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Tomato seed biopriming with water extracts from *Anabaena minutissima, Ecklonia maxima* and *Jania adhaerens* as a new agro-ecological option against *Rhizoctonia solani*

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ABSTRACT

In recent years, the use of synthetic pesticides in agriculture has been restricted for environmental pollution issues. Alternatives to chemicals for plant disease control are highly recommended by the recent EU legislation. We tested tomato seed treatment with water extracts from *Anabaena minutissima, Ecklonia maxima,* and *Jania adhaerens* for their biocontrol activity against the fungal plant pathogen *Rhizoctonia solani*. Algae were characterized into their contents in macro and microelements and into their functional groups by using FT-IR spectroscopy. The extracts were applied at 0.0, 2.5, 5.0, and 10.0 mg/mL concentrations on tomato seeds against the pathogen, in *in vitro* experiments and under greenhouse conditions. To estimate the efficiency of treatment in priming plant defence response, plant chitinase activity was measured and the different distribution of functional groups of roots was determined by FT-IR spectroscopy. Increases of germination and seedling dry weight for treated seeds without pathogen challenge were observed. The extracts reduced disease severity and increased seedling dry weight both in *in vitro* and greenhouse experiments at all concentrations. All extracts also increased stem seedling calibre under greenhouse conditions. The plant chitinase activity was increased by all extracts. The aromatic rings assigned to lignin changed with the treatment. We concluded that, although our experiments were based on a small scale, algae and cyanobacteria water extracts could provide a potential tool for the *R. solani* control on tomato plants, by contributing to the reduction of synthetic product input in the environment.

1. Introduction

Rhizoctonia solani J.G. Kühn (teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk.) is a Basidiomycota soil-borne pathogen with a worldwide distribution (Baker, 1970). Soil-borne pathogens include several other fungi, such as *Verticillium* spp., *Fusarium* spp., *Phytophthora* spp., *Pythium* spp., *Sclerotinia* spp. and *Sclerotium rolfsii*, which significantly reduce the quality and yield of many agricultural crops (Suwannarach et al., 2015; De Cal et al., 2004). *Rhizoctonia solani* causes serious plant disease by attacking primarily the roots and lower stems of plants. Damping-off of seedlings, root rot and leaf blight are the main symptoms (Baker, 1970). The pathogen can survive in the soil in the form of mycelium or resistant structures (sclerotia) under unfavourable environmental conditions for several years, and on debris from various plants such as tomato (Cao et al., 2004; Suwannarach et al., 2015), cucumber (Huang et al., 2012), corn (Ogoshi, 1987), cotton (Howell et al., 2000), rice (Groth and Bond, 2006), strawberry (De Cal et al., 2004), French bean (Sinobas et al., 1994) and several weeds (Suwannarach et al., 2015). The complete control of *R. solani* disease is not possible, because of the wide host-range and the long-term survival of sclerotia also in fallow soils in the absence of susceptible hosts and because no highly effective strategies are available against soil-borne pathogens (Bell and Sumner, 1987). Indeed, the 1107/2009 EU regulation has restricted the placing on the market of synthetic pesticides for soil treatment, because of their side effects against non-target organisms or the whole environment, such in the case of the methyl bromide/ozone layer issue (Bell, 1996; Ristaino and Thomas, 1997). Alternative approaches include cultural practices such as crop rotation with unsusceptible plant species, the removal of infected debris from the field and biological control with antagonistic microorganisms (Huang et al., 2012). A few tomato cultivars partially resistant to *R. solani* are available with commercially acceptable horticultural traits and peroxidase enzymes seem to be involved in the resistance mechanism (Nikraftar et al., 2013). The development

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of bio-based strategies to control plant pathogens by reducing synthetic product input in the environment has been encouraged by the Directive 2009/128/EC and its implementation that promotes specific actions to support the establishment of sustainable agriculture. Bio-based strategies include the use of natural compounds that may act against the pathogens both directly and indirectly through the activation of plant defence responces. Indeed, plants intimately interact with the pathogens in the complex nature environments. To protect themselves against pathogen colonization, plants have evolved a variety of defense mechanisms, including constitutive and inducible resistance strategies. Among the inducible ones, plants prevent penetration and restrict the growth of pathogens by various mechanisms, such as cell death, oxidative burst, defense genes that code for the expression of pathogenesis related proteins (*e.g.* chitinases), phytoalexins, cell wall thickness which include lignin deposition (Agrios, 2005). Thus, lignin accumulation is considered a first-line protection against invasive pathogens.

Algae and cyanobacteria have the potential to be exploited as a new bio-based mean to control plant pathogens, especially at this time when organic farming and pressure to reduce phytosanitary products are increasing. Nowadays only the active substance laminarin from the alga *Laminaria digitata* is allowed under Reg. (EC) No 1107/2009 and already authorised in many European countries for its commercialization as phytosanitary products. On the contrary, many products based on algae or cyanobacteria extracts are commercially available as biostimulants (Ertani et al., 2018). Algae and microalgae are characterized by high levels of bioactive compounds such as polysaccharides, phytohormones, polyphenols, proteins, tocopherols and pigments such as carotenoids (carotene, xanthophyll), chlorophylls and phycobilins (allophycocyanin, phycocyanin, phycoerythrin) with antioxidant activity (Craigie, 2011; Michalak and Chojnacka, 2015; Righini et al., 2018). Among these compounds, polysaccharides and proteins have also shown antimicrobial properties against plant pathogenic bacteria and fungi (Kulik, 1995; Prasanna et al., 2008; Righini and Roberti, 2019; Vera et al., 2011).

Some studies have investigated several kinds of extracts and their application to plants. The techniques for applying extracts include leaf spraying, soil drenching, and seed treatments (Arioli et al., 2015; Godlewska et al., 2019; Righini et al., 2018). Seed priming improves germination and may induce an immune response that protects plants from both abiotic and biotic stresses (Mahmood et al., 2016; Song et al., 2017). Seed priming, which starts the germination process without radicle protrusion, is termed biopriming when any biological compound is applied trough seeds hydration (Ashraf and Foolad, 2005; Mahmood et al., 2016). Seed biopriming has been already observed with the bacteria or fungi application or their derived compounds (Jensen et al., 2004; Mahmood et al., 2016; Singh et al., 2016; Song et al., 2017). Currently, extracts from several kinds of seaweeds (*Gracilaria corticata*, *Kappaphycus alvarezii, Ulva lactuca, U. reticulata*, *Padina pavonica,* and *Sargassum johnstonii)* as biopriming agents, improved the speed of seed germination, seedling growth, seed vigour index and enhanced the antioxidant activity of tomato, aubergine, onion and common cabbage seeds (Patel et al., 2017, 2018). Besides, seaweed suspension of *Ascophyllum nodosum* and *Laminaria hyperborean* also induced the priming of seed germination on lettuce (Möller and Smith, 1998). Recently, it has been shown that the seed priming led to trigger the plant defence in cucumber and pepper against *Pseudomonas syringae* pv. *lachrymans* (Song et al., 2017). The application of algae extracts to seeds during the priming process might be an important tool for controlling seed-borne diseases, as well as improve the overall performance of the plant.

On the basis of the studies mentioned above, we have investigated if tomato seed treatment with water extracts from the two algae *Ecklonia maxima* and *Jania adhaerens* and the cyanobacterium *Anabaena minutissima* could be considered as biopriming treatment. To our knowledge, no study has been carried out on seed biopriming with algae and cyanobacteria that considered also the possibility to protect plants from the attack of soil-borne pathogens. Therefore, water extracts from *E. maxima*, *J. adhaerens* and *A. minutissima* were applied on tomato seed and studied for their: (i) biostimulant effect on seedlings; (ii) control activity of *Rhizoctonia solani* disease on seedlings under laboratory and greenhouse conditions; (iii) elicitation of plant defence responses.

2. Materials and methods

2.1. Algae and cyanobacterium

The cyanobacterium *Anabaena minutissima* strain BEA 0300B, the brown alga *Ecklonia maxima,* and the red alga *Jania adhaerens* were provided by the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria. The cyanobacterium was lyophilized. The algae were dried thallus, therefore they were ground to a fine powder (particle size < 500 μm) with mortar and pestle before using.

2.2. Elemental and FT-IR analyses

Total carbon and nitrogen contents of algae and cyanobacteria were carried out by an elemental analyzer (CHNS-O EA 1110 Thermo Fisher Scientific). Metal contents were determined in finely ground lyophilized algae by microwave-assisted acid digestion (Milestone, Shelton, CT, USA) with HNO_3 suprapure (Carlo Erba, Italy) and H_2O_2 (30 %). Mineralized samples were then diluted with ultrapure water and each element was assayed *via* Inductive Coupled Plasma-Optic Emission Spectroscopy (ICP-OES). All analyses were performed in triplicate.

The FT-IR spectra were recorded using an ALPHA FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with an ATR (attenuated total reflectance) sampling device containing a diamond crystal. The absorbance spectra were recorded from 4000 cm^{-1} to 400 cm^{-1}, at a spectral resolution of 4 cm^{-1}, with 100 scans co-added and averaged. A background spectrum of air was scanned under the same instrumental conditions before each series of measurements. Spectra were processed with the Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH).

2.3. Water extracts preparation

Powder of *A. minutissima, E. maxima,* and *J. adhaerens* was suspended in sterile distilled water (10.0 mg/mL) under continuous stirring at 300 rpm with an heating magnetic stirrer (Arex Digital, Velp Scientifica, Italy) for 12 h at 50 °C and then filtered through sterile filter paper (Roberti et al., 2015) to obtain the water extracts which were named as ANA, ECK, and JAN, respectively. In all experiments, each water extract was used at three concentrations, 2.5, 5.0, and 10.0 mg/mL. Concentrations of 2.5 and 5.0 mg/mL were prepared by serial dilution (1:2) with sterile distilled water.

2.4. Pathogen isolation and identification and plant material

The fungus *Rhizoctonia solani* DAFS3001 (RS) belongs to the collections of the Department of Agricultural and Food Sciences, University of Bologna. The pathogen was isolated from tissues of tomato plants showing symptoms of root and crown rot. Pieces of tissue were surface disinfected with 1.5 % sodium hypochlorite (NaClO) for 2 min, rinsed two times in sterile distilled water, dried on sterile filter paper and then placed on 90 mm diameter Petri dishes containing potato dextrose agar (PDA 3.9 %, Biolife S.r.l., MI, Italy) supplemented with 60 mg/L of streptomycin sulphate (Sigma – Aldrich, St Louis, MO, USA). The pres-

ence of RS from tomato tissues was then examined after incubation at 25 °C in the dark for 7 d, using a light microscope (Carl Zeiss mod. ZM, Germany) at ×500 magnification. The fungus was preliminary identified based on morphological features (Sneh et al., 1991) and the pathogenicity was verified through inoculation of 7-day-old colony portions on tomato root of ten-day-old seedlings grown at 25 °C on filter paper, and waiting for the symptom appearance. To confirm the morphological identification, genomic DNA was extracted from the strain with a protocol based on the CTAB (2 %) detergent (Alkadri et al., 2013); the internal transcribed spacer region (ITS) of the nuclear rDNA was amplified using primers ITS4 and ITS5 (White et al., 1990) and cloned following the protocol described by (Bernicchia et al., 2012). Sequencing work was done by a commercial company (Eurofins - Germany).

For all experiments, the tomato cv. Marmande (L'Ortolano, Savini Vivai, Italy) was used as a model plant, firstly because it is among the crops which are most responsive to priming (Nawaz et al., 2013) and secondly for its faster growth.

2.5. Effect of seed priming with water extracts on seed germination and seedling dry weight in in vitro bioassay

Firstly, it was examined if the concentrations of water extracts used through seed priming application could have any effects on seed germination and seedling growth. Tomato seeds were firstly surface disinfected with ethanol (70 %) for 5 min and then with 1.5 % NaClO solution for 3 min, then rinsed with sterile distilled water three times (Mbega et al., 2012 with modification). Immediately after disinfection, the seeds were treated by immersion in a 600-µL aliquot of each extract at 2.5, 5.0, and 10.0 mg/mL at room temperature overnight in the dark. Sterile distilled water was used as control. Treated seeds were dried on sterile filter paper in a laminar airflow cabinet for 10 min and then sown on the edge of a half-cut PDA medium in Petri plates (5 seeds per dish). Twenty plates (n = 20) were considered for each treatment and the control. Plates were incubated vertically at 24–25 °C for 48 h in a growth chamber with a 12 h/12 h day/night photoperiod. The percentage of seed germination was recorded for each plate 9 d after treatment. Seeds were considered germinated once the radicle protruded more than 2 mm (Hernández-Herrera et al., 2016). After germination assessment, all plates were randomly divided into two groups of 120 plates each, 10 plates (n = 10) for each treatment and the control: one group was used for dry weight determination in absence of pathogen inoculation and the other group was used for pathogen inoculation (§ 2.6). For dry weight determination, seedlings from the first group were removed from the medium thirty days after sowing, and then they were hoven dried at 60 ± 5 °C for 48 h. The experiment was repeated twice.

2.6. Effect of seed priming with water extracts against R. solani in vitro

Rhizoctonia solani plugs cut from a 10-day-old colony were inoculated (2 plugs per dish) on PDA medium of the second group of 120 plates after 15 d from the seeding. The plugs were put at a distance of about 5 mm from each seedling root. Dishes were incubated vertically in a growth chamber at the same conditions mentioned above. Fifteen days after inoculation, seedlings were carefully removed and necrosis root symptoms were visually assessed using a five-point scale, where: 0, absence of necrosis (0 % of symptoms); 1, very slight root necrosis (up to 3 % of root with symptoms); 2, slight necrosis (4–30 % of root with symptoms); 3, moderate root necrosis (31–70 % of root with symptoms); 4, severe root necrosis (> 71 % of root with symptoms). Plant dry weight was determined after oven drying at 60 ± 5 °C for 48 h. The experiment was repeated twice.

2.7. Effect of seed priming with water extracts against R. solani in the greenhouse pot experiment

Tomato seeds were treated as described above with ANA, ECK, and JAN at concentrations of 2.5, 5.0 and 10.0 mg/mL. Thirty seeds were sown in each plastic pots (11.5 × 14 × 9.5 cm) in a substrate consisting of a mixture of peat and sand (7:3 w/w) inoculated with RS. For inoculation, 10-day-old colonies of RS grown on PDA medium were homogenized with sterile distilled water and then mixed with the substrate (2 % w/w, pathogen/substrate). The inoculated substrate was covered with a plastic film and incubated at 25 °C for two days in a growth chamber before the sowing. Seeds treated with water and sown in growing substrate not infected with the pathogen were used as a negative control. Seeds treated with water and sown in substrate infected with RS were used as a positive control. Three pots (n = 3) per treatment and the controls were considered. Plants were grown under greenhouse conditions at 24–26 °C (day), 20–22 °C (night), with a 12 h/12 h day/night photoperiod, 70 % relative humidity. Ten days after sowing, the seedling emergence was recorded, and then the average emergence for each pot was calculated. Four weeks after inoculation, seedlings were removed, the stem calibre was measured, and necrosis root symptoms were visually assessed by using a five-point scale as reported above for *in vitro* assay. To determine the dry weight, all seedlings were dried in a hoven at 60 ± 5 °C for 72 h. The experiment was repeated twice.

2.8. Effect of seed priming with water extracts on plant chitinase activity

Tomato seeds were treated with each extract as reported above. Sixty seeds for each extract concentration (3 replicates of 20 seeds each, n = 3) were sown on sterile filter paper in aluminium trays (23 × 10 × 5 cm), then irrigated with 15 mL of sterile distilled water. The control consisted of seeds treated with water. The trays were covered with transparent film and incubated at 24–25 °C for two days with a 12 h/12 h day/night photoperiod. Fifteen days after sowing, seedlings were harvested, weighed, and snap-frozen in liquid nitrogen. Frozen tissues were grounded to a fine powder by using a pre-chilled mortar and pestle, then total proteins were extracted with 20 mM sodium acetate buffer pH 5.2 (1 mL/g of fresh weight) added with 1 % polyvinylpolypyrrolidone (Sigma – Aldrich, St Louis, MO, USA) under continuous gentle stirring at 4 °C for 90 min (Roberti et al., 2015). After incubation, the crude extracts were centrifuged twice at 12,000 rpm for 20 min at 4 °C, and then the supernatant was filtered using a GV Millex® Syringe Filter Unit (pore size 0.22 µm, Millipore Corporation, USA). Protein concentration in the crude extract was determined by the protein–dye-binding method of Bradford (1976) in a 96 wells microplate (Greiner CELLSTAR®), by using bovine serum albumin (Bio-Rad Laboratories, Inc.) as the standard. Chitinase activity of each sample was assayed in duplicate following the procedure of the plate assay in agarose gel (1 %) containing 0.01 % glycol chitin in a 14 cm diameter glass Petri dish (Bargabus et al., 2004). Gel plugs were cut with a 4 mm hole diameter cork borer to obtain wells. Forty µg of proteins of each sample were added into wells. As chitinase standard, *Streptomyces griseus* (Sigma – Aldrich, St Louis, MO, USA) was used. After incubation at 37 °C for 24 h, 50 mL of 500 mM Tris–HCl (pH 8.9) containing 0.01 % fluorescent brightener was added to each dish. Ten min later, the dishes were rinsed three times with distilled water, flooded with water, maintained for 24 h in the dark, and then observed under 302 nm UV light source to visualize non-fluorescent lytic zones corresponding to the enzyme activity on a fluorescent background. Images of gels were taken with a digital camera, then the area (mm^2) of chitinase activity was calculated with the Quantity One 1-

D analysis software v. 4.6.6 (Bio-Rad Laboratories, Inc. Hercules, CA, USA, 2003). The experiment was repeated twice.

2.9. FT-IR and C, N analyses of roots

Tomato seedlings were treated and grown as reported above in the previous paragraph. Three trays (n = 3) of 20 seeds each were considered for each treatment and the untreated control. After 15 days from treatment, five roots were randomly harvested from each tray and lyophilized. FT-IR spectra and C and N content were determined on lyophilized roots according to the method reported in paragraph 2.2.

Overlapping peaks were resolved using a peak fitting a nalysis in the spectral region from 1780 to 1490 cm^{-1} by using the Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH). The overlapping bands were resolved with a Gaussian function and the best fitting p arameters w ere o btained b y minimization of the reduced Chi-square (χ^2). Good agreement between experimental and calculated profiles was obtained, with coefficients of determination, R^2, ranging from 0.999 to 0.988, and the standard error, SE, from 0.001 to 0.003. All data are expressed as percentage area.

Table 1
Average results for the elemental composition (± SD, dry mass) of the algae and cyanobacteria.

Elements	*A. minutissima*	*E. maxima*	*J. adhaerens*
%			
C	42.46 ± 2.20*	22.03 ± 3.30*	8.09 ± 0.55*
N	9.44 ± 0.41*	1.32 ± 0.05*	1.44 ± 0.01*
mg/kg			
P	11.13 ± 1.04*	2.17 ± 0.06*	1.08 ± 0.02*
K	7.96 ± 0.30*	<LLD	7.19 ± 0.43*
S	3.92 ± 0.18*	7.87 ± 0.32*	4.79 ± 0.11*
Ca	4.53 ± 0.15*	18.31 ± 0.23*	140 ± 10.48*
Mg	5.38 ± 0.20*	5.79 ± 0.71*	23.71 ± 0.98*
μg/kg			
Fe	1969 ± 38.85*	526 ± 37.84*	1181 ± 29.43*
Cu	12.09 ± 1.03*	3.05 ± 0.76*	5.93 ± 0.61*
Zn	41.25 ± 1.65*	29.70 ± 2.90*	38.04 ± 2.01*
Mn	258 ± 4.54*	8.11 ± 2.47*	51.18 ± 1.89*

<LLD: below detection limit.
* Significant differences in a line according to LSD test ($p < 0.05$).

2.10. Statistical analysis

All experiments were arranged in a complete randomized design. Data obtained from *in vitro* and *in vivo* experiments were analyzed by two-way ANOVA, and means were separated by Fisher's least significant difference (LSD) test ($p < 0.05$). Factors for ANOVA analysis were: extract (treatment) and extract concentration. All analyses were performed with GraphPad Prism software, v. 4.03, 2005.

3. Results

3.1. Characterization of algae and cyanobacterium and pathogen identification

In general, the algae are rich in macro and micronutrients even though their concentrations varied considerably among species (Table 1). Especially, ANA showed the highest contents of macronutrients (C, N, and P) (42.46 %, 9.44 %, and 11.13 mg/kg, respectively) whilst ECK recorded the highest content in S (7.87 mg/kg). Mg was observed in all the species, but the higher level was found in JAN (23.71 mg/kg). This element is a key mineral of the chlorophyll molecule. JAN displayed the highest content in Ca (140 mg/kg). This kind of microalgae has a significant role in calcium carbonate ($CaCO_3$) production (McCoy and Kamenos, 2015). Other micronutrients such as Fe, Cu, Zn, and Mn were markedly more concentrated in ANA (1969, 12.09, 41.25, and 258 μg/kg, respectively).

FT-IR spectra of ANA, ECK, and JAN were characterized by the clear bands (Fig. 1) assigned to specific molecular structures such as proteins (3072, 1650 and 1540 cm^{-1}), alginates (1600 and 1400 cm^{-1}) (Gómez-Ordóñez and Rupérez 2011), carbohydrates (from 1180 to 950 cm^{-1}), lipids (from 2956 to 2860 cm^{-1}), phosphorus compounds (1245 cm^{-1}) and carbonate (1404, 873 and 714 cm^{-1}) (Dean et al., 2012). ANA was characterized by the typical bands of proteins as also supported by the high content in total N whilst ECK was dominated by the presence of alginates and carbohydrates and JAN by the carbonate.

The fungal sequence was submitted to NCBI and the strain belongs to *Rhizoctonia solani* AG-4 HG-I.

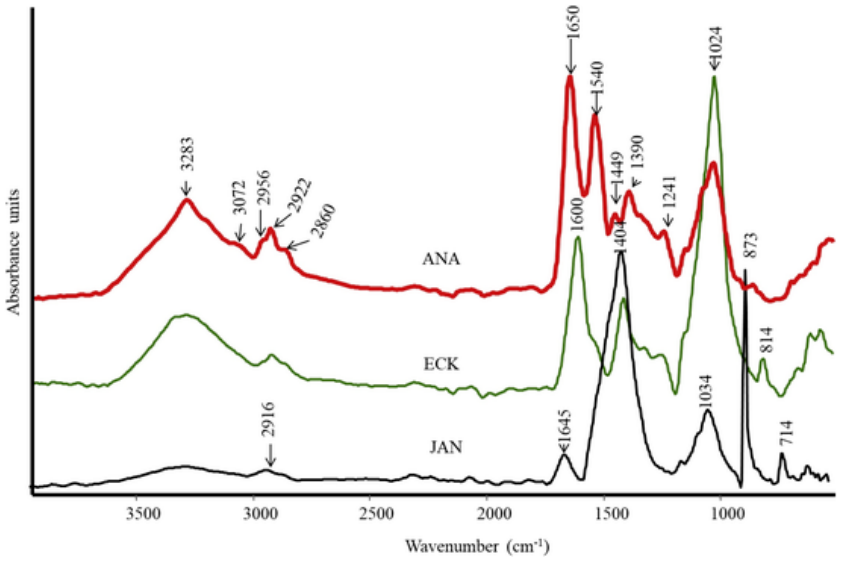

Fig. 1. FT-IR spectra of *Anabaena* minutissima (ANA), *Ecklonia* maxima (ECK) and *Jania adhaerens* (JAN).

3.2. Effect of seed priming with water extracts on seed germination and seedling dry weight in in vitro bioassay

The effect of seed treatment with different concentrations of the extracts on the percentage of tomato seed germination is reported in Table 2. Two-way ANOVA indicated a significant interaction between extract and concentration factors. All concentrations of extracts increased the percentage of seed germination. The highest significant increase of seed germination was observed for ANA at 10.0 mg/mL, by 19.3 % in comparison to the untreated sample. ECK showed a similar and higher effect in increasing seed germination at 5.0 and 10.0 mg/mL than at 2.5 mg/mL, while JAN enhanced the percentage of seed germination more efficiently at 5.0 mg/mL than at the other concentrations. Within concentrations, ECK and JAN showed a similar effect in increasing seed germination at 5.0 and 10.0 mg/mL. No differences were obtained among extracts at 2.5 mg/mL.

The effect of priming of seeds with each extract on the dry weight of seedlings is reported in Table 3. Two-way ANOVA indicated a significant interaction between extract and concentration factors. Dry weight was significantly increased by all extracts at all concentrations. For ANA, the maximum increase of seedling dry weight was obtained at 10.0 mg/mL by 53.5 %, for ECK at both 5.0 and 10.0 mg/mL (by 32.3 % and 40.8 %, respectively) and for JAN at 5.0 mg/mL by 63.8 %, compared to untreated control. Within concentrations, at 5.0 mg/mL JAN showed the highest increased of dry weight, whereas at 2.5 mg/mL it increased dry weight more than ANA. At 10.0 mg/mL ANA increased more than JAN.

Table 2
Effect of seed treatment with water extracts on tomato seed germination (%) *in vitro*.

Extract	Concentration (mg/mL)			
	0.0	2.5	5.0	10.0
A. minutissima	80.0 ± 2.2 A	82.2 ± 1.3 B	89.3 ± 1.8 aC	95.4 ± 5.0 bD
E. maxima	80.5 ± 2.2 A	83.4 ± 2.4 B	92.3 ± 2.9 bC	92.5 ± 2.7 aC
J. adhaerens	80.1 ± 2.7 A	83.4 ± 2.4 B	93.6 ± 1.8 bD	91.7 ± 2.7 aC

Concentration and its interaction with extract are significant according to factorial ANOVA ($p < 0.05$). $F(3, 240) = 347.96$, $p < 0.05$ (for concentration factor), $F(6, 240) = 8.51$, $p < 0.05$ (for interaction). Means ($n = 20$) ± SD followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($p < 0.05$). The absence of lower-case letters indicates no significant differences, according to LSD test ($p < 0.05$).

Table 3
Effect of seed treatment with water extracts on tomato seedling dry weight (mg) *in vitro*.

Extract	Concentration (mg/mL)			
	0.0	2.5	5.0	10.0
A. minutissima	7.1 ± 0.3 A	7.9 ± 0.6 aB	8.0 ± 0.2 aB	10.9 ± 1.5 bC
E. maxima	7.1 ± 0.7 A	8.3 ± 0.1 abB	9.4 ± 1.3 bC	10.0 ± 1.1 abC
J. adhaerens	6.9 ± 0.6 A	8.9 ± 1.1 bB	11.4 ± 0.7 cD	9.7 ± 0.4 aC

Extract, concentration, and their interaction are significant according to factorial ANOVA ($p < 0.05$). $F(2, 120) = 8.19$, $p < 0.05$ (for extract factor), $F(3, 120) = 88.10$, $p < 0.05$ (for concentration factor), $F(6, 120) = 14.64$, $p < 0.05$ (for interaction). Means ($n = 10$) ± SD followed by different lower-case letters in a column and by different upper-case letters in a line are significantly different according to LSD test ($p < 0.05$). The absence of lower-case letters indicates no significant differences, according to LSD test ($p < 0.05$).

3.3. Effect of seed priming with water extracts against R. solani in vitro

Seed priming with ANA, ECK, and JAN showed that the disease index of seedling infected by RS was significantly influenced by the extract, the concentration and by their interaction as indicated by two-way ANOVA analysis (Fig. 2). All extracts at all concentrations reduced the disease index compared to the respective untreated control up to a maximum of 66.0 % for ANA at 10.0 mg/mL. At this concentration, ANA was more efficient than JAN and ECK in reducing disease index. ANA and JAN at 2.5 and 5.0 mg/mL reduced disease index in a similar way and more than ECK.

For what concerns the effect of the seed priming with the three extracts on the dry weight of infected seedlings (Fig. 3), two-way ANOVA indicated that extract, concentration, and their interaction were significant ($p < 0.05$). The extracts at all concentrations significantly increased seedling dry weight compared to the respective untreated control. In particular, ANA increased dry weight in a range of 187.5–195.0 %. Furthermore, all ANA concentrations increased seedling dry weight more than the other extracts concentrations.

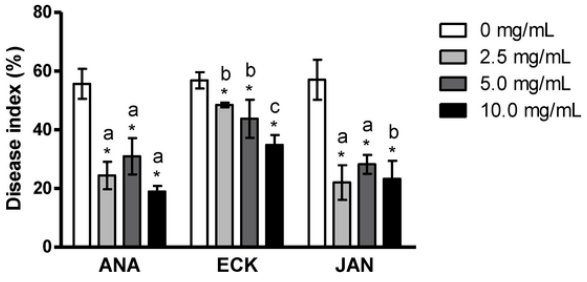

Fig. 2. Effects of tomato seed priming with different concentrations (0.0, 2.5, 5.0, 10.0 mg/mL) of water extracts from *Anabaena minutissima* (ANA), *Ecklonia maxima* (ECK), and *Jania adhaerens* (JAN) on disease index percentage of *R. solani* on seedling root rot, *in vitro*. Treatment factor, concentration factor, and treatment × concentration interaction are significant according to two way ANOVA. $F(2, 120) = 101.26$, $p < 0.05$ (for treatment factor), $F(3, 120) = 230.62$, $p < 0.05$ (for concentration factor), $F(6, 120) = 15.66$ $p < 0.05$ (for interaction). Columns are mean values ($n = 10$) ± SD. The asterisk indicates significant disease index reduction caused by each extract concentration towards the corresponding control (0.0 mg/mL) and different letters indicate significant differences within each concentration, according to LSD test ($p < 0.05$).

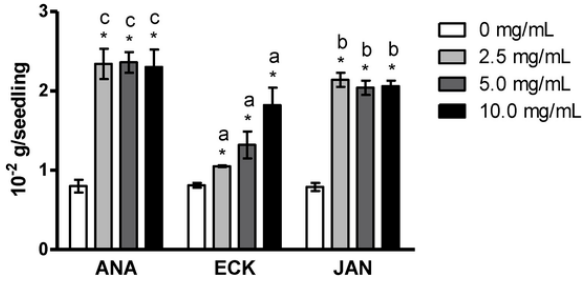

Fig. 3. Effects of tomato seed priming with different concentrations (0.0, 2.5, 5.0, 10.0 mg/mL) of water extracts from *Anabaena minutissima* (ANA), *Ecklonia maxima* (ECK), and *Jania adhaerens* (JAN) on the dry weight of seedlings infected by *R. solani*, *in vitro*. Extract, concentration, and their interaction are significant according to factorial ANOVA. $F(2, 120) = 300.65$, $p < 0.05$ (for extract factor), $F(3, 120) = 573.29$, $p < 0.05$ (for concentration factor), $F(6, 120) = 57.66$, $p < 0.05$ (for interaction). Columns are mean values ($n = 10$) ± SD. The asterisk indicates significant dry weight increase caused by each extract concentration towards the corresponding control (0.0 mg/mL) and different letters indicate significant differences within each concentration, according to LSD test ($p < 0.05$).

3.4. Effect of seed priming with water extracts against R. solani in the greenhouse pot experiment

To further verify the effect of seed priming against *R. solani* root rot on tomato seedlings, the pathogen was inoculated in a substrate in pots under greenhouse conditions. The effect of treatments applied at different concentrations against the disease is reported in Fig. 4.

Two-way ANOVA indicated that for emergence, disease severity, seedling calibre and dry weight the concentration factor was significant ($p < 0.05$), whereas the extract factor and the interaction between the two factors were not significant. The improvement of seedling emergence was positively related to extract concentration (Fig. 4A). The 10.0 mg/mL concentration showed the highest emergence increase by 31.4 %. All treatments reduced the disease index up to a maximum of 58.4 % at 10.0 mg/mL, compared with the control (Fig. 4B). All extracts increased seedling calibre with respect to the control (Fig. 4C). In particular, this increase was positively related to extract concentration by reaching a value of 65.0 % at 10.0 mg/mL. Seedling dry weight was significantly increased by all concentrations (Fig. 4D). The 10.0 mg/mL concentration showed a value statistically higher than 2.5 mg/mL concentration and similar to 5.0 mg/mL. The increase of seedling dry weight obtained with 10.0 mg/mL concentration was 114.8 % compared to untreated control.

3.5. Effect of seed priming with water extracts on plant chitinase activity

Seed priming with ANA, ECK, and JAN at different concentrations significantly enhanced the chitinase activity of seedlings (Fig. 5). Indeed, two-way ANOVA analysis showed that extract and concentration factors and their interaction were significant ($p < 0.05$). Within concentrations, the chitinase activity was markedly increased by JAN at 2.5 and 5.0 mg/mL. At 10.0 mg/mL, the enzyme activity of JAN was similar to that of ANA. At all concentrations, ECK showed the lowest chitinase activity.

3.6. Determination of total carbon, nitrogen, and functional groups

Tomato roots treated with ANA, ECK, and JAN at different concentrations showed an increase in total carbon and nitrogen (Table 4) compared to untreated plants. In detail, an accumulation in total nitrogen in plant roots supplied with ANA and ECK at 2.5, 5 and 10.0 mg/mL (+21 %, +22 %, 20 % and +20.3 %, +20.5 % and 13.6 % respectively) was observed. By contrast, the treatment with JAN has enhanced progressively the total nitrogen (from 12 % to 18 %) in roots. Algae extracts have generally a great impact on the nitrogen metabolism of plants, confirming their high potential application as biostimulants (Ertani et al., 2018).

The spectra of tomato roots untreated and treated with ANA, ECK, and JAN extracts at 5.0 and 10.0 mg/mL are shown in Fig. 6. Spectra of tomato roots that were treated with low doses of algae ex-

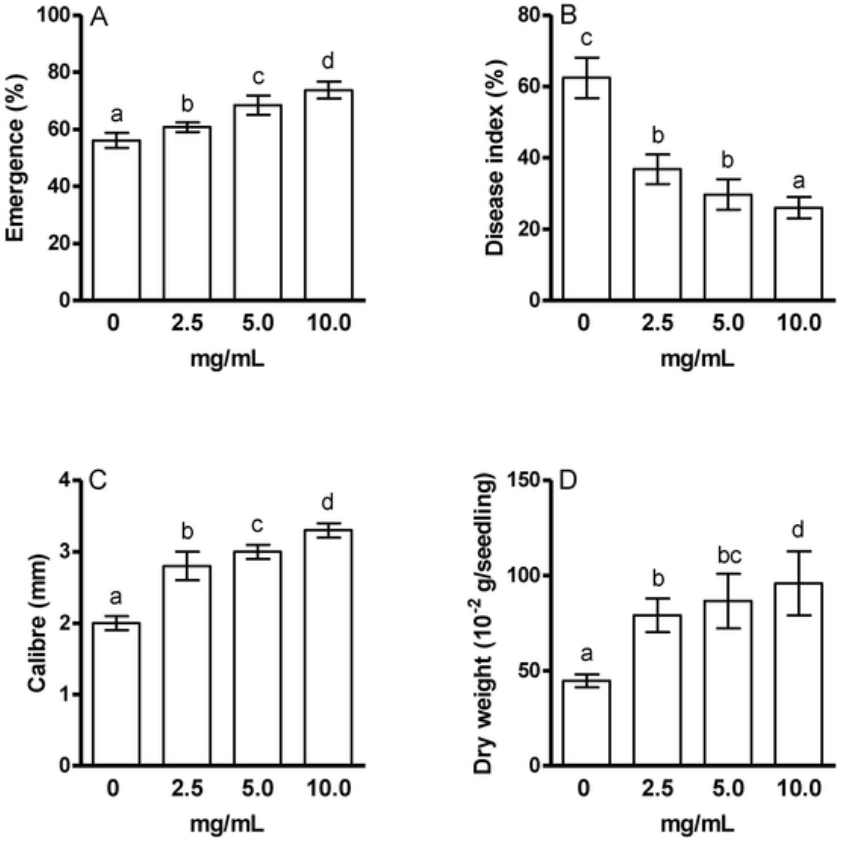

Fig. 4. Effect of tomato seed priming with different concentrations (0.0, 2.5, 5.0, 10.0 mg/mL) of water extracts from *Anabaena minutissima*, *Ecklonia maxima*, and *Jania adhaerens* on emergence (A), disease index (B), dry weight (C), and stem calibre (D) of seedlings grown in substrate infected with *Rhizoctonia solani*. For emergence, disease index, stem calibre, and dry weight, the concentration factor is significant according to factorial ANOVA ($p < 0.05$). For emergence, $F(3, 36) = 75.8$, disease index, $F(3, 36) = 122.0$, stem calibre, $F(3, 36) = 105.9$ and dry weight, $F(3, 36) = 33.9$. Extract and interaction extract × concentration are not significant according to two way ANOVA ($p > 0.05$). Columns are mean values ($n = 3$) ± SD. Different letters indicate significant differences according to LSD test ($p < 0.05$).

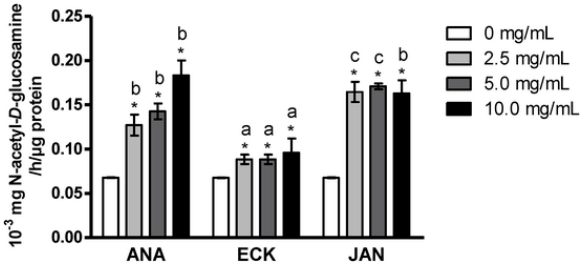

Fig. 5. *N*-acetyl-D-glucosamine activity determined in protein extract of tomato seedlings following seed priming with water extracts from *Anabaena minutissima* (ANA), *Ecklonia maxima* (ECK), and *Jania adhaerens* (JAN) at different concentrations (0.0, 2.5, 5.0, 10.0 mg/mL). Extract, concentration, and their interaction are significant according to factorial ANOVA ($p < 0.05$). $F(2, 36) = 108.29$, $p < 0.05$ (for extract factor), $F(3, 36) = 113.17$ $p < 0.05$ (for concentration factor), $F(6, 36) = 17.59$, $p < 0.05$ (for interaction). Enzyme activity was defined as the amount of enzyme that liberates 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour. Columns are mean values ($n = 3$) ± SD. The asterisk indicates significant differences towards untreated control within each extract (0.0 mg/mL) and different letters indicate significant differences within each concentration according to LSD test ($p < 0.05$).

Table 4
Total carbon and nitrogen content in tomato root plants treated with *A. minutissima*, *E. maxima*, and *J. adhaerens* extracts at different concentrations.

Treatments (mg/mL)	C (%) Mean ± SD	N (%) Mean ± SD
0.0	38.29 ± 0.11	2.34 ± 0.13
A. minutissima		
2.5	39.09 ± 0.28	2.85 ± 0.05
5.0	39.26 ± 1.52	2.85 ± 0.07
10.0	38.96 ± 1.32	2.76 ± 0.09
E. maxima		
2.5	39.74 ± 0.35	2.81 ± 0.01
5.0	38.94 ± 0.77	2.82 ± 0.22
10.0	39.74 ± 0.76	2.66 ± 0.02
J. adhaerens		
2.5	38.28 ± 0.02	2.61 ± 0.03
5.0	39.95 ± 0.12	2.72 ± 0.05
10.0	39.77 ± 0.41	2.74 ± 0.38

tracts (2.5 mg/mL) are not reported, because spectral pattern was very similar to that at 5.0 mg/mL. In general, the spectra displayed the most significant changes in the region between 1800–1200 cm^{-1}. The most important peaks appeared at: ~1733 cm^{-1} due to CO═ stretching vibration of ester in lipids (*i.e.*, cutin) and also cell wall pectin (Schulz and Baranska, 2007); amide I (~1640 cm^{-1}) and amide II (~1540 cm^{-1}) in proteins (Naumann, 2009); ~1515 cm^{-1} and assigned to aromatic skeletal vibration in lignin; ~1458 and 1417 cm^{-1} due to CH_3 and CH_2 bending motion, respectively; ~1378 cm^{-1} was assigned to O–CH_3 and CH— symmetric deformation; ~1327 cm^{-1} was due to aryl ring breathing with CO— stretching in lignin; ~1244 cm^{-1} was assigned to amide III plus C—O stretching coupled with C—C vibrations (Rao, 1963).

More detailed information on the spectra was obtained by the Gaussian curve fitting procedure applied to the investigated region. The percentage area of each functional group can be considered representative of the treatments with the algae extracts at different doses (Fig. 7). In this section, the results concerning the aromatic compound of lignin (~1515 cm^{-1}) after treatment with extracts were described (Fig. 7). In untreated root tomato, the percentage area of lignin (~1515 cm^{-1}) accounted for 3 %, while with ANA extracts treatment, it increased by 8.2 % and 6.5 % at the doses of 2.5 and 5.0 mg/mL, respectively. On the other hand, no effect was seen at 10.0 mg/mL (4 %) compared to the untreated one. With ECK treatment, it increased by 5.6 % and 8 % at the doses of 5.0 and 10.0 mg/mL, respectively. For the JAN treatment was found a progressive increase, by 7 %, 8 %, and 6 % at doses of 2.5, 5.0, and 10.0 mg/mL, respectively.

4. Discussion

The demand for environmentally sustainable agriculture in the context of climate change requires an innovative agricultural approach. Nowadays, plant pest control solutions are highly recommended by European standards (Reg. EC 1107/2008; Dir. 128/2009). The effects of seaweed extracts and cyanobacteria on plants have been reviewed in detail (Khan et al., 2009; Osman et al., 2010; Pereira et al., 2009; Righini et al., 2018; Shukla et al., 2019; Singh, 2014). However, the role of algae and cyanobacteria extracts has been recently considered as a potential tool in the integrated management of plant diseases (Arioli et al., 2015; O' Keeffe et al., 2019; Prasanna et al., 2013; Righini and Roberti, 2019). Among the application techniques to plants, seed priming allows seeds to absorb substances from the extract (Godlewska et al., 2019) that can improve different plant characteristics and also helps plants to overcome biotic and abiotic stress (El-Mougy et al., 2012). In particular, algae and cyanobacteria extracts are rich in macro- and micronutrients for plants, *e.g.* vitamins, amino acids and phytohormones (auxins, cytokinins, and gibberellins), as well as in compounds with antimicrobial activity including polysaccharides (Calvo et al., 2014; Rivas-Garcia et al., 2018; Singh, 2014; Vera et al., 2011).

For the first time, we have evaluated if tomato seed treatment with water extracts from the BEA 0300B strain of cyanobacterium *Anabaena minutissima* and the macroalgae *Ecklonia maxima* and *Jania adhaerens* could promote seed germination, seedling emergence and dry weight, acting as seed priming and simultaneously control the soil-borne pathogen *Rhizoctonia solani*. This study was divided into three experimental phases. The effect of seed treatment with extracts was evaluated, in the first phase, on seedlings grown on agarized medium, in the second one, on seedlings grown in a substrate under greenhouse conditions. The third phase consisted of a study of possible mechanisms of action of the extracts including eliciting of seedling defence responses. Overall, our findings showed that tomato seed treatment with the water extracts might be considered as a biopriming treatment, because it produces beneficial effects by increasing seed germination, seedling emergence and dry weight, enhancing the chitinase activity and the deposition of lignin in root tissues. We think that these effects may be collectively involved in the *R. solani* disease control.

The cyanobacterium *A. minutissima* and the macroalgae *E. maxima* and *J. adhaerens* used in this study showed a distinct chemical composition in nutrients, which could also explain their different action in eliciting plant physiological responses. Concerning the micronutrients, Fe, Cu, Zn, and Mn were markedly more concentrated in *A. minutissima* (1969, 12.1, 41.25 and 258 µg/kg, respectively). Their initial nutrient composition would be considered a real supplementation of micronutrients necessary for the growth of the treated plants. In particular, iron is present in a chelated form in algae, a condition favorable to iron uptake by tomato plants grown in sandy or calcareous soils, where a deficiency of Fe is expected (Cerdán et al., 2013). Mn is another important micronutrient for plant growth that is involved in the lignin biosynthesis and pathogen defense system (Alejandro et al., 2020). However, it cannot be excluded that the variability in algae and cyanobacteria chemical composition also depends on the species and where they have been harvested. In particular, we found several functional groups attributable to specific molecular structures that characterized *A. minutissima* for the high protein content also supported by N content, *E. maxima* for the presence of alginates and carbohydrates, and finally, *J. adhaerens* for the very high level of carbonate. All these compounds were considered to have several biological activities, including the stimulation of natural defence responses in plants (Hernández-

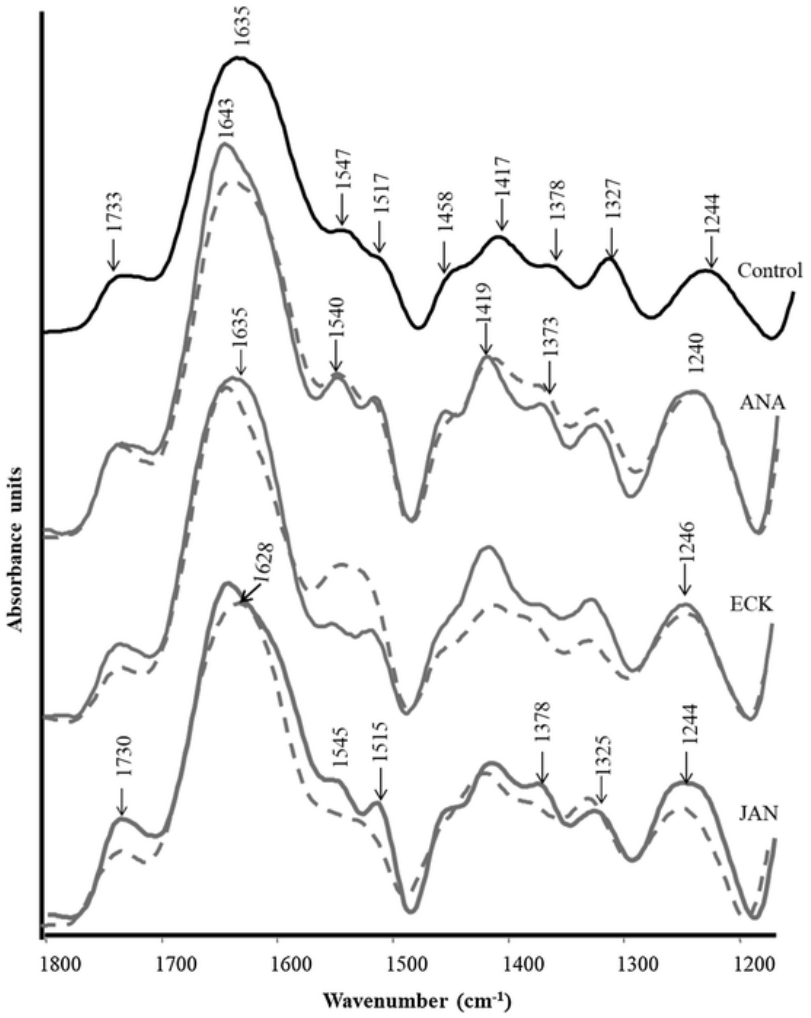

Fig. 6. FTIR spectra of freeze dry tomate roots from seeds primed with *Anabaena minutissima* (ANA), *Ecklonia maxima* (ECK) and *Jania adhaerens* (JAN) extracts at the concentrations of 5.0 (grey line) and 10.0 mg/mL (grey dashed line). Untreated sample (control) is located on the top (black line).

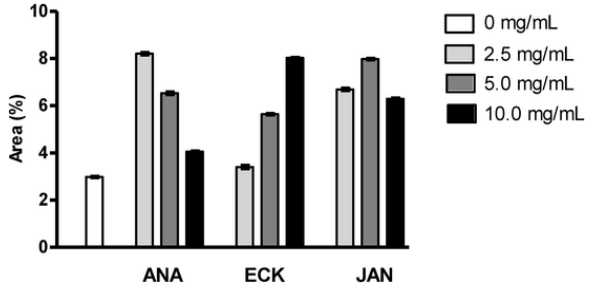

Fig. 7. Effects of tomato seed priming with different concentrations (0.0, 2.5, 5.0, 10.0 mg/mL) of water extracts from *Anabaena minutissima* (ANA), *Ecklonia maxima* (ECK), and *Jania adhaerens* (JAN) on the accumulation of lignin compounds in tomato roots. Columns are mean values (n = 3) ± SD of peaks areas at 1510 cm^{-1} processed by using curve fitting. The error bars correspond to the standard deviation.

Herrera et al., 2016). We found that all extracts exhibited a very positive influence on the total nitrogen accumulation in the roots with a more marked impact related to the *A. minutissima* and *E. maxima* extracts than *J. adhaerens*. Algae and cyanobacteria extracts were extensively studied for their beneficial effects on plants (Calvo et al., 2014; Craigie, 2011; Essa et al., 2015; Obana et al., 2007; Prasanna et al., 2013; Righini et al., 2018). They can enhance several plant parameters, such as seed germination, root development, and help seed and the future plant to overcome both abiotic and biotic stresses (El-Mougy et al., 2012).

The application of extracts by seed treatment can lead to the optimal absorption of nutrients and growth regulators (Scott, 1998). Overall, we obtained that *E. maxima*, *J. adhaerens* and *A. minutissima* extracts applied by seed treatment increased tomato seed germination and seedling emergence. Similarly, seed soaking in extracts from other brown algae, *Sargassum wightii* and *Ascophyllum nodosum*, increased black-eyed pea seed germination (Sivasankari et al., 2006) and bean seedlings emergence (Carvalho et al., 2013). It is well known that extracts from brown algae are rich of many plant hormones such as abscisic acid, auxins, cytokinins, gibberellins and brassinosteroids that can affect seed and plant metabolism, influencing their growth and development (Craigie, 2011; Khan et al., 2009; Stirk et al., 2014). The beneficial effects obtained in our study might be due to the presence of hormones in the water extracts. Indeed, gibberellins promote seed germination, organ differentiation, shoot growth, stem elongation leaf expansion. A positive interaction between gibberellins with auxins to stimulate cell expansion, differentiation and root elongation. Brassinosteroids promote cell division, elongation, stem, root and vascular differentiation, while cytokinins are associated with nutrient mobilization (Khan et al., 2009; Stirk et al., 2014).

Concerning red algae, to our knowledge, no study has been carried out on their biostimulant effect by seed treatment. However, positive effects on chickpea and maize plant growth were obtained by spray

and soil treatments respectively with an extract from *Jania rubens* (Abdel Latef et al., 2017; Safinaz and Ragaa, 2013) and on tomato by foliar application of *Kappaphycus alvarezii* (Zodape et al., 2011, 2009). The effects of the red algae are related to their content in glycine betaine, plant growth regulators, carrageenans, phenolic compounds, micronutrients that play a major role in improving crop productivity in addition to enhance abiotic stress tolerance (Prasad et al., 2010; Trivedi et al., 2018). About cyanobacteria, it is known that they can produce growth-promoting substances such as auxins, cytokinins, and gibberellins (Singh, 2014) whose beneficial effects on plants are the same above reported for algae. Even in this case, hormones may be involved in the increase of seed germination, seedling emergence and dry weight we have obtained by tomato seed treatment with *A. minutissima* water extract. Indeed, in line with our results, some authors reported the biostimulant effects of *Anabaena* sp. on several crops. For example, *Anabaena variabilis* extract applied on seeds of *Hordeum vulgare* L. and *Trigonella foenum-graecum* caused an increase of seed germination, as well as of shoot length, fresh and dry weight of seedlings (Ismail and Abo-Hamad, 2017). Wheat seed treatment with *A. variabilis* increased germination rate and stimulated the growth of the plants (Kumar and Kaur, 2014); sorghum and sunflower seed soaking in *Anabaena oryzae* extract increased seed germination, shoot and root length and fresh weight (Essa et al., 2015).

In general, the effect of the extracts was also visible in the FT-IR spectra of the roots through the Amide I, the typical band of proteins (Fig. S1). In *A. minutissima* treatment, Amide I increased by 37 % at the highest dose. Remarkable was the effect of the *E. maxima* extract which gave a 50 % increase of Amide I at the highest dose. In the treatment with *J. adhaerens*, Amide I increased by 39 % and 44 % at doses of 2.5 and 5.0 mg/mL, respectively. This type of response can be attributed to the characteristic biostimulant effect of algae, as also described by Ertani et al. (2018).

It is noteworthy that the extracts also stimulated the accumulation of lignin compounds as revealed by the FT-IR spectra of tomato roots (Fig. 7). In the treated roots, lignin compounds ranged from 5 % to 8 % compared to the untreated roots (3 %). This finding can be considered an important effect since lignin is involved in plants' defence against abiotic and biotic stress (Agrios, 2005). This remark suggests that when seeds are treated with algal extracts two principal metabolic pathways may be stimulated, *i.e.* the primary N metabolism and the secondary metabolism involved in the lignin synthesis (Ertani et al., 2018).

In response to the *R. solani* challenge, all extracts reduced root rot disease and increased seedling dry weight in the *in vitro* assay. The disease control depended both on the extracts and their concentrations, while all the extracts showed a similar effect against the pathogen in the pot experiment. In the *in vitro* assay, the extract from *Anabaena* sp. reduced the disease at all doses, more significantly at the 10 mg/mL dose.

Moreover, *A. minutissima* extract caused a high increase of chitinase activity, particularly at 10 mg/mL, which is a marker of plant defence response (ISR). This is consistent with what already observed by Roberti et al. (2015) on zucchini cotyledons. A spray application of water extract from the same *Anabaena* strain increased chitinase, β-1,3-glucanase, and peroxidase activities in cotyledon tissues and reduced the *Podosphaera xanthii* disease. Similarly, *A. variabilis* and *A. laxa* caused in tomato tissues an increment of defence enzymes activities, such as PAL, PPO, chitosanase, and β-1,3 glucanase (Prasanna et al., 2008). The resistance induction mechanism is likely elicited by extract compounds such as polysaccharides. Indeed, *A. minutissima* polysaccharides (previously *Anabaena* sp.) applied as a pre-harvest treatment on strawberry fruits reduced grey mould disease caused by *Botrytis cinerea* which was inoculated after the treatment (Righini et al., 2019).

The disease control exerted by the extract from the red alga *J. adhaerens* seems to be due to the induction of plant defences, indeed a high plant chitinase activity at all doses was observed. The same extract applied on cucumber cotyledonary leaves differentially induced the expression of pathogenesis-related genes, particularly chitinases through the involvement of the jasmonic acid pathway (Righini et al., 2020). It is well known that extracellular matrix of red seaweeds contains sulphated galactans as major (Aruna et al., 2010; Damonte et al., 2004; Matsuhiro et al., 2005; Pujol et al., 2006; Souza et al., 2012) which are elicitors of plant defence responses against pathogens, as in the case of *Phytophthora parasitica* var. *nicotianae* on tobacco plants (Mercier et al., 2001). On tomato seedlings infected by *Macrophomina phaseolina*, Agarwal et al. (2016) demonstrated that an extract from the red alga *K. alvarezii* increased the transcription of pathogenesis-related genes such as PR-1b1, PR-3, and PR-4 and the endogenous concentration of salicylic acid and the cytokinin zeatin. A high concentration of cytokinin leads to salicylic acid accumulation and activation of defence gene expression (Argueso et al., 2012).

Concerning the extract from the brown alga *E. maxima*, as in the case of *J. adhaerens*, the control of *R. solani* seemed to be due to the induction of plant defences. A correlation between the disease control and the induction of plant defences was shown by Jayaraman et al. (2011) with an extract from the brown alga *Ascophyllum nodosum* applied on cucumber plants as spray treatment and/or root drench. Indeed, these treatments reduced the disease caused by *F. oxysporum*, *Alternaria cucumerinum,* and *B. cinerea* and enhanced the activities of various defence-related enzymes such as chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase. Among these enzymes, chitinases and β-1,3-glucanases can degrade the fungal cell wall components and their increase is considered a molecular marker of plant induced resistance (van Loon et al., 2006). Klarzynski et al. (2003) showed that the sulphated fucans, which are polysaccharides occurring both in red and brown algae, are elicitors of plant defence responses against pathogens by inducing systemic accumulation of both salicylic acid and pathogenesis-related proteins in the tobacco leaves.

In the majority of literature, the extracts from algae and cyanobacteria were mainly obtained by using organic solvents, whereas little research has been carried out by using water extracts notably against plant pathogens (Roberti et al., 2015, 2016). Therefore, data obtained in the present study provide deep insights into the relationship of tomato seed priming with *R. solani* disease control.

In conclusion, this study shows that seed treatment with water extracts from *A. minutissima*, *E. maxima,* and *J. adhaerens* was active against *R. solani* root rot by working indirectly by seed biopriming through the involvement of plant defence responses helping the plant to withstand the pathogen. Once these effects will be verified on tomato plants in a larger scale experiment, these extracts may provide a useful preventative tool to apply in environmentally-friendly disease management, reducing the potential adverse environmental effects of hazardous pesticides.

CRediT authorship contribution statement

Hillary Righini: Conceptualization, Methodology, Data curation, Writing - original draft. **Ornella Francioso:** Methodology, Data curation. **Michele Di Foggia:** Methodology. **Antonio Prodi:** Methodology. **Antera Martel Quintana:** Resources. **Roberta Roberti:** Conceptualization, Data curation, 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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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2021.109921.

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