nutrient deficiency and toxicity are nutritional disorders that can affect plant growth [56]. In particular, the correction of N deficiency requires the application of N fertilizers with can have a negative impact from an economic and environmental point of view [57]. A more sustainable agricultural production under nutritional stress conditions could be reached through the improvement of genotypes for a higher nitrogen use efficiency [58] or the application of biostimulants. These substances allow plants to better tolerate stress conditions and ameliorate the nutrient efficiency [59]. In this context, we evaluated if maize seedlings treated with microalgae preparations were able to better respond to low N (0.1 mM) conditions. In these experiments, only the CS-derived preparations were considered because they showed the most effective biostimulant activity on the maize root apparatus (Fig. 1). MA treatment was adopted for comparison also in this case. For each treatment (MA, CSW, CS + B), a negative control was set-up growing maize seedlings in a nutrient solution containing an equal amount of N

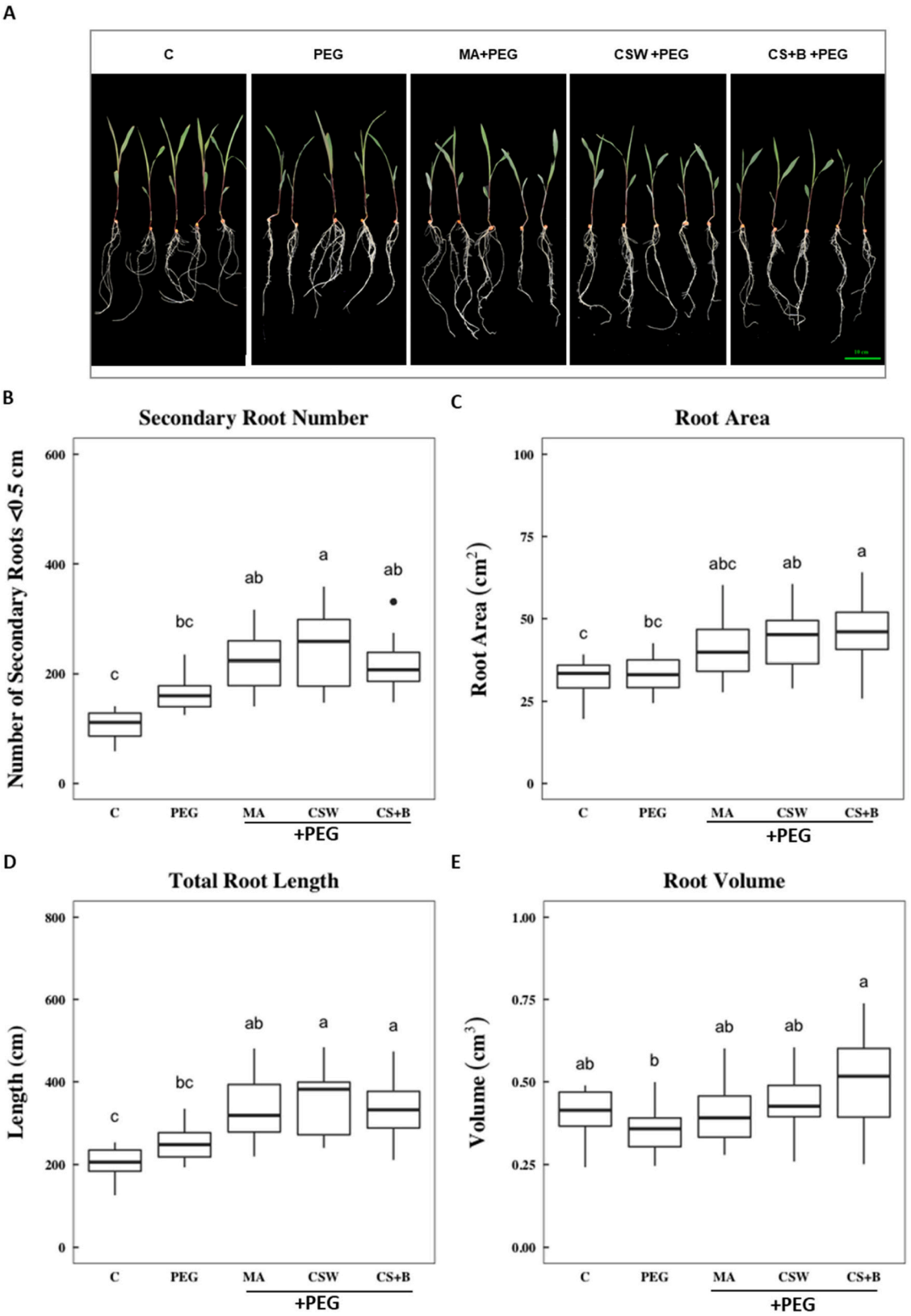

Fig. 3. Phenotypic characterization of hydroponically grown maize plants under PEG-induced drought stress after 7 days of treatment: nutrient solution without PEG6000 (C), PEG treatment (PEG), PEG with MA (MA), PEG with CS whole culture (CSW) and PEG with CS broken cells (CS + B). (A) Maize seedlings at the end of the experiment, scale bar refers to 10 cm. Total number of secondary roots <0.5 cm (B), root surface area (C), root length (D) and volume (E) of primary seminal and lateral roots of maize measured by the WinRHIZO™ software. The boxes represent the interquartile range (IQR) with the median line inside the boxes and the whiskers that represent 1.5 times the IQR ($n = 4$ plants, $N = 3$ independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

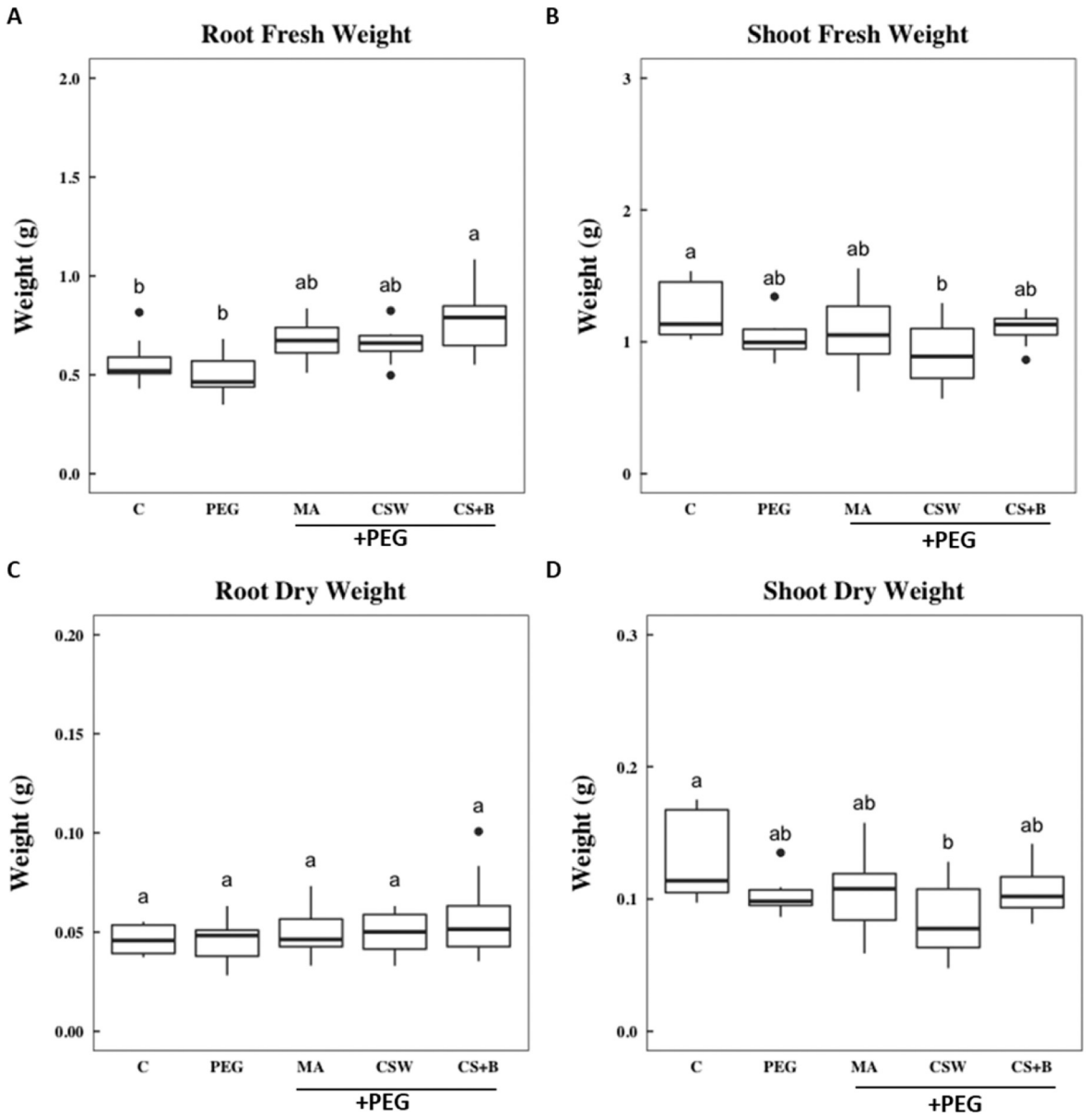

Fig. 4. Fresh (FW) and dry (DW) weight of hydroponically grown maize plants under PEG6000 induced drought stress after 7 days of treatments with: CS whole culture (CSW), CS broken cells (CS + B), MA extract and the standard solution with (PEG) and without PEG6000 (C). Fresh (A, B) and dry (C, D) weight of roots (A, C) and shoots (B, D) of maize seedlings measured at the end of the experiment. The boxes represent the interquartile range (IQR) with the median line inside the boxes and the whiskers that represent 1.5 times the IQR ($n = 3$, $N = 3$). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

provided by the algal preparations as inorganic N form (MA C, CSW C and CS + B C). In addition, the same comparisons were carried out on maize plants grown under high N (10 mM). It is important to note that the high N condition is not comparable to the N replete condition reported in Fig. 1, where the N content was 25 μM $NH_4H_2PO_4$.

Plants under low N were visibly stressed, as confirmed by the reduced root and shoot fresh weight and reduced relative chlorophyll content (Supplementary Fig. S4–6).

The effects of the CSW and CS + B treatments were evident under low N condition, but not in plant grown in high N (Fig. 5). Both CSW and CS + B positively affected the roots paraments with increased number of secondary roots, total roots area and roots length compared to their negative controls (CSW C and CS + B C). Moreover, CS + B treatment caused a significant increase in the fresh root weight compared to its control (Supplementary Fig. S5A). Differently, no significant differences were observed between plants treated with the commercial biostimulant product (MA) and its control (Fig. 5A, C, E).

Photosynthetic parameters were weakly affected by the application of the different biostimulant preparations (Supplementary Figs. S6 and S7) in both N conditions. The CS + B treatment led to a higher leaf temperature differential in absolute value only in the case of the high N supply (Supplementary Fig. S7D). Interestingly, in low N condition CSW-treated plants were characterized by a higher accumulation of Mn^{2+} in both roots and shoots and Cu^{2+} in roots compared to its control (Table 2). Differently, similar macro- and micronutrient concentrations in maize root and shoot tissues compared to their respective controls were measured in the case of MA and CS + B (Table 2).

4. Discussion

In this work the potential biostimulant properties of two green algae species, *C. reinhardtii* and *C. sorokiniana*, were tested on maize plants

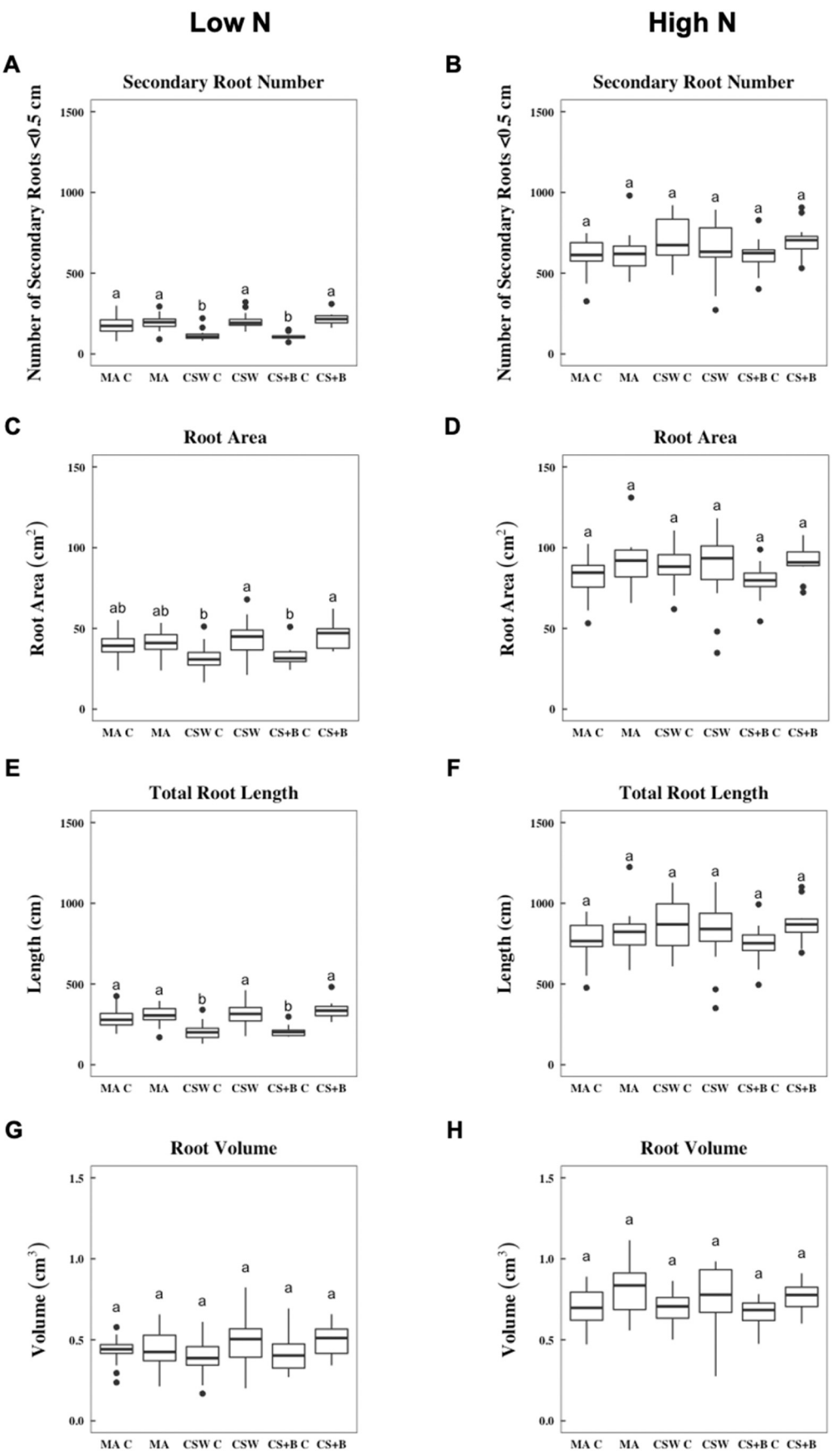

Fig. 5. Phenotypic characterization of hydroponically grown maize plants under low and high N after 7 days of treatment: MA C (control of MA), MA, CSW C (control of CSW), CSW, CS + B C (control of CS + B) and CS + B. Total number of secondary roots < 0.5 cm (A, B), root surface area (C, D), root length (E, F) and volume (G, H) of primary seminal and lateral roots of maize plants grown under low (A, C, E, G) and high (B, D, F, H) N measured by the WinRHIZO™ software. The boxes represent the interquartile range (IQR) with the median line inside the boxes and the whiskers that represent 1.5 times the IQR ($n = 4$ plants, $N = 3$ independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

Table 2
Macro- and micro- nutrients amount in maize roots and shoots after 7 days under low N and treated with: CS extracts, MA, and their N-adjusted relative controls.

Treatment	Mg	P	K	Ca	Mn	Fe	Cu	Zn
	mg/g				μg/g			
Roots								
MA C	0,971 ± 0,269 a	6713 ± 1,69 a	20,225 ± 7425 a	1185 ± 0,366 a	18,951 ± 14,497 ab	154,66 ± 30,687 a	12,389 ± 3,86 ab	43,214 ± 15,49 a
MA	0,873 ± 0,366 a	5526 ± 2033 a	18,191 ± 6211 ab	0,899 ± 0,224 a	24,707 ± 10,252 a	159,939 ± 40,803 a	11,566 ± 4442 ab	45,424 ± 16,522 a
CSW C	0,941 ± 0,254 a	6825 ± 2358 a	15,773 ± 7425 ab	1152 ± 0,538 a	6546 ± 4089 b	135,228 ± 45,091 a	7,4 ± 2443 b	42,799 ± 10,961 a
CSW	0,81 ± 0,433 a	6299 ± 2093 a	18,513 ± 6211 ab	0,826 ± 0,128 a	23,522 ± 6764 a	128,132 ± 47,51 a	12,973 ± 4,06 a	49,458 ± 6996 a
CS + B C	0,81 ± 0,207 a	4742 ± 0,968 a	11,01 ± 2,92 b	0,994 ± 0,437 a	8,15 ± 2612 b	136,476 ± 40,325 a	7818 ± 2082 b	33,659 ± 10,721 a
CS + B	0,938 ± 0,437 a	5672 ± 1,93 a	17,694 ± 5731 ab	0,983 ± 0,382 a	16,01 ± 5777 ab	118,415 ± 26,185 a	9324 ± 2566 ab	33,579 ± 10,474 a
Shoots								
MA C	2076 ± 0,291 a	6684 ± 0,718 bc	60,468 ± 12,99 a	1272 ± 0,253 a	16,716 ± 3357 a	90,269 ± 13,656 a	5304 ± 0,793 a	34,548 ± 10,107 a
MA	2143 ± 0,5 a	6139 ± 0,701 bc	64,301 ± 16,563 a	1263 ± 0,407 a	20,743 ± 6371 a	84,452 ± 28,384 a	4487 ± 1279 a	35,362 ± 8143 a
CSW C	1778 ± 0,299 a	8129 ± 1337 a	47,875 ± 9786 ab	1235 ± 0,386 a	10,621 ± 2606 b	80,803 ± 24,356 a	4,78 ± 0,868 a	28,071 ± 4404 ab
CSW	1,77 ± 0,191 a	5809 ± 0,549 bc	55,667 ± 14,328 ab	0,983 ± 0,3 a	17,101 ± 3.029 a	68,584 ± 14,238 a	4418 ± 0,666 a	30,038 ± 3525 ab
CS + B C	1,76 ± 0,389 a	7137 ± 1389 ab	40,712 ± 7629 b	1503 ± 0,601 a	10,707 ± 1914 b	76,442 ± 17,27 a	4403 ± 0,913 a	23,604 ± 3541 b
CS + B	1689 ± 0,275 a	5699 ± 0,708 c	54,89 ± 14,752 ab	1379 ± 0,449 a	16,312 ± 4523 ab	67,921 ± 9762 a	4067 ± 0,984 a	27,916 ± 6503 ab

Macro- (Mg, P, K, Ca) and micronutrients (Mn, Fe, Cu, Zn) content in roots and shoots of hydroponically grown maize plants under low N after 7 days of treatment: MA C (control of MA), MA, CSW C (control of CSW), CSW, CS + B C (control of CS + B) and CS + B. The average values are reported ± SD ($n = 3$ plants, $N = 3$ independent experiments). Statistical analysis of data was performed using the one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Letters denote statistically significant variations ($p < 0.05$).

grown in hydroponics conditions. Algae biomass, cultivated in lab-scale photobioreactors was indeed used to formulate putative biostimulants which were compared with a commercial biostimulant product based on macroalgae extract. The use of microalgae as biostimulants has been recently investigated in the case of several species, obtaining in general positive results [4,10,20,49,50]. For instance, Kholssi et al. [24] showed that CS suspensions induced an increase of roots and shoots biomass in wheat, while both CR and CS have reported to have a biostimulant effect on tomato plants enhancing growth and nutrient uptake [20]. The possible use of microalgae as biostimulants is a novel promising sector which allows the exploitation of their carbon fixation properties to produce a biomass by which to improve crops productivity, fitness and resilience. Despite being the market of biostimulants dominated by macroalgae extracts [60], the possibility to produce biostimulants from microalgae biomass has an emerging appeal toward improved sustainability [4]. Indeed, while macroalgae are essentially harvested in specific sites, it is easier to cultivate microalgae locally compared to fields where biostimulants are required. Moreover, it is possible to design dedicated industrial processes to exploit microalgae photosynthesis to reduce CO_2 emission and valorize waste products. Finally, in the biorefinery process to produce high value products from microalgae, it could be interesting to exploit the possibility to use the biomass produced in excess, or its residual fraction upon extraction processes, as biostimulant.

In this work microalgae cells were added in the nutrient solution as a lyophilized powder obtained from intact whole cells or cells physically treated to partially disrupt their wall: the biostimulant properties of algal extracts have been indeed usually associated to the release of specific peptides or proteins, which could be enhanced by partial disruption of the cell wall. Alternatively, polysaccharides or hormones secreted or present in microalgal biomass were reported to be induce a biostimulant effect on plants [61]. The results obtained using either physically treated or untreated cells demonstrate that CS had a clear positive effect on roots formation in maize plants, while the biostimulant properties of CR were less evident. It is important to note that plants treated with CS were characterized not only by increased root area and root volume, as in the case of MA, but also by increased total root length and, in the case of CS + B, increased secondary roots number. Physical treatment of microalgae cells has thus only a limited effect on the biostimulant properties of the biomass adopted for plants treatment. It is worth to note that treated or untreated cells were freeze dried before using them for plants treatment, likely partially disrupting the cell during lyophilization. The possibility to obtain a biostimulant activity of microalgae cells even in absence of pretreatments specifically aimed to disrupt the cell wall, allow designing a more sustainable process for the formulation of these products.

The results here presented suggest strongest biostimulant properties of CS cells compared to MA and data concerning the root length are in line with the results previously reported in the case of tomato seedlings treated with CS extracts [20]. However, in the case of tomato, both CS and CR treatments induced an increase in root length relative to the untreated control with a major effect due to the application of *Chlamydomonas reinhardtii* than *Chlorella sorokiniana* [20]. In addition, *Chlamydomonas reinhardtii* treatment caused an increased shoot and root dry biomass. In our experiment carried out on maize seedlings, no significant differences were observed between the different conditions in fresh (FW) and dry (DW) weight of both roots and shoots (Supplementary Fig. S8). The differences could be ascribed to the experimental conditions such as plant species, algae preparation, growing conditions, type of application, dose and time of treatments.

No major effect on photosynthetic activity and chlorophyll content was measured in the case of the maize plants treated with CS, CR or MA. Anyway, the effects on chlorophyll content and photosynthetic parameters can be related to several variables as the plant species and the experimental conditions (e.g. application method and time). In general, a positive impact was recorded on chlorophyll content in response to the treatment of different plant species when treated with plant-derived biostimulants [62–66] which was previously related to delayed leaf senescence [3]. Similar effects were recorded in the case of *Brassica rapa* and maize treated with seaweeds [67,68] and maize with microalgae polysaccharides [61]. Anyway, no significant differences in leaf pigments content was observed when lettuce was sprayed with plant-

derived protein hydrolysates [69] and when maize seedlings were treated with an animal protein hydrolysate containing free amino acids and small peptides [70]. In the case herein reported, maize plants were harvested in a juvenile state, where a possible positive effect of biostimulant in delaying senescence could not be assessed.

Changes in root architecture leading to an increase in absorptive surface can improve the ability of plants to acquire water and nutrients, in particular under nutrient deficiency [71]. Although the effect of the availability on primary root length and the length, number and density of lateral roots depend on nutrients [72], the algae treatment by-passes nutrient-specific response of root apparatus. The increased leaf temperature differential in absolute value observed here in algae treated samples can be associated to an improved stomatal conductance which is in line with an improved root formation induced by the biostimulants properties of the algal biomass used. Interestingly, it was reported that *Ascophyllum nodosum* seaweed extract can positively affect the leaf surface temperature of soybean under drought stress [73]. Similar results could be obtained in presence of PEG, which reduces the water availability for plants. In presence of PEG both CSW and CS + B treatments led to a significant improvement of the total root area and length. Again, the improved root formation caused an increased leaf temperature differential in absolute value, suggesting increased stomata conductance. These results agree with the positive effects exerted by microalgae on root growth recorded in tomato plants grown under water stress [25]. The improved roots formation in CSW and CS + B was likely improving water acquisition allowing for a safer stoma opening. This response could favor the plants under stress conditions. One of the main limiting factors in agriculture is the N starvation, affecting the whole crop development and production [74]. Indeed, crops productivity is heavily dependent on inorganic nitrogenous fertilizers whose use has negative economic and environmental impacts [75]. In the last years, the improvement of N use efficiency has become essential for a more sustainable agriculture [76]. It was reported that microbial biostimulants can be a sustainable tool to obtain yield stability in response to low N and P availability [77].

Accordingly, the effects of CSW and CS + B on root apparatus were recorded not only under normal growth condition (Fig. 1) but also under stress conditions such as the low N availability (Fig. 4). In N deficiency, CS treatment caused an improved root formation increasing the number of secondary roots, total roots area and roots length. Interestingly, the biostimulant activity in N deficiency was evident only in the case of microalgae CS cells, while no evident effect could be detected in MA treated plants (Fig. 4A, C, E). We can hypothesize that the MA product is suited to boost plant productivity only in presence of the required nutrients, while in the case of microalgae-based treatment plants were generally induced to be more resistant to abiotic stresses, likely improving the mechanisms of specific nutrient acquisitions, as reported in Table 2. In line with this finding, it was reported that a seaweed extract does not affect the growth parameters of okra in response to N deficiency [78]. The microalgae-based biostimulants seem to be more effective than the macroalgae ones to stimulate plant growth in response to low N stress. It is interesting to note that biostimulatory activity of CS cells in low N conditions induced an increased accumulation of Mn^{2+} in both roots and shoots, and Cu^{2+} in the roots (Table 2). Previously it was reported that $Mn^{2+}$and Cu^{2+} assimilation are linked to N metabolism: plants grown in Mn deficiency are indeed characterized by reduced N assimilation with inhibited activities of N-metabolism-related enzymes, such as nitrate reductase, glutamine synthetase, and glutamic-oxaloacetate transaminase [79]. Similarly, Cu deficiency has been reported to negatively influence N assimilation and several N-deprivation induced microRNAs were shown to target genes involved in Cu homeostasis. As previously discussed, plant biostimulant derived by animal matrixes can improve the micronutrient uptake by maize possibly through the positive effects on the expression of genes encoding to the metal transport systems of roots [45,54]. Considering the relation between Mn^{2+} and Cu^{2+} assimilation and homeostasis with N-metabolism, it is possible to speculate that the positive effects observed upon treatment with microalgae-based biomass in low N condition might be partially related to improved Mn^{2+} and Cu^{2+} assimilation. However, this observation requires additional experimental evidence to be supported.

5. Conclusions

In conclusion, this work demonstrates the biostimulatory activity of microalgae biomass on maize plants grown in hydroponic conditions. The positive effect for root development were in the case of CS similar to the biostimulant properties of the commercial product based on macroalgae extract in N replete conditions. Differently, in N deficiency CS based treatments were having even a more evident stimulatory effect on plants roots compared to the MA case. Further research efforts are required in order to investigate the molecule(s) involved in the biostimulant properties herein observed, with putative candidates as peptides, polysaccharides or phytohormones. It is worth to note that the biostimulant properties of CS and CR- based microalgae biomass should be also investigated in soil to propose their use in traditional agriculture application. Indeed, we are currently investigating the biostimulant properties of CS and CR in soil cultivation, which will be the subject of a future dedicated work. Anyway, the data herein reported already allow proposing the use of CS treatment for hydroponic cultivation of plants. Considering the increasing interest for hydroponic cultivation of plant species with biomedical, pharmaceutical or nutraceutical use, their high value in the market could also be relevant to cover the additional costs of microalgae-based biostimulant production. Microalgae cultivation is thus an appealing industrial process where bio-commodities can be produced aiming toward a bio-sustainable economy, including biostimulants for improving plant resilience toward stress conditions.

CRediT authorship contribution statement

Flavio Martini, Giorgia Beghini: Investigation, Data curation, Validation, Visualization, Writing - original draft; **Laura Zanin**: Investigation, Data curation, Writing - review & editing; **Zeno Varanini**: Conceptualization, Supervision, Methodology, Writing - review & editing; **Anita Zamboni**: Investigation, Data curation, Supervision, Methodology, Writing - review & editing; **Matteo Ballottari**: Conceptualization, Funding acquisition, Supervision, Methodology, Validation, Visualization, Project administration, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2021.102515.

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