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Inhibitory activity of aqueous extracts from *Anabaena minutissima*, *Ecklonia maxima* and *Jania adhaerens* on the cucumber powdery mildew pathogen in vitro and in vivo

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Abstract

Aqueous extracts from *Anabaena minutissima* BEA 0300B (ANA), *Ecklonia maxima* (ECK) and *Jania adhaerens* (JAN) were evaluated for their antifungal effect against powdery mildew disease caused by *Podosphaera xanthii* on cucumber detached cotyledons and seedlings. All the extracts were sprayed on detached cotyledons at 2.5, 5.0 and 10.0 mg dry biomass mL^{-1} water and those of ANA and JAN at 5.0 and 10.0 mg mL^{-1} on seedlings before pathogen challenge (10^6 spores mL^{-1}). ANA and JAN at 5.0 and 10.0 mg mL^{-1} reduced infected area and fungal sporulation on both detached cotyledons and seedlings. ANA and JAN at 5.0 and 10.0 mg mL^{-1} were also evaluated for their elicitation of seedling defence responses, 1, 2 and 3 days before *P. xanthii* inoculation. Treatments reduced disease symptoms depending on extract, concentration and application time. Both extracts differentially induced the expression of PR genes, which may have concurred to pathogen control. At all times, ANA mainly induced AePR3 and PR1 genes, at 5.0 and 10.0 mg mL^{-1}, respectively, while JAN mainly induced AePR3 and PR4 at 5.0 mg mL^{-1}. This suggests that both ANA and JAN activated the expression of genes within the jasmonic acid and salicylic acid pathway. Proteins, phycobiliproteins, chlorophylls, carotenoids and antioxidant activities determined in the extracts could be involved in the antifungal effect or induction of plant systemic resistance. These results demonstrate that aqueous extracts from algae and cyanobacteria may be considered for further studies as a bio-based strategy for sustainable disease management.

Keywords Aqueous extracts · Cyanobacteria · Algae · Antifungal activity · Biocontrol · Plant-induced resistance

Introduction

The use of natural compounds is recommended for plant disease control because of the increasing demand by consumers for safe food with low levels of synthetic pesticide residues. The use of natural alternatives wherever possible is a preferred approach to control plant pathogens and to enhance food safety, which have been encouraged by the EC Regulation No. 1107/2009 and by the European Directive 128/2009/EC. These laws seek to promote sustainable agriculture. Algae and cyanobacteria are natural sources of antifungal compounds such as polysaccharides, phenols, cyclic peptides and terpenes (de Cano et al. 1990; Burja et al. 2001; Vera et al. 2011; Righini et al. 2018). Most antifungal studies using algal and cyanobacterial extracts focus on human pathogens (Cheel et al. 2016; Pérez et al. 2016; Boutin et al. 2019; Kidgell et al. 2019; Torres et al. 2019), so overall the literature is lacking studies addressing plant pathogens despite several published studies demonstrating the efficacy of algal and cyanobacterial extracts on turnip (Stephenson 1965), tomato (Jiménez et al. 2011; Chaudhary et al. 2012; Prasanna et al. 2013), strawberry (Righini et al. 2018), cucumber (Jaulneau et al. 2011; Jayaraman et al. 2011), zucchini (Roberti et al. 2015, 2016) and common bean and grapevine (Jaulneau et al. 2011).

Worldwide, powdery mildew is one of the major destructive fungal diseases of cucurbits, both in open field and under greenhouse conditions (Cohen et al. 1996; Kobori et al. 2004; Gengotti and Brunelli 2007; Davis et al. 2007; Pérez-García

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et al. 2009; Kousik et al. 2011; Lebeda et al. 2011). Currently, the disease is mostly controlled by using chemical fungicides, but growers are seeking sustainable solutions. The disease may be caused by different Ascomycota fungi, *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff, *Golovinomyces cichoracearum* (DC.) V.P. Heluta and *Sphaerotheca fuliginea* (Schltdl.) Pollacci. Among these species, *P. xanthii* is considered the main causal agent of cucurbits powdery mildew in many countries of Europe and around the world (Pérez-García et al. 2009; McCreight et al. 2012; Cerkauskas and Ferguson 2014). The disease is visually distinguishable by typical symptoms consisting of whitish powdery mass mainly composed by mycelium and mitospores (Martínez-Cruz et al. 2014). Symptoms are present on leaf surfaces, petioles and stems, occasionally on fruits (Pérez-García et al. 2009). When the environmental conditions are favourable, fungal colonies coalesce may cover the entire upper surface of the leaves leading to chlorosis and early host tissue senescence (Keinath and Dubose 2004). The infected fruits may be malformed and sunburned and sometimes may ripen prematurely (Pérez-García et al. 2009).

Few studies have examined the protectant activity of extracts from cyanobacteria and algae against powdery mildew on cucurbits. Powdery mildew caused by *S. fuliginea* on cucumber was reduced by an extract of *Ulva armoricana* (Jaulneau et al. 2011), while on squash, a commercial product based on laminarin from *Laminaria digitata* reduced *P. xanthii* disease severity (Zhang et al. 2016). On zucchini, the disease caused by *P. xanthii* was controlled by application of extracts from the cyanobacterium *Anabaena minutissima* (formerly *Anabaena* sp.) and other algal species (Roberti et al. 2015, 2016) and from commercial compound based on *Ascophyllum nodosum* extract (Roberti et al. 2011). On cucumber, Jaulneau et al. (2011) showed that the extract of *U. armoricana* elicits a reporter gene regulated by a defence-gene promoter in a transgenic tobacco line, while on zucchini, *A. minutissima* extract was able to enhance enzyme activities correlated to plant-induced resistance, such as chitinase, glucanase and peroxidase and in particular some of their acidic isoforms (Roberti et al. 2015).

In this research, we have undertaken a deeper investigation of our previous study and evaluated the preventative effects of extracts from the cyanobacterium *Anabaena minutissima* and from the algae *Ecklonia maxima* and *Jania adhaerens* against powdery mildew of cucumber. In particular, the purpose of this study was to evaluate (i) the antifungal activity of the extracts on detached cotyledons in vitro; (ii) their antifungal activity on seedlings under greenhouse conditions; (iii) the plant-induced systemic resistance to the pathogen through a bioassay conducted under greenhouse conditions; and (iv) the molecular mechanisms associated in induced resistance involving the expression of pathogenesis related (PR) proteins in cotyledonary tissue of seedlings grown under greenhouse conditions.

Materials and methods

Aqueous extract preparation and characterization

Lyophilized *Anabaena minutissima* BEA 0300B and dry thallus of both *Ecklonia maxima* and *Jania adhaerens* were provided by the Spanish Bank of Algae, University of Las Palmas de Gran Canaria. Dry thallus was ground to fine powder with mortar and pestle. Aqueous extracts of *A. minutissima* (ANA), *E. maxima* (ECK) and *J. adhaerens* (JAN) were obtained by suspending each powder in sterile distilled water (10.0 mg mL^{-1}) under continuous stirring at 50 °C for 12 h and then filtered through a sterile filter paper before use (Roberti et al. 2015). Concentrations of 5.0 and 2.5 mg mL^{-1} were prepared by serial dilution (1:2) with sterile distilled water.

Proteins, chlorophylls, carotenoids, phycobiliproteins and antioxidant activities were assessed by colorimetric determination starting from 10 mg lyophilized aqueous extract. Therefore, prior to the analysis, the extracts were frozen at − 20 °C and then lyophilized with a FreeZone Freeze Dryers (Labconco Corporation, USA). Chlorophylls, carotenoids and phycobiliproteins were expressed as μg mg^{-1} lyophilized extract.

For protein determination, lyophilized aqueous extracts were dissolved in 2 mL of NaOH (0.1 M) essentially as described by Parimi et al. (2015). Samples were incubated for 1 h in an Eppendorf Thermomixer R Mixer at 42 °C and then centrifuged for 10 min at 10,000×*g*. Protein concentration was determined in supernatant by Lowry colorimetric method (Lowry et al. 1951), using bovine serum albumin (Bio-Rad Laboratories, Inc.) as a standard. The experiment was repeated three times ($n = 3$).

For the determination of chlorophylls and carotenoids, the methods of Wellburn (1994) and Lichtenthaler and Buschmann (2001) were used. The experiment was repeated three times ($n = 3$).

For phycobiliproteins, lyophilized extracts were mixed with 8–10 glass beads for 5 min, and then 1 mL of phosphate buffer was added and mixed (Retsch MM400) for 5 min at 30 Hz. After 3 h of incubation in a thermomixer at 23 °C at 450 rpm, samples were centrifuged for 15–20 min at 10,000×*g*. The absorbance at 652, 615 and 562 nm was measured in the supernatants. Phosphate buffer was used as blank. Phycobiliproteins content of the lyophilized extracts was calculated using the following equations (Bennett and Bogorad 1973):

$$\text{Phycocyanin (PC)}\ \left(\text{mg mL}^{-1}\right) = [A_{615} - (0.474 \cdot A_{652})]/5.34$$

$$\text{Allophycocyanin (APC)}\ \left(\text{mg mL}^{-1}\right) = [A_{652} - (0.208 \cdot A_{615})]/5.09$$

$$\text{Phycoerythrin}\ \left(\text{mg mL}^{-1}\right) = [A_{562} - (2.41 \cdot \text{PC}) - (0.849 \cdot \text{APC})]/9.62$$

The experiment was repeated three times ($n = 3$).

For free-radical scavenging activity (antioxidant activity), lyophilized extracts were analysed by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) essentially as described by Shanab et al. (2012). Extracts were added with 0.5 mL of MeOH (100%) and vortex-mixed for 5 min at room temperature. After further addition of 0.5 mL of MeOH, samples were incubated in the dark for 3 h in a thermomixer at 23 °C at 450 rpm. After centrifugation, 150 μL of supernatant was added to 150 μL of DPPH (1.27 mM in MeOH 90%) and 1.35 mL of MeOH (90%). MeOH (90%) + DPPH was used as control and MeOH (90%) only as blank. Absorbance at 517 nm was measured after 30 min at room temperature. The DPPH scavenging effect was calculated as follows:

$$\%\text{scavenging activity} = \left(A_{\text{control}} - \left(A_{\text{sample}} - A_{\text{blank}}\right)/A_{\text{control}}\right) \times 100.$$

The experiment was repeated three times ($n = 3$).

Pathogen inoculum and plant material

Podosphaera xanthii isolate was obtained from cucumber plants var. Le Généreux (Vilmorin Jardin, St. Quentin Fallavier Cedex, France) naturally infected with powdery mildew grown under a plastic tunnel located in the Po Valley, province of Bologna, Northern Italy. The pathogen was identified based on morphological features of spore germination (Zaracovitis 1965) and the presence of fibrosin bodies (Lebeda 1983). Infected leaves were randomly collected, and a spore suspension was made in distilled water (5×10^4 spores mL^{-1}). The inoculum was sprayed on 15-day-old cucumber seedlings var. Le Généreux grown until cotyledonary leaves were fully expanded, by using a handheld spray atomizer at the rate of 20 mL per seedling (Mercier et al. 2014; Kousik et al. 2018). Seedlings were grown in a substrate consisting of a sterile mixture of peat moss, sand and vermiculite (2:1:1, v:v:v) in pots (10 cm Ø) at 24–26 °C (day), 20–22 °C (night), 70% relative humidity, and 14-h photoperiod under greenhouse conditions. The pathogen inoculum was renewed weekly by replacing the old plants with newly inoculated plants throughout the experiments. Non-inoculated plants, for both inoculum renewal and the experiments, were kept in an isolated sector of the greenhouse under the same conditions described above.

Antifungal activity by detached cotyledon assay

Detached cucumber cotyledons were used as a model system to study the antifungal activity of aqueous extracts (Moret et al. 2009). Cotyledons were randomly excised at the base of their laminae from 15-day-old healthy seedlings, sterilized with sodium hypochlorite (1% active Cl) for 90 s, washed three times with sterile distilled water and dried with adsorbent sterile paper under a sterile flow cabinet. Four cotyledons were placed in each polystyrene Petri plate (13 cm Ø) on agarized medium containing Difco agar 1.5%, glucose 10 g L^{-1}, benomyl 1 g L^{-1}, prochloraz 10 mg L^{-1} and streptomycin sulphate 0.5 g L^{-1}. Cotyledons were arranged with the abaxial surface in contact with the medium, and then they were sprayed with 4 mL of ANA, ECK and JAN extracts at three concentrations each, 2.5, 5.0 and 10.0 mg mL^{-1}. Chitosan was used as a positive control (4 mL, 10.0 mg mL^{-1}), and it was prepared from crab-shell chitosan (Sigma Chemical Co., USA) following the procedure of Romanazzi et al. (2002). Chitosan was used because it is known to act directly against fungi. Negative control cotyledons were sprayed with 4 mL of sterile distilled water. After the cotyledons were air-dried for 30 min, they were inoculated on the adaxial surface with six 10-μL drops of a spore suspension of *P. xanthii* (1×10^6 spores mL^{-1}), according to Moret et al. (2009) with modifications. Three replicates (plates; $n = 3$) were considered for each extract concentration and for the controls. Plates were incubated in a completely randomized experimental design in a growth chamber at 24–25 °C in the dark for 48 h and then shifted to 14-h photoperiod. Nine days after pathogen inoculation, disease symptoms were evaluated visually, and disease severity was expressed as percentage of inoculated area showing white, powdery spots. Cotyledons from each plate were then washed with distilled water (5 mL per cotyledon) to remove the spores. Four 10-μL drops of the spore suspension were observed at a microscope (Zeiss, GmbH, Germany, × 300 magnification). All spores were counted and the number of spores per mm^2 of inoculated area (sporulation density) was calculated for each plate ($n = 3$). The experiment was repeated twice.

Antifungal activity by seedling assay

Fifteen-day-old seedlings were treated with ANA and JAN extracts at 5.0 and 10.0 mg mL^{-1} and chitosan at 10.0 mg mL^{-1} as positive control (2 mL per seedling). Two millilitres of distilled water were sprayed on negative control seedlings. Once the cotyledonary leaves were dried, 2 mL of a spore suspension of *P. xanthii* (1×10^6 spores mL^{-1}) were sprayed on treated and control seedlings. Three seedlings ($n = 3$) for each treatment and for the controls were arranged. The seedlings were distributed in a completely randomized experimental design on a shelf of the greenhouse and grown at 24–26 °C (day), 20–22 °C (night), 70% relative humidity, and 14-h photoperiod. For disease evaluation, the percentage of cotyledonary area showing symptoms and the pathogen sporulation were determined 9 days after pathogen inoculation as above described for detached cotyledon assay. The experiment was repeated twice.

Systemic-induced resistance bioassay

For this assay 15-day-old seedlings at the cotyledonary stage were considered. In order to demonstrate the induction of plant systemic resistance, treatments were applied at site spatially separated from *P. xanthii* inoculation according to the model system of De Meyer et al. (1998). Seedling treatments with ANA and JAN (5.0 and 10.0 mg mL^{-1}), chitosan as positive control (10.0 mg mL^{-1}) and water as negative control were applied on one of the two cotyledons (1 mL of extract, chitosan or water per cotyledon), while the other cotyledon was left untreated by covering it with an aluminium sheet. Chitosan was used because it elicits the jasmonic acid (JA) pathway mostly and is able to activate some genes involved in the salicylic acid (SA) pathway. The sheet was removed 4 h after treatment. One, 2 and 3 days after treatment (DAT), 1 mL of *P. xanthii* spore suspension (1×10^6 spores mL^{-1}) was sprayed on each untreated cotyledon. Four seedlings (one seedling in a pot; $n = 4$) for each treatment and the controls were considered. They were randomly distributed on a shelf of the greenhouse under the same conditions reported above. Disease was evaluated as percentage of area showing symptoms, 9 days after pathogen inoculation. The experiment was repeated twice.

Expression of PR protein genes

Seedling treatments were performed as reported above for the induced resistance assay. The ANA and JAN extracts were separately applied at the concentration of 5.0 and 10.0 mg mL^{-1}. Chitosan at 10 mg mL^{-1} was used as a positive control, since it is a well-known potent elicitor of plant defence responses, and it was prepared as described above. Non-treated cotyledons were collected from 3 seedlings (one seedling in a pot; $n = 3$) for each treatment and the controls. Cotyledon tissues were ground to a fine powder in liquid N_2, and cellular RNA was extracted with TRIZOL (Invitrogen), according to manufacturer's instructions. The RNA pellets were quantified by using a spectrophotometer (Nanovue, GE Healthcare Life Sciences), and the same amount of total RNA (500 ng) was reverse transcribed by using random primers and the reagents provided with the Superscript VILO Master Mix for RT-qPCR (Thermo Fisher). The cDNA mixture (2 μL) was used in real-time PCR analysis in a LightCycler Instrument (Roche Molecular Biochemicals) by means of the QuantiTect SYBR Green PCR kit (TaKaRa) with the following protocol: initial activation of HotStart Taq DNA polymerase at 95 °C for 10 s, followed by amplification (40 cycles: 95 °C for 5 s followed by annealing step at 59 °C kept for 20 s). The protocol was concluded by melting curve analysis to check amplicon specificity. Primer sequences used in the experiment are given in Table 1. The amount of mRNA was normalized for Actin-7 expression in each sample and referred to untreated control sample. The experiment was repeated twice.

Statistical analysis

All experiments were arranged in a complete randomized design. Data obtained from antifungal activity assays were analysed by two-way ANOVA, while those obtained from induced resistance bioassay, gene expression of PR proteins and determination of chlorophylls, carotenoids, proteins, phycobiliproteins and antioxidant activity were analysed by one-way ANOVA. Means were separated by Fisher's least significant difference (LSD) test ($p < 0.05$). Data are reported as mean values ± standard deviation. Analysis was performed with the software Statgraphic Plus Version 2.1 (Statistical Graphics Corp., USA).

Results

Antifungal activity

The preliminary detached cotyledon assay performed with ANA, ECK and JAN at three concentrations (2.5, 5.0 and 10.0 mg mL^{-1}) showed some reduction of the infected area and of the sporulation (Table 2). For both parameters, two-way ANOVA indicated a significant interaction between extract and concentration factors. *Anabaena* sp. extract significantly reduced both infected area and sporulation at 5.0 (by 58.9% and 79.7%, respectively) and 10.0 mg mL^{-1} (by 58.5% and 72.8%, respectively) towards the control (0.0 mg mL^{-1}). *Jania adhaerens* extract reduced the infected area by 25.5 and 33.0% and the pathogen sporulation by 44.0 and 48.3% at 5.0 and 10.0 mg mL^{-1}, respectively, whereas ECK did not exert

Table 1 Primer sequences used in PCR amplification

Gene name	Primer sequence (5′-3′)
Actin-7	TCCACGAGACTACCTACAACTC GCTCATACGGTCAGCGAT
PR1	ATTGGATGCGGTGACTTCGT ATCTGTGTGTAATGGCCGCA
PR2 (β-1,3-glucanase)	TGTGGTTGGAGATTCGTGGG TCGCAACGTCCCGTTTAAGA
AePR3 (Acidic endochitinase)	TTATTCACTCTCCTCCGCCG CCTGAGCTAGTACGTCCCAG
BePR3 (Basic endochitinase)	GCGCCATTCGATGACGAAAA GATCCACATAACCCCGACCC
PR4 (Endochitinase)	GCCGACAAGCCTTTGGAATG TTCCGAAGCTCCCGTTTCAG
PR5	GAATCCGCCGTCTACACCAT CCTTGTCGAAGTCACAGCCT

any effect on both parameters at all concentrations. Overall, ANA was more effective than JAN. Some effective treatments, such as ANA and JAN at 5.0 and 10.0 mg mL^{-1}, displayed an antifungal activity higher than or equal to chitosan treatment, used as a positive control. The extract from ANA reduced the infected area by an average of 36.5% with respect to chitosan and both extracts at 5.0 and 10.0 mg mL^{-1} reduced sporulation by an average of 70.0 and 25.4% with respect to chitosan, respectively.

The antifungal activity assay has also been carried out with seedlings under greenhouse conditions (Table 3). For this assay, we have used ANA and JAN at 5.0 and 10.0 mg mL^{-1}, because they proved to be the most effective treatments in the previous assay. Two-way ANOVA indicated a significant interaction between extract and concentration factors for both infected area and sporulation (Table 3). As in the detached cotyledon assay, extracts from ANA and JAN at all doses significantly reduced both the infected area and the pathogen sporulation with respect to the control. *Jania adhaerens* extract showed higher efficacy than ANA for both infected area (by 22.2%) and sporulation (by 66.1%) at 10.0 mg mL^{-1}, while no difference was observed between the two extracts at 5.0 mg mL^{-1}. Our results also showed that ANA and JAN at both concentrations reduced the sporulation more than chitosan by 42.0 and 38.6% at 5.0 mg mL^{-1}, respectively, and by 31.9 and 76.9% at 10.0 mg mL^{-1}, respectively (Table 3). Both extracts at 10.0 mg mL^{-1} reduced the infected area as well as chitosan.

We have searched for compounds in aqueous extracts potentially active as antifungal. Figure 1 shows that protein content and antioxidant activity were higher in ANA than in JAN, while the lowest were in ECK. Likewise, extract components such as carotenoids, chlorophylls and phycobiliproteins were more abundant in ANA than in JAN and scarcely present or absent in ECK (Fig. 1).

Systemic-induced resistance

The systemic-induced resistance bioassay showed that the efficacy of treatments was variable depending on the time (DAT) and on the concentration (Fig. 2). At 1 DAT, application of ANA at 10.0 mg mL^{-1} and JAN at 5.0 mg mL^{-1} significantly reduced the percentage of infected area with respect to the control. The reduction of infected area by JAN at 5.0 mg mL^{-1} was not statistically different from that of chitosan at 1DAT and higher than chitosan at 2DAT. At 2DAT, even JAN at 10.0 mg mL^{-1} reduced the infected area as well as chitosan. At 3DAT, ANA and JAN at 5.0 mg mL^{-1} significantly reduced the disease with respect to the control and similarly to chitosan. The specific assay on gene expression of PR proteins showed an upregulation of PR1, PR2, PR3 (both acidic and basic endochitinases), PR4 and PR5 depending on extract, its concentration and application time (Fig. 3). Overall, we observed a co-upregulation of almost all genes by ANA and JAN after 1, 2 and 3DAT compared with the control. The treatment with JAN significantly enhanced the transcription level of all genes at 1 and 2DAT, except for BePR3 (basic endochitinases) at 1DAT. Actually BePR3 gene was generally little modulated by all these treatments. Spraying JAN at the lowest concentration was enough to cause the maximum induction of all gene expressions at 2DAT, and it significantly increased the expression of PR2, PR5 and

t2.1 **Table 2** Detached cotyledon assay: effect of treatment with extracts from *Anabaena minutissima* BEA 0300B (ANA), *Ecklonia maxima* (ECK) and *Jania adhaerens* (JAN) on infected area by *Podosphaera xanthii* and fungal sporulation

t2.2	Extract	Concentration (mg mL^{-1})			
t2.3		0.0	2.5	5.0	10.0
t2.4	Infected area (%)				
t2.5	*ANA*	56.4 ± 11.1 B	59.9 ± 12.1 aB	23.2 ± 2.4 aA*	23.4 ± 2.3 aA*
t2.6	*ECK*	57.1 ± 10.5	55.3 ± 4.9 a	55.8 ± 3.8 c	59.9 ± 1.6 c
t2.7	*JAN*	56.0 ± 9.5 B	141.7 ± 10.4 bC	41.7 ± 3.1 bA°	37.5 ± 2.6 bA°
t2.8	Sporulation (spores mm^{-2})				
t2.9	*ANA*	2431.2 ± 175.0 B	2304.2 ± 102.5 aB	494.2 ± 76.1 aA*	660.0 ± 58.4 aA*
t2.10	*ECK*	2418.7 ± 183.9	2464.2 ± 106.1 a	2575.4 ± 351.1 c	2328.6 ± 280.8 c
t2.11	*JAN*	2571.0 ± 457.7 B	6667.2 ± 424.1 bC	1438.8 ± 215.7 bA*	1329.9 ± 223.3 bA*

For both infected area and sporulation, extract and concentration factors and their interaction are significant according to factorial ANOVA ($p < 0.05$). For infected area: F (2, 36) = 45.4, $p < 0.05$ (for extract factor); F (3, 36) = 76.3, $p < 0.05$ (for concentration factor); F (6, 36) = 39.8, $p < 0.05$ (for interaction). For sporulation: F (2, 36) = 110.2, $p < 0.05$ (for extract factor); F (3, 36) = 169.7, $p < 0.05$ (for concentration factor); F (6, 36) = 84.6, $p < 0.05$ (for interaction). Means ($n = 3$) ± 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- or upper-case letters indicates no significant difference, according to LSD test ($p < 0.05$). The asterisk means antifungal activity higher than chitosan (positive control); the degree sign means antifungal activity equal to that of chitosan according to one-way ANOVA ($p < 0.05$). Chitosan: infected area = 36.7% ± 1.3; sporulation 1856.7 ± 58.6

t3.1 **Table 3** Antifungal activity by seedling assay: effect of treatment with extracts from *Anabaena minutissima* BEA 0300B (ANA) and *Jania adhaerens* (JAN) on infected area by *Podosphaera xanthii* and fungal sporulation under greenhouse conditions

t3.2 Extract	Concentration (mg mL^{-1})		
t3.3	0.0	5.0	10.0
t3.4 Infected area (%)			
t3.5 *ANA*	85.0 ± 3.8 C	65.7 ± 5.1 B	58.9 ± 4.9 bA°
t3.6 *JAN*	80.6 ± 4.4 C	69.5 ± 0.4 B	45.8 ± 5.4 aA°
t3.7 Sporulation (spores mm^{-2})			
t3.8 *ANA*	43,955.3 ± 6777.7 B	19,611.1 ± 2045.2 A*	23,055.6 ± 867.4 bA*
t3.9 *JAN*	46,987.0 ± 3956.0 C	20,765.1 ± 557.8 B*	7822.1 ± 258.0 aA*

For both infected area and sporulation, extract and concentration factors and their interaction are significant according to factorial ANOVA ($p < 0.05$). For infected area: F (1, 18) = 5.0, $p < 0.05$ (for extract factor); F (2, 18) = 74.1, $p < 0.05$ (for concentration factor); F (2, 18) = 5.7, $p < 0.05$ (for interaction). For sporulation: F (1, 18) = 5.5, $p < 0.05$ (for extract factor); F (2, 18) = 140.2, $p < 0.05$ (for concentration factor); F (2, 18) = 13.6, $p < 0.05$ (for interaction). Means ($n = 3$) ± 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- or upper-case letters indicates no significant difference, according to LSD test ($p < 0.05$). The asterisk means antifungal activity higher than chitosan (positive control); the degree sign means antifungal activity equal to that of chitosan according to one-way ANOVA ($p < 0.05$). Chitosan: infected area = 52.8% ± 1.4; sporulation 33,832.7 ± 2231.9

AePR3 more than chitosan (Fig. 3). These effects could be correlated to the disease control obtained in the bioassay at 1 and 2DAT where JAN at 5.0 mg mL^{-1} significantly reduced the percentage of infected area with respect to the control (Fig. 2). The application of JAN at 5.0 mg mL^{-1} also caused a significant reduction of the disease with respect to chitosan at 2DAT. At 3DAT, 5.0 mg mL^{-1} of JAN increased the expression of both PR4 and AePR3 (Fig. 3) consistently with the disease control in the systemic-induced resistance bioassay.

The ANA extract simultaneously upregulated the expression up to a maximum of two PR genes. In particular, ANA at 5.0 mg mL^{-1} upregulated AePR3 at all DAT and PR1 at 3DAT, in line with the control of the disease observed in the systemic-induced resistance bioassay at 3DAT. In addition, ANA at 10.0 mg mL^{-1} upregulated the expression of two different genes at each DAT. Consistently with the disease control obtained in bioassay with ANA at 5.0 mg mL^{-1} (3DAT) and ANA at 10.0 mg mL^{-1} (1 and 2DAT), the treatment at both concentrations upregulated PR1 expression.

On cucumber, we observed a similar behaviour of JAN aqueous extract for powdery mildew control in the detached cotyledon and seedling assays and in the resistance induction bioassay. Moreover, JAN elicited the expression of plant defence genes more than ANA.

Discussion

This study was carried out to investigate the effect of extracts from the red alga *J. adhaerens*, the brown alga *E. maxima* and the cyanobacterium *A. minutissima* BEA 0300B against *P. xanthii* on cucumber. The study was divided into two experimental phases. In the first phase, the antifungal activity of the extracts was evaluated and in the second one their eliciting activity of seedling defence responses.

Antifungal activity

The extracts from *A. minutissima* and *J. adhaerens* showed antifungal activity by reducing the percentage of symptomatic area and sporulation density in both in vitro and in vivo assay. Considering that *P. xanthii* is an obligate parasite, we used the detached cotyledonary leave assay to verify the efficacy of the extracts against the pathogen in vitro. Under these experimental conditions, the two highest doses (5.0 and 10.0 mg mL^{-1}) of *A. minutissima* and *J. adhaerens* were similarly effective in reducing both infected area and sporulation density. Under greenhouse conditions, the high extract dose was more effective than the low dose, because the disease severity was very high, more than 80% of infected area. However, detached cotyledon assay could be a suitable model system for a preliminary study to verify a possible effect of exogenous substances application as published by other authors (Ullanat and Jayabaskaran 2002; Moret et al. 2009; Gafni et al. 2015). Consistently, in a preliminary screening on zucchini detached cotyledons, 5.0 mg mL^{-1} of aqueous extract from *A. minutissima* 0300B strain and the red alga *Corallina* sp., belonging to the same family of *J. adhaerens* (Corallinaceae), reduced symptoms of *P. xanthii* (Roberti et al. 2016). The same aqueous extract from *A. minutissima* and the extract from *J. adhaerens* (formerly *Jania* sp.) at 5.0 and 10.0 mg mL^{-1} were also effective in reducing the colony growth of the not obligate plant pathogen *Botrytis cinerea* (Righini et al. 2019). Other in vitro studies on cyanobacteria reported the antifungal activity of different *Anabaena* species against non-obligate fungal plant pathogens such as

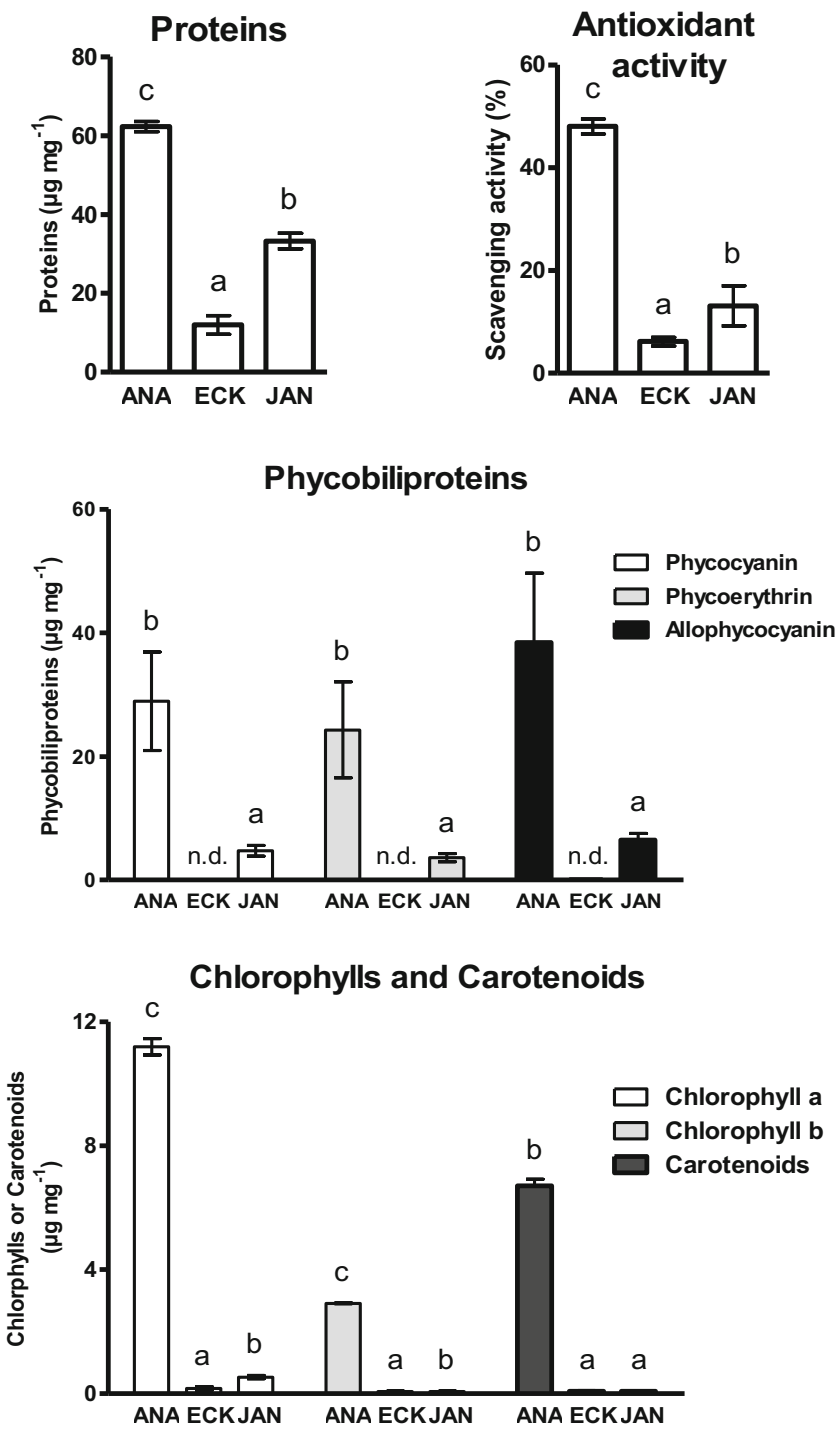


Fig. 1 Antioxidant activity and content of proteins, phycobiliproteins, chlorophylls and carotenoids in the extracts of *Anabaena minutissima* BEA 0300B (ANA), *Ecklonia maxima* (ECK) and *Jania adhaerens* (JAN). Bars represent means (n = 3) ± SD. Different letters within each determination indicate significant differences according to LSD test ($p < 0.05$); n.d., not detectable

Alternaria solani, *Fusarium moniliforme*, *Pythium aphanidermatum*, *P. debaryanum*, and *Rhizoctonia bataticola* (Prasanna et al. 2008; Radhakrishnan et al. 2009). Further studies have examined the efficacy of cyanobacteria against plant diseases. On tomato, substrate treatment with *Anabaena variabilis* and *A. oscillarioides* extracts, obtained with organic solvents, were effective against *Pythium debaryanum*, *Fusarium oxysporum* f.sp. *lycopersici*, *F. moniliforme* and *Rhizoctonia solani* (Chaudhary et al. 2012), while aqueous filtrates of *A. variabilis* and *A. laxa* fresh cultures amended to the growing substrate reduced plant wilt caused by *F. oxysporum* f.sp. *lycopersici* (Prasanna et al. 2013).

Cyanobacteria are a source of bioactive compounds that can act directly against fungi. Indeed, water-soluble polysaccharides extracted from the same extract of *A. minutissima* have shown antifungal activity against *B. cinerea* colony

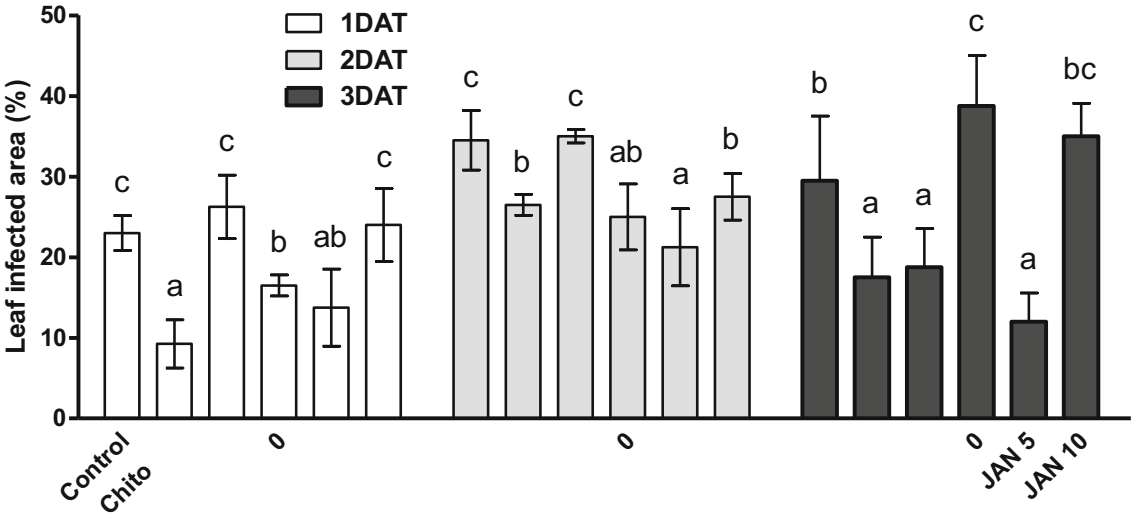
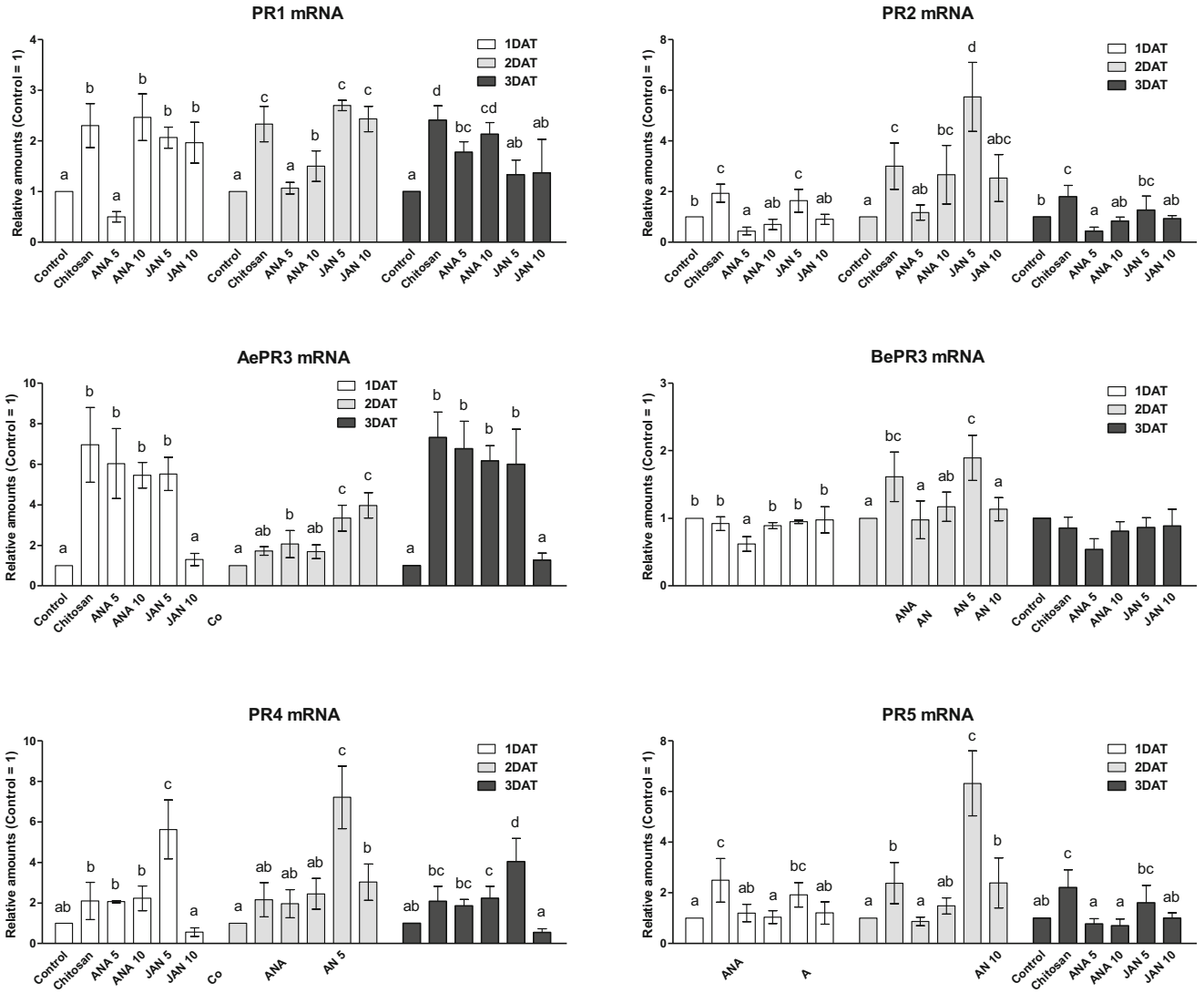


Fig. 2 Systemic-induced resistance bioassay: effect of treatment with extracts from *Anabaena minutissima* BEA 0300B (ANA) and *Jania adhaerens* (JAN) on the percentage of cotyledonary leaf area infected by *Podosphaera xanthii*. Inoculation with *P. xanthii* was carried out at 1, 2 or 3 days after the treatment (1, 2, 3DAT) with water (control), chitosan (10.0 mg mL^{-1}), and ANA or JAN (ANA 5, JAN 5 = 5.0 mg mL^{-1}; ANA 10, JAN 10 = 10.0 mg mL^{-1}). Disease was evaluated 9 days after pathogen inoculation. Bars represent means (n = 4) ± SD. Different letters within each DAT indicate significant differences according to LSD test ($p < 0.05$)

growth and colony-forming units (Righini et al. 2019). Several hydrolytic enzymes were also identified in cyanobacteria extracts, and the presence of chitosanases and endoglucanases were correlated to the antifungal activity against several plant pathogens (Gupta et al. 2010, 2011; Prasanna et al. 2010). Chitosanases are known to hydrolyse the β-1,4-glycosidic bonds that link *N*-acetyl glucosamine residues of chitin which is the main component of fungal cell wall. In addition to enzymes, cyanobacteria are also able to produce phenolic compounds that inhibited *Candida albicans* colony growth (de Cano et al. 1990). Marrez and Sultan (2016) demonstrated that 2,6-di-*t*-butyl-4-methyl phenol, hexadecanoic acid and methyl ester were the main responsible of the antifungal activity of the cyanobacterium *Microcystis aeruginosa* against *Aspergillus flavus*, *A. niger*, *Fusarium verticillioides* and *F. proliferatum*. Moreover, the terpenoid noscomin extracted from *Nostoc commune* showed antibacterial activity against human bacterial pathogens (Jaki et al. 2000).

Although a lot of scientific literature on the effectiveness of brown and green algae extracts against plant fungal diseases is published (Hamed et al. 2018), only a few findings are available on red algae efficacy. A study reported that *Porphyra umbilicalis* crude extract used on wounded fruits controlled *B. cinerea* grey mould on strawberries, *Monilia laxa* brown rot on peaches and *Penicillium digitatum* green mould on lemons when the extract was applied few hours after conidia inoculation (De Corato et al. 2017). In addition, the mycelial growth of the plant pathogen *Macrophomina phaseolina* was inhibited by an extract from the red alga *Gracilaria edulis* (Ambika and Sujatha 2015). Red algae synthetize several bioactive compounds that have shown antifungal activity. Water-soluble polysaccharides of *J. adhaerens* played an important role in reducing *B. cinerea* spore germination, thereby reducing the pathogen inoculum potential (Righini et al. 2019). Among polysaccharides, carrageenans, the major components of the extracellular matrix of red seaweeds, showed antifungal activity against *Alternaria* sp. and *Aspergillus* sp. (Soares et al. 2016). In addition to water-soluble polysaccharides, some authors identified lipids and phenolic compounds in crude extracts of *P. umbilicalis* whose antifungal activity was mainly ascribed to a direct toxicity of fatty acids such as palmitic acid (De Corato et al. 2017). Among phenols, the bromophenol bis(2,3-dibromo-4,5-dihydroxybenzyl) ether extracted from the red alga *Rhodomela confervoides* inhibited the mycelial growth, spore germination and the germ tube elongation of *B. cinerea* (Liu et al. 2014).

We measured some compounds in the aqueous extracts of *A. minutissima*, *E. maxima* and *J. adhaerens*, such as proteins, carotenoids, chlorophylls and phycobiliproteins, which are known for their antioxidant activity (Lanfer-Marquez et al. 2005; Shalaby 2011; Guedes et al. 2013; de Jesús Bonilla-Ahumada et al. 2018; Chentir et al. 2018). Carotenoids, chlorophylls and phycobiliproteins were more abundant in *A. minutissima* than in *J. adhaerens*, while they were scarcely present or absent in *E. maxima*. Still, the antioxidant activity was higher in *A. minutissima* than in *J. adhaerens*, while it was lower in *E. maxima*. Considering that *E. maxima* did not show any effect against *P. xanthii*, it is reasonable to assume that these components may be correlated to the antifungal activity of *A. minutissima* and *J. adhaerens* as stated by other authors. Machado et al. (2014) showed a significant increase in antifungal activity against *Colletotrichum gloeosporioides* of the red alga *Palisada*



Q3 **Fig. 3** Effect of treatment with extracts from *Anabaena minutissima* (ANA) and *Jania adhaerens* (JAN) on PR genes expression in cotyledonary leaves. Treatments were performed with water (control), chitosan (10.0 mg mL^{-1}) and ANA or JAN (ANA 5, JAN 5 = 5.0 mg mL^{-1}; ANA 10, JAN 10 = 10.0 mg mL^{-1}). After 1, 2 or 3 days of treatment (1, 2, 3DAT), transcript levels were assessed by real-time PCR quantitative reverse transcription-polymerase chain reaction. Bars represent means ($n = 3$) the fold change of PR genes in elicited seedlings relative to the control. Each error bar represents SD. Different letters within each DAT indicate significant differences according to LSD test ($p < 0.05$); absence of letter means no significant difference

flagellifera, which could be positively correlated with the change in concentration and ratios of chlorophyll *a* and accessory pigments such as carotenoids and phycobiliproteins. Other examples of correlation between antioxidant compounds with antimicrobial activity concern bacterial human pathogens (Shanmugam et al. 2017; Karpiński and Adamczak 2019).

Systemic resistance induction

Plants enhance their defensive capacity in response to appropriate stimuli through two forms of induced resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR involves accumulations of PR proteins and salicylic acid (SA), whereas ISR depends on jasmonic acid (JA) and ethylene (ET) pathways (Derksen et al. 2013; Ghannam et al. 2013; Pettongkhao et al. 2019). In our study, we included the investigation of the expression levels of PR1, PR2 and PR5, as members of SA-dependent PR genes, and PR3 and PR4 as members of JA-dependent PR genes, thus giving an overview of both resistance forms. Although more genes may be included in further studies, our work provides a first indication of the mechanisms modulated by the aqueous extracts from *Anabaena* and *Jania* species. Concerning the potential of aqueous extracts of *A. minutissima* and *J. adhaerens* to induce systemic resistance in cucumber seedlings under greenhouse conditions, a bioassay for powdery mildew control and a specific assay on gene expression of PR proteins in cotyledons were

conducted. In the bioassay, due to the spatial separation of treatment and pathogen inoculation, the disease control was attributed to plant-induced systemic resistance in which different expression of various PR protein genes is involved. For the bioassay, we adopted a modified model system already described by De Meyer et al. (1998). These authors concluded that the application of the fungal biocontrol agent *Trichoderma harzianum* T39 in the soil before *B. cinerea* inoculation on leaves of tomato, lettuce, pepper, bean and tobacco plants induced systemic resistance as verified by the disease suppression. *Anabaena minutissima* extract differentially induced the expression of PR genes, which may have concurred to pathogen control. Plant responses to *A. minutissima* treatment are JA and SA dependent. Jasmonic acid responsive genes (PR3) were mainly induced by *A. minutissima* extract at 5.0 mg mL^{-1} while SA responsive genes (PR1) at 10.0 mg mL^{-1}. Singh (2014), reviewing several articles on the role of elicitors molecules of cyanobacteria in the plant tolerance against biotic or abiotic stress, reported that their metabolites can promote the gene expression of the host and thereby help plants to counteract a pathogen invasion. The induction of seedling resistance by *A. minutissima* is in accordance with Prasanna et al. (2013), who demonstrated the increase of defence enzymes activities such as phenylalanine ammonia-lyase, polyphenol oxidase, chitosanase and β-1,3 glucanase in tomato plants grown in substrate treated with filtrates of *A. variabilis* and *A. laxa* fresh cultures. Also in our previous study, *A. minutissima* (formerly *Anabaena* sp.) at 5.0 mg mL^{-1} increased the total chitinase activity, particularly three chitinase acid isoforms at 1, 2 and 3 days after treatment (Roberti et al. 2015). Contrary to what was expected, *A. minutissima* at 10.0 mg mL^{-1} was not effective against the disease 3 days after treatment. The accumulation of several compounds produced by the cyanobacterium may be responsible of a seedling physiological stress. Indeed, *A. minutissima* showed high content of proteins, phycobiliproteins, chlorophylls and carotenoids and the highest antioxidant activity that may represent a challenge for the plant.

The extract from the red seaweed *J. adhaerens* appears to show mostly a biostimulant behaviour, since it was effective in reducing powdery mildew disease and eliciting PR defence genes more at 5.0 mg mL^{-1} than at 10.0 mg mL^{-1}. It is known that seaweed extracts have biostimulant effect on plants by involving the synthesis of several plant hormones (Khan et al. 2009). Still, they are bioactive at very low concentrations as reviewed by Khan et al. (2009). The highest concentration (10.0 mg mL^{-1}) of *J. adhaerens* could cause a stress to seedling that may affect the transcriptional activities of different components in different signalling pathways as in case of JA metabolic pathway (Yang et al. 2019). When 5.0 mg mL^{-1} of *J. adhaerens* were applied, almost all genes were coordinately expressed along with disease reduction. Among these genes, AePR3 and PR4 were always upregulated, while P1, PR2 and PR5 were expressed at the first and second time after treatment. This suggests that *J. adhaerens* extract activated the expression of genes within the SA and JA signalling pathways.

The elicitation of seedling defence response could be attributed to several compounds in the extracts of *A. minutissima* and *J. adhaerens*. In particular, the polysaccharides from red algae carrageenans can increase the expression of defence-related genes such as PR1 and PR5 in chilli plants (Mani and Nagarathnam 2018) as well as PR1, PR2, PR3 and PR5 (Ghannam et al. 2013), PR3 and proteinase inhibitors (Mercier et al. 2001) in tobacco leaves. The resistance induction we obtained in this study could also be attributed to the presence of proteins such as phycobiliproteins that were identified in the aqueous extracts. The phycobiliprotein phycocyanin showed an early elicitation of capsaicin and anthocyanin in *Capsicum frutescens* and *Daucus carota* cell cultures respectively (Ramachandra Rao et al. 1996). Capsaicin plays an important role in plant defence besides its antimicrobial effect (Marini et al. 2015).

Conclusion

Many available studies both in vitro and in vivo are focused on plant pathogen control by extracts obtained with organic solvents such as methanol, ethanol and acetone (Kulik 1995; Arunkumar et al. 2010; Righini et al. 2018). On the contrary, aqueous extracts have not been thoroughly investigated, even though they may be more sustainable for plant disease control, because they do not contain solvent residues. In conclusion, this study shows that treatments with aqueous extracts from *A. minutissima* and *J. adhaerens* were effective against *P. xanthii* on cucumber seedlings, by working both directly to the pathogen and indirectly through the involvement of plant defence responses helping plant to withstand the pathogen. Since powdery mildew is one of the most important limiting factors for cucurbit production and mostly controlled by the use of chemical fungicides, the studied extracts obtained with water may be important sustainable tools to further investigate on plants. Their application could be considered in environmentally friendly disease management, reducing the adverse environmental effects of pesticides, once their biocontrol activity will be verified in a larger scale experiment.

Authors' contributions Concept and design of the study: HR and RR. Acquisition of data: HR, AS, RR, SC, and SD. Providing and characterization of the cyanobacterium and the algae: AMQ. Interpretation and analysis of the data: HR, RR, FF, and SC. Drafting of the article: HR, RR, and FF. Critical revision and important intellectual content: RR, HR, FF, SC, and SD. Final approval of the article: all authors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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