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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

Hillary Righini¹ · Annalisa Somma¹ · Silvia Cetrullo² · Stefania D'Adamo² · Flavio Flamigni² · Antera Martel Quintana³ · Roberta Roberti¹

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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 (10⁶ 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

✉ Roberta Roberti
roberta.roberti@unibo.it

¹ Department of Agricultural and Food Sciences, University of Bologna, Viale G. Fanin, 40, 40127 Bologna, Italy

² Department of Biochemical and Neuromotor Sciences, University of Bologna, Via Imerio, 48, 40126 Bologna, Italy

³ Banco Español de Algas, Instituto de Oceanografía y Cambio Global, IOCAG, Universidad de Las Palmas de Gran Canaria, Telde, 35214 Las Palmas, Canary Islands, Spain

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):

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

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

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

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

164 For free-radical scavenging activity (antioxidant activity),
 165 lyophilized extracts were analysed by using 1,1-diphenyl-2-
 166 picrylhydrazyl (DPPH) essentially as described by Shanab
 167 et al. (2012). Extracts were added with 0.5 mL of MeOH
 168 (100%) and vortex-mixed for 5 min at room temperature.
 169 After further addition of 0.5 mL of MeOH, samples were
 170 incubated in the dark for 3 h in a thermomixer at 23 °C at
 171 450 rpm. After centrifugation, 150 µL of supernatant was
 172 added to 150 µL of DPPH (1.27 mM in MeOH 90%) and
 173 1.35 mL of MeOH (90%). MeOH (90%) + DPPH was used
 174 as control and MeOH (90%) only as blank. Absorbance at
 175 517 nm was measured after 30 min at room temperature.
 176 The DPPH scavenging effect was calculated as follows:

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

179

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

181 Pathogen inoculum and plant material

182 *Podosphaera xanthii* isolate was obtained from cucumber
 183 plants var. Le Génereux (Vilmorin Jardin, St. Quentin
 184 Fallavier Cedex, France) naturally infected with powdery mil-
 185 dew grown under a plastic tunnel located in the Po Valley,
 186 province of Bologna, Northern Italy. The pathogen was iden-
 187 tified based on morphological features of spore germination
 188 (Zaracovitis 1965) and the presence of fibrosin bodies
 189 (Lebeda 1983). Infected leaves were randomly collected,
 190 and a spore suspension was made in distilled water (5×10^4
 191 spores mL⁻¹). The inoculum was sprayed on 15-day-old cu-
 192 cumber seedlings var. Le Génereux grown until cotyledonary
 193 leaves were fully expanded, by using a handheld spray atom-
 194 izer at the rate of 20 mL per seedling (Mercier et al. 2014;
 195 Kousik et al. 2018). Seedlings were grown in a substrate
 196 consisting of a sterile mixture of peat moss, sand and vermic-
 197 ulite (2:1:1, v:v:v) in pots (10 cm Ø) at 24–26 °C (day), 20–
 198 22 °C (night), 70% relative humidity, and 14-h photoperiod
 199 under greenhouse conditions. The pathogen inoculum was
 200 renewed weekly by replacing the old plants with newly inoc-
 201 ulated plants throughout the experiments. Non-inoculated
 202 plants, for both inoculum renewal and the experiments, were
 203 kept in an isolated sector of the greenhouse under the same
 204 conditions described above.

205 Antifungal activity by detached cotyledon assay

206 Detached cucumber cotyledons were used as a model system
 207 to study the antifungal activity of aqueous extracts (Moret
 208 et al. 2009). Cotyledons were randomly excised at the base
 209 of their laminae from 15-day-old healthy seedlings, sterilized
 210 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 strep-
 tomycin 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 cotyle-
 dons 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 suspen-
 sion 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, $\times 300$ magnification). All spores were
 counted and the number of spores per mm² of inoculated area
 (sporulation density) was calculated for each plate ($n = 3$). The
 experiment was repeated twice.

244 Antifungal activity by seedling assay

245 Fifteen-day-old seedlings were treated with ANA and JAN
 246 extracts at 5.0 and 10.0 mg mL⁻¹ and chitosan at
 247 10.0 mg mL⁻¹ as positive control (2 mL per seedling). Two
 248 millilitres of distilled water were sprayed on negative control
 249 seedlings. Once the cotyledonary leaves were dried, 2 mL of a
 250 spore suspension of *P. xanthii* (1×10^6 spores mL⁻¹) were
 251 sprayed on treated and control seedlings. Three seedlings
 252 ($n = 3$) for each treatment and for the controls were arranged.
 253 The seedlings were distributed in a completely randomized
 254 experimental design on a shelf of the greenhouse and grown
 255 at 24–26 °C (day), 20–22 °C (night), 70% relative humidity,
 256 and 14-h photoperiod. For disease evaluation, the percentage
 257 of cotyledonary area showing symptoms and the pathogen
 258 sporulation were determined 9 days after pathogen inoculation
 259 as above described for detached cotyledon assay. The exper-
 260 iment was repeated twice.

261 Systemic-induced resistance bioassay

262 For this assay 15-day-old seedlings at the cotyledonary stage
 263 were considered. In order to demonstrate the induction of
 264 plant systemic resistance, treatments were applied at site spa-
 265 tially separated from *P. xanthii* inoculation according to the
 266 model system of De Meyer et al. (1998). Seedling treatments
 267 with ANA and JAN (5.0 and 10.0 mg mL⁻¹), chitosan as
 268 positive control (10.0 mg mL⁻¹) and water as negative control
 269 were applied on one of the two cotyledons (1 mL of extract,
 270 chitosan or water per cotyledon), while the other cotyledon
 271 was left untreated by covering it with an aluminium sheet.
 272 Chitosan was used because it elicits the jasmonic acid (JA)
 273 pathway mostly and is able to activate some genes involved in
 274 the salicylic acid (SA) pathway. The sheet was removed 4 h
 275 after treatment. One, 2 and 3 days after treatment (DAT), 1 mL
 276 of *P. xanthii* spore suspension (1 × 10⁶ spores mL⁻¹) was
 277 sprayed on each untreated cotyledon. Four seedlings (one
 278 seedling in a pot; *n* = 4) for each treatment and the controls
 279 were considered. They were randomly distributed on a shelf of
 280 the greenhouse under the same conditions reported above.
 281 Disease was evaluated as percentage of area showing symp-
 282 toms, 9 days after pathogen inoculation. The experiment was
 283 repeated twice.

284 Expression of PR protein genes

285 Seedling treatments were performed as reported above for the
 286 induced resistance assay. The ANA and JAN extracts were
 287 separately applied at the concentration of 5.0 and
 288 10.0 mg mL⁻¹. Chitosan at 10 mg mL⁻¹ was used as a positive
 289 control, since it is a well-known potent elicitor of plant de-
 290 fence responses, and it was prepared as described above. Non-
 291 treated cotyledons were collected from 3 seedlings (one seed-
 292 ling in a pot; *n* = 3) for each treatment and the controls.
 293 Cotyledon tissues were ground to a fine powder in liquid
 294 N₂, and cellular RNA was extracted with TRIZOL
 295 (Invitrogen), according to manufacturer's instructions. The
 296 RNA pellets were quantified by using a spectrophotometer
 297 (Nanovue, GE Healthcare Life Sciences), and the same
 298 amount of total RNA (500 ng) was reverse transcribed by
 299 using random primers and the reagents provided with the
 300 Superscript VILO Master Mix for RT-qPCR (Thermo
 301 Fisher). The cDNA mixture (2 µL) was used in real-time
 302 PCR analysis in a LightCycler Instrument (Roche Molecular
 303 Biochemicals) by means of the QuantiTect SYBR Green PCR
 304 kit (TaKaRa) with the following protocol: initial activation of
 305 HotStart Taq DNA polymerase at 95 °C for 10 s, followed by
 306 amplification (40 cycles: 95 °C for 5 s followed by annealing
 307 step at 59 °C kept for 20 s). The protocol was concluded by
 308 melting curve analysis to check amplicon specificity. Primer
 309 sequences used in the experiment are given in Table 1. The
 310 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 de-
 sign. 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 ex-
 tract and concentration factors. *Anabaena* sp. extract signifi-
 cantly 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	TCCACGAGACTACCTACAACCT GTCATACGGTCAGCGAT
<i>PR1</i>	ATTGGATGCGGTGACTTCGT ATCTGTGTGTAATGGCCGCA
<i>PR2</i> (β-1,3-glucanase)	TGTGGTTGGAGATTCGTGGG TCGCAACGTCCCCTTTAAGA
<i>AePR3</i> (Acidic endochitinase)	TTATTCACTCTCTCCGCCG CCTGAGCTAGTACGTCCCAG
<i>BePR3</i> (Basic endochitinase)	GCGCCATTTCGATGACGAAAA GATCCACATAACCCCGACCC
<i>PR4</i> (Endochitinase)	GCCGACAAGCCTTTGGAATG TTCCGAAGCTCCCCTTTTCAG
<i>PR5</i>	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)		
		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

399 AePR3 more than chitosan (Fig. 3). These effects could be
400 correlated to the disease control obtained in the bioassay at 1
401 and 2DAT where JAN at 5.0 mg mL⁻¹ significantly reduced
402 the percentage of infected area with respect to the control
403 (Fig. 2). The application of JAN at 5.0 mg mL⁻¹ also caused
404 a significant reduction of the disease with respect to chitosan
405 at 2DAT. At 3DAT, 5.0 mg mL⁻¹ of JAN increased the ex-
406 pression of both PR4 and AePR3 (Fig. 3) consistently with the
407 disease control in the systemic-induced resistance bioassay.

408 The ANA extract simultaneously upregulated the expres-
409 sion up to a maximum of two PR genes. In particular, ANA at
410 5.0 mg mL⁻¹ upregulated AePR3 at all DAT and PR1 at
411 3DAT, in line with the control of the disease observed in the
412 systemic-induced resistance bioassay at 3DAT. In addition,
413 ANA at 10.0 mg mL⁻¹ upregulated the expression of two
414 different genes at each DAT. Consistently with the disease
415 control obtained in bioassay with ANA at 5.0 mg mL⁻¹
416 (3DAT) and ANA at 10.0 mg mL⁻¹ (1 and 2DAT), the treat-
417 ment at both concentrations upregulated PR1 expression.

418 On cucumber, we observed a similar behaviour of JAN
419 aqueous extract for powdery mildew control in the detached
420 cotyledon and seedling assays and in the resistance induction
421 bioassay. Moreover, JAN elicited the expression of plant de-
422 fence genes more than ANA.

423 Discussion

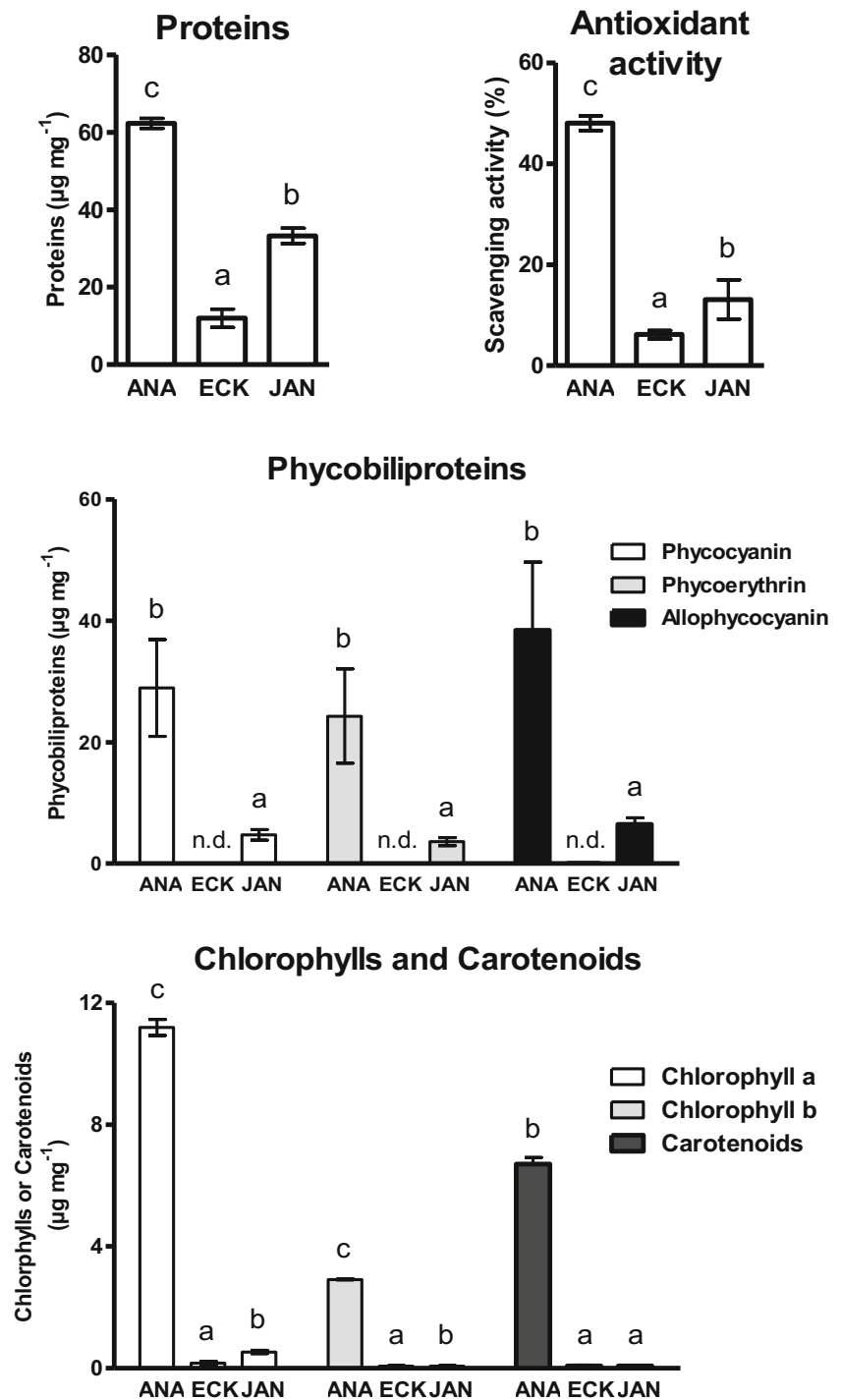
424 This study was carried out to investigate the effect of extracts
425 from the red alga *J. adhaerens*, the brown alga *E. maxima* and
426 the cyanobacterium *A. minutissima* BEA 0300B against
427 *P. xanthii* on cucumber. The study was divided into two ex-
428 perimental phases. In the first phase, the antifungal activity of

the extracts was evaluated and in the second one their eliciting
activity of seedling defence responses.

Antifungal activity

431 The extracts from *A. minutissima* and *J. adhaerens* showed
432 antifungal activity by reducing the percentage of symptomatic
433 area and sporulation density in both in vitro and in vivo assay.
434 Considering that *P. xanthii* is an obligate parasite, we used the
435 detached cotyledonary leave assay to verify the efficacy of the
436 extracts against the pathogen in vitro. Under these experimen-
437 tal conditions, the two highest doses (5.0 and 10.0 mg mL⁻¹)
438 of *A. minutissima* and *J. adhaerens* were similarly effective in
439 reducing both infected area and sporulation density. Under
440 greenhouse conditions, the high extract dose was more effec-
441 tive than the low dose, because the disease severity was very
442 high, more than 80% of infected area. However, detached
443 cotyledon assay could be a suitable model system for a pre-
444 liminary study to verify a possible effect of exogenous sub-
445 stances application as published by other authors (Ullanat and
446 Jayabaskaran 2002; Moret et al. 2009; Gafni et al. 2015).
447 Consistently, in a preliminary screening on zucchini detached
448 cotyledons, 5.0 mg mL⁻¹ of aqueous extract from
449 *A. minutissima* 0300B strain and the red alga *Corallina* sp.,
450 belonging to the same family of *J. adhaerens* (Corallinaceae),
451 reduced symptoms of *P. xanthii* (Roberti et al. 2016). The
452 same aqueous extract from *A. minutissima* and the extract
453 from *J. adhaerens* (formerly *Jania* sp.) at 5.0 and
454 10.0 mg mL⁻¹ were also effective in reducing the colony
455 growth of the not obligate plant pathogen *Botrytis cinerea*
456 (Righini et al. 2019). Other in vitro studies on cyanobacteria
457 reported the antifungal activity of different *Anabaena* species
458 against non-obligate fungal plant pathogens such as
459

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 ($n = 3$) \pm SD. Different letters within each determination indicate significant differences according to LSD test ($p < 0.05$); n.d., not detectable



460 *Alternaria solani*, *Fusarium moniliforme*, *Pythium*
 461 *aphanidermatum*, *P. debaryanum*, and *Rhizoctonia bataticola*
 462 (Prasanna et al. 2008; Radhakrishnan et al. 2009). Further
 463 studies have examined the efficacy of cyanobacteria against
 464 plant diseases. On tomato, substrate treatment with *Anabaena*
 465 *variabilis* and *A. oscillarioides* extracts, obtained with organic
 466 solvents, were effective against *Pythium debaryanum*,
 467 *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 polysac-
 charides extracted from the same extract of *A. minutissima*
 have shown antifungal activity against *B. cinerea* colony

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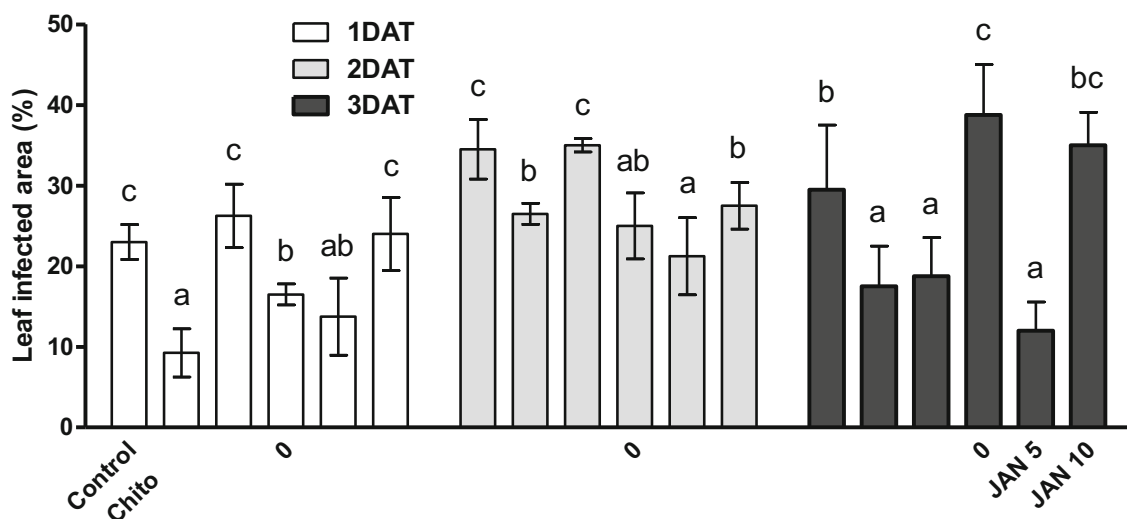


Fig. 2 Systemic-induced resistance bioassay: effect of treatment with extracts from *Anabaena minutissima* BEA 0300B (ANA) and *Jania adhaerens* (JAN) on the percentage of cotyledonary leaf area infected by *Podosphaera xanthii*. Inoculation with *P. xanthii* was carried out at 1, 2 or 3 days after the treatment (1, 2, 3DAT) with water (control),

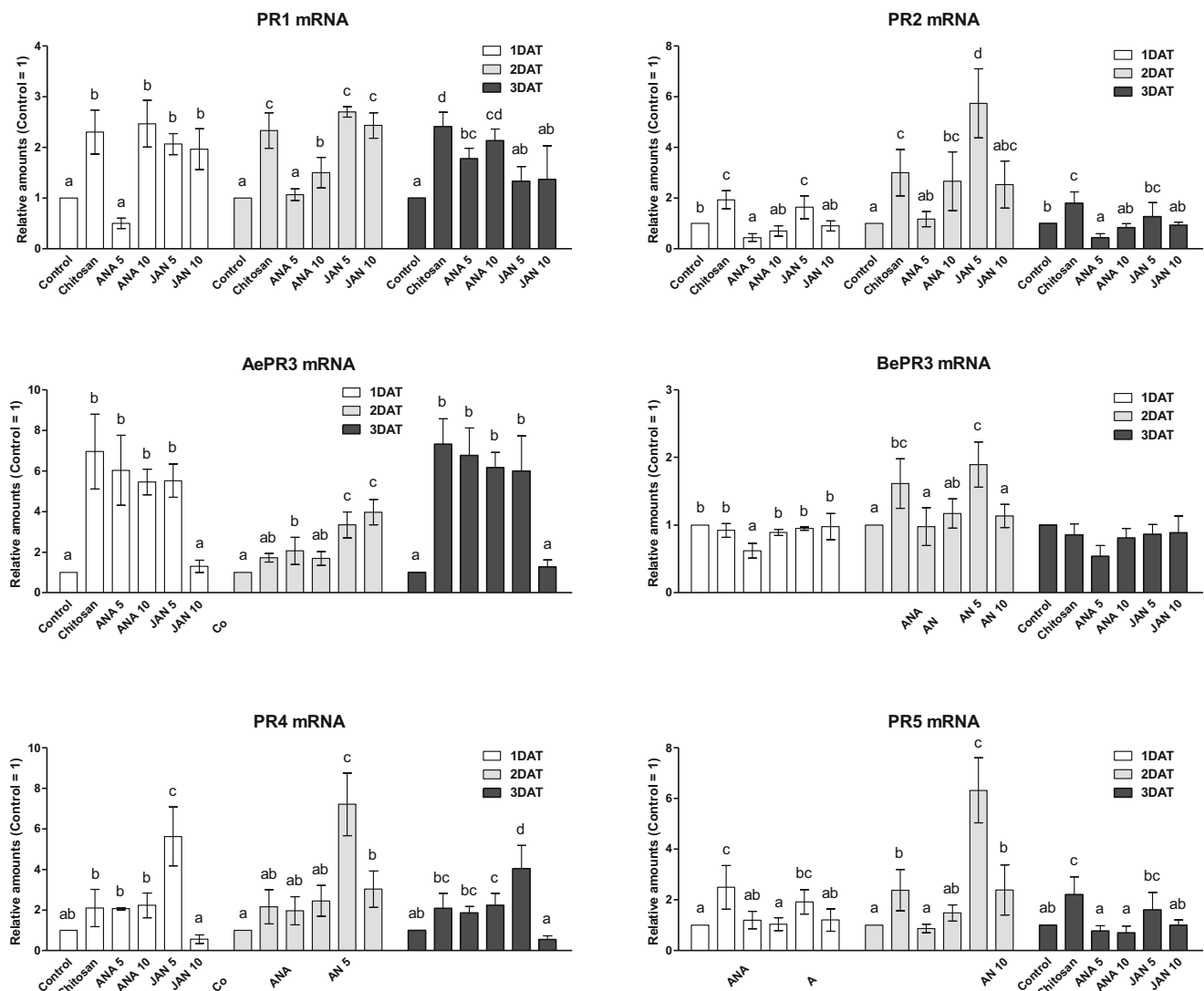
chitosan (10.0 mg mL^{-1}), and ANA or JAN (ANA 5, JAN 5 = 5.0 mg mL^{-1} ; ANA 10, JAN 10 = 10.0 mg mL^{-1}). Disease was evaluated 9 days after pathogen inoculation. Bars represent means ($n = 4$) \pm SD. Different letters within each DAT indicate significant differences according to LSD test ($p < 0.05$)

476 growth and colony-forming units (Righini et al. 2019).
 477 Several hydrolytic enzymes were also identified in
 478 cyanobacteria extracts, and the presence of chitosanases and
 479 endoglucanases were correlated to the antifungal activity
 480 against several plant pathogens (Gupta et al. 2010, 2011;
 481 Prasanna et al. 2010). Chitosanases are known to hydrolyse
 482 the β -1,4-glycosidic bonds that link *N*-acetyl glucosamine res-
 483 idues of chitin which is the main component of fungal cell
 484 wall. In addition to enzymes, cyanobacteria are also able to
 485 produce phenolic compounds that inhibited *Candida albicans*
 486 colony growth (de Cano et al. 1990). Marrez and Sultan
 487 (2016) demonstrated that 2,6-di-*t*-butyl-4-methyl phenol,
 488 hexadecanoic acid and methyl ester were the main responsible
 489 of the antifungal activity of the cyanobacterium *Microcystis*
 490 *aeruginosa* against *Aspergillus flavus*, *A. niger*, *Fusarium*
 491 *verticillioides* and *F. proliferatum*. Moreover, the terpenoid
 492 noscomin extracted from *Nostoc commune* showed antibacte-
 493 rial activity against human bacterial pathogens (Jaki et al.
 494 2000).

495 Although a lot of scientific literature on the effectiveness of
 496 brown and green algae extracts against plant fungal diseases is
 497 published (Hamed et al. 2018), only a few findings are avail-
 498 able on red algae efficacy. A study reported that *Porphyra*
 499 *umbilicalis* crude extract used on wounded fruits controlled
 500 *B. cinerea* grey mould on strawberries, *Monilia laxa* brown
 501 rot on peaches and *Penicillium digitatum* green mould on
 502 lemons when the extract was applied few hours after conidia
 503 inoculation (De Corato et al. 2017). In addition, the mycelial
 504 growth of the plant pathogen *Macrophomina phaseolina* was
 505 inhibited by an extract from the red alga *Gracilaria edulis*
 506 (Ambika and Sujatha 2015). Red algae synthesize several bio-
 507 active compounds that have shown antifungal activity. Water-

soluble polysaccharides of *J. adhaerens* played an important
 role in reducing *B. cinerea* spore germination, thereby reduc-
 ing 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

540 *flagellifera*, which could be positively correlated with the
541 change in concentration and ratios of chlorophyll *a* and acces-
542 sory pigments such as carotenoids and phycobiliproteins.
543 Other examples of correlation between antioxidant compo-
544 und compounds with antimicrobial activity concern bacterial human
545 pathogens (Shanmugam et al. 2017; Karpiński and
546 Adamczak 2019).

547 Systemic resistance induction

548 Plants enhance their defensive capacity in response to appropri-
549 ate stimuli through two forms of induced resistance, systemic
550 acquired resistance (SAR) and induced systemic resistance
551 (ISR). SAR involves accumulations of PR proteins and salicylic

552 acid (SA), whereas ISR depends on jasmonic acid (JA) and
553 ethylene (ET) pathways (Derksen et al. 2013; Ghannam et al.
554 2013; Pettongkhao et al. 2019). In our study, we included the
555 investigation of the expression levels of PR1, PR2 and PR5, as
556 members of SA-dependent PR genes, and PR3 and PR4 as
557 members of JA-dependent PR genes, thus giving an overview
558 of both resistance forms. Although more genes may be included
559 in further studies, our work provides a first indication of the
560 mechanisms modulated by the aqueous extracts from
561 *Anabaena* and *Jania* species. Concerning the potential of aque-
562 ous extracts of *A. minutissima* and *J. adhaerens* to induce sys-
563 temic resistance in cucumber seedlings under greenhouse con-
564 ditions, a bioassay for powdery mildew control and a specific
565 assay on gene expression of PR proteins in cotyledons were

566 conducted. In the bioassay, due to the spatial separation of
 567 treatment and pathogen inoculation, the disease control was
 568 attributed to plant-induced systemic resistance in which differ-
 569 ent expression of various PR protein genes is involved. For the
 570 bioassay, we adopted a modified model system already de-
 571 scribed by De Meyer et al. (1998). These authors concluded
 572 that the application of the fungal biocontrol agent *Trichoderma*
 573 *harzianum* T39 in the soil before *B. cinerea* inoculation on
 574 leaves of tomato, lettuce, pepper, bean and tobacco plants in-
 575 duced systemic resistance as verified by the disease suppres-
 576 sion. *Anabaena minutissima* extract differentially induced the
 577 expression of PR genes, which may have concurred to patho-
 578 gen control. Plant responses to *A. minutissima* treatment are JA
 579 and SA dependent. Jasmonic acid responsive genes (PR3) were
 580 mainly induced by *A. minutissima* extract at 5.0 mg mL⁻¹ while
 581 SA responsive genes (PR1) at 10.0 mg mL⁻¹. Singh (2014),
 582 reviewing several articles on the role of elicitors molecules of
 583 cyanobacteria in the plant tolerance against biotic or abiotic
 584 stress, reported that their metabolites can promote the gene
 585 expression of the host and thereby help plants to counteract a
 586 pathogen invasion. The induction of seedling resistance by
 587 *A. minutissima* is in accordance with Prasanna et al. (2013),
 588 who demonstrated the increase of defence enzymes activities
 589 such as phenylalanine ammonia-lyase, polyphenol oxidase,
 590 chitinase and β -1,3 glucanase in tomato plants grown in
 591 substrate treated with filtrates of *A. variabilis* and *A. laxa* fresh
 592 cultures. Also in our previous study, *A. minutissima* (formerly
 593 *Anabaena* sp.) at 5.0 mg mL⁻¹ increased the total chitinase
 594 activity, particularly three chitinase acid isoforms at 1, 2 and
 595 3 days after treatment (Roberti et al. 2015). Contrary to what
 596 was expected, *A. minutissima* at 10.0 mg mL⁻¹ was not effec-
 597 tive against the disease 3 days after treatment. The accumula-
 598 tion of several compounds produced by the cyanobacterium
 599 may be responsible of a seedling physiological stress. Indeed,
 600 *A. minutissima* showed high content of proteins,
 601 phycobiliproteins, chlorophylls and carotenoids and the highest
 602 antioxidant activity that may represent a challenge for the plant.

603 The extract from the red seaweed *J. adhaerens* appears to
 604 show mostly a biostimulant behaviour, since it was effective in
 605 reducing powdery mildew disease and eliciting PR defence
 606 genes more at 5.0 mg mL⁻¹ than at 10.0 mg mL⁻¹. It is known
 607 that seaweed extracts have biostimulant effect on plants by
 608 involving the synthesis of several plant hormones (Khan et al.
 609 2009). Still, they are bioactive at very low concentrations as
 610 reviewed by Khan et al. (2009). The highest concentration
 611 (10.0 mg mL⁻¹) of *J. adhaerens* could cause a stress to seedling
 612 that may affect the transcriptional activities of different compo-
 613 nents in different signalling pathways as in case of JA metabolic
 614 pathway (Yang et al. 2019). When 5.0 mg mL⁻¹ of
 615 *J. adhaerens* were applied, almost all genes were coordinately
 616 expressed along with disease reduction. Among these genes,
 617 AePR3 and PR4 were always upregulated, while P1, PR2 and
 618 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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668 **Compliance with ethical standards**669 **Conflict of interest** The authors declare that they have no conflict of
670 interest.671 **References**

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