Seed Priming With Anabaena Minutissima Extract Ameliorates the Stress Response of Tomato Plantlets to Soilborne Pathogens

Hillary Righini  
University of Bologna

Stefania Galletti  
stefania.galletti@crea.gov.it

Council for agricultural research and economics

Stefano Cianchetta  
Council for agricultural research and economics

Antera Martel Quintana  
University of Las Palmas de Gran Canaria

Omella Francioso  
University of Bologna

Roberta Roberti  
University of Bologna

Research Article

Keywords: Cyanobacteria, microalgae, plant disease control, Pythium ultimum, Rhizoctonia solani, induced resistance

Posted Date: April 5th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4189107/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Additional Declarations: No competing interests reported.
Abstract

This study assessed the effectiveness of the treatment of tomato seeds with an aqueous extract of the dried biomass of the microalgae *Anabaena minutissima* in protecting the plants from diseases caused by soilborne pathogens like *Rhizoctonia solani* and *Pythium ultimum*. The extract, obtained by an autoclave-assisted method at 100°C, was rich in proteins and carbohydrates (56.6 and 26.9% of total solids). Preliminary tests were performed revealing: i) a moderate direct activity toward pathogen *in vitro* growth, with ± 9% stimulation/inhibition, depending on the pathogen; ii) a stimulatory/inhibitory activity toward seedling growth, depending on the dose; iii) no effect on the mycelial growth by root exudates of treated plantlets. Then, a greenhouse experiment was set up to test the response of tomato plants in substrates artificially inoculated with the single pathogens, after seed treatment with the extract at different doses (0, 2.5, 5, and 10 mg mL\(^{-1}\)). The treatment generally increased the percentage of standing plants and restored plant development up to the level of healthy controls. Moreover, the disease incidence and severity progressively reduced at increasing doses. Finally, the seed treatment significantly increased some markers of induced systemic resistance like endochitinase and glucanase activity, in hypocotyls of 14-day-old seedlings, compared to non-treated controls. Besides, the treatment increased epicotyls’ carotenoid and chlorophyll a and b content. Overall, these results demonstrate that seed priming with *A. minutissima* aqueous extract is a promising eco-friendly tool to ameliorate tomato plant responses towards soilborne pathogens, stimulating plant growth and activating induced resistance mechanisms.

INTRODUCTION

The *Anabaena* species are gram-negative photosynthetic cyanobacteria belonging to the *Nostocales* order (Anagnostidis and Komarek 1990; Curtis 2019). These cyanobacteria are often found in freshwater ecosystems such as lakes, ponds, and slow-moving rivers. However, they can also thrive in brackish water and a variety of terrestrial habitats (Righini et al. 2022a). These microorganisms possess ecological significance because they can fix atmospheric nitrogen, converting it into ammonia through specialized cells known as heterocysts. This conversion enables plants and other organisms in their vicinity to readily utilize nitrogen (Curtis 2019). Cyanobacteria are also effective producers of bioactive substances including enzymes, lipids, pigments, proteins, and compounds of significant value in medical, pharmaceutical, and biotechnological fields. Their remarkable productivity in this regard has earned them the nickname of ‘miniature factories’ (Galhano et al. 2011). According to the literature, within cyanobacteria, the order *Nostocales* ranks second in secondary metabolite production after the order *Oscillatoriales* (Tripathi et al. 2021). Specific bioactive compounds from *Anabaena* spp., namely anatoxins, hassallidins, chlorophylls, flavonoids, and phycobiliproteins, have demonstrated their effectiveness in antibacterial, antifungal, antineoplastic, and antioxidant capacities (Hamouda et al. 2017; Mandhata et al. 2023).

The bioactive compounds derived from *Anabaena* species are also being explored in agriculture due to their potential to provide protection from phytopathogens, which are responsible for severe economic
losses. The use of sustainable approaches or techniques like this represents a tool to reduce the risk and impact of chemical pesticides on human health and the environment.

On tomato fruits, *A. minutissima* phycobiliproteins exerted antifungal activity against *Botrytis cinerea* causing grey mold disease (Righini et al. 2021a). Moreover, other compounds from *A. minutissima*, such as polysaccharides, protected strawberry fruits in pre-harvest phase from grey mold disease in terms of fruit-infected area and pathogen sporulation reduction (Righini et al. 2019).

Cyanobacteria, their extracts, or derived compounds have been also applied as a seed treatment to promote both plant growth and health, with a technique known as seed priming (Paparella et al. 2015). (Sharma et al. 2020) emphasized the potential of cyanobacteria for improving nutrient mobilization at the seed stage. They also noted their ability to influence the abundance and activities of soil microbial communities, leading to improved growth and vigor of maize plants.

In a recent study (Sneha et al. 2024) applying *Nostoc* sp. and *Neowestiellopsis* sp. on wheat seed, obtained enhanced growth rate, relative water content, chlorophyll, carotenoid, and photosynthetic activity under drought stress. Additionally, plants subjected to priming exhibited reduced accumulation of free radicals and lipid peroxidase products, alongside increased activity of antioxidant enzymes. Under *Pythium ultimum* stress, priming of cucumber seeds with sonicated cyanobacteria extracts from different species of the Hungarian Mosonmagyaróvár Algal Culture Collection (MACC) and Spanish Bank of Algae (SBA) prevented damping-off, and specifically, the SAB-M465 strain was identified as an effective biocontrol agent (Toribio et al. 2021).

On tomato, *A. minutissima* phycobiliproteins applied as seed treatment have shown a priming effect on seedling growth and protection against root rot caused by the fungal pathogen *Rhizoctonia solani* (Righini et al. 2023). Besides, in the same pathosystem an aqueous extract at 50°C from this cyanobacterium, increased seed germination and seedling growth and reduced root rot severity as a result of increased plant defense responses (Righini et al. 2021b).

Among extraction methods, the autoclave-assisted one has recently been applied to improve the extraction of protein from different algal biomasses (O’ Connor et al. 2020). This approach is technically attractive due to its ease of use and fast processing times, as well as the absence of degradation products caused by prolonged chemical hydrolysis. Therefore, autoclave-assisted aqueous extractions seem to be advantageous for the recovery of biomacromolecules. Extracts of cyanobacteria have an important impact on biocontrol and have proven to enhance plant primary metabolic pathways due to their high nitrogen content and other bioactive molecules (Righini et al. 2023). However, the efficacy of extracts obtained by autoclave-assisted methods has not been well studied in seed priming. Moreover, seed priming with environmentally friendly substances aligns with new European Regulations on the sustainable use of pesticides.

The main objective of the study was to evaluate the effectiveness of the aqueous extract obtained through an autoclave-assisted method from the cyanobacterium *A. minutissima* applied as a seed
treatment to prime tomato (*Lycopersicon esculentum* L.) plants against the pathogens *R. solani* and *P. ultimum*. Initially, the direct activity of the extract on pathogens’ growth was evaluated *in vitro*, and then its bio-stimulating activity on seedlings was assessed in microcosms. The efficacy of seed priming with the extract was then evaluated in greenhouse-grown plants in the presence of both pathogens. Finally, the activation of plant defense responses involved in counteracting stresses from pathogens and related plant physiological changes were investigated.

**MATERIALS AND METHODS**

**Cyanobacterium strain and preparation of the extract**

The freeze-dried biomass of *A. minutissima* (BEA 0300B), originally isolated from a biofilm sample collected on the Fuerteventura coast, Canary Islands, was provided by the Banco Español de Algas, University of Las Palmas, Spain. The strain was cultivated in photobioreactors and the biomass was dehydrated as previously described (Righini et al. 2021a). This strain does not produce cyanotoxins, as previously assessed (Roberti et al. 2015).

For the preparation of the *A. minutissima* extract (AME), the biomass was suspended in sterile distilled water at 20 mg mL$^{-1}$ concentration, through an autoclave-assisted method (100°C, 1 bar, 20 min), at the University of Bologna. The obtained extract was centrifuged twice for 20 min at 5000 rpm with a Beckman Coulter Allegra (21R Centrifuge, Inc., Krefeld, Germany). The supernatant (extract) was collected under a sterile flow cabinet and frozen until use.

**Chemical and spectroscopic analyses of the aqueous extract of Anabaena minutissima**

Total C and nitrogen contents of AME were determined by a CHN elemental analyzer (CHN Elemental Analyser 1110, Thermo Scientific GmbH, Dreieich, DE) on 10 mL samples after freeze-drying, in duplicates. The analyses were performed in triplicate. The total solid content of AME was measured gravimetrically. Moisture and ash were analyzed with the TG-DTA92B thermogravimetric instrument (SETARAM, France). A quantity of 6 mg of lyophilized extract was placed in an alumina crucible and heated in a temperature range of 25°C to 750°C at a rate of 10°C min$^{-1}$. The oven atmosphere consisted of ultra-zero grade air at a flow rate of 130 mL min$^{-1}$. The analysis was performed in triplicate.

The total carbohydrate content was determined by the anthrone method which is suitable for carbohydrate determination in the presence of proteins (Fagen et al. 1954). The assay was performed at a microscale (Laurentin & Edwards, 2003) and modified as follows: anthrone reagent was prepared daily by dissolving anthrone (ACS reagent 97%, N° 319899, Sigma-Aldrich) 2 mg mL$^{-1}$ in ice-chilled 98% H$_2$SO$_4$ (84727, Sigma-Aldrich). The assay was performed in a rigid 96-well Polypropylene PCR Microplate (Corning® Thermowell® 96-well Product Number 6551, Corning, NY, USA). Samples and standards (50 µL/well) were cooled by maintaining the plate in a 0°C water bath (containing approx. 30% ice by volume), ice cold anthrone reagent (200 µL) was then carefully added and mixed. As standards several
dilutions (0; 0.1; 0.2; 0.3; 0.4; 1.0 mg/mL) of pure glucose were used. The plate was covered with adhesive aluminum foil for microplates (VWR 60941-112, VWR International, Radnor, Pennsylvania, USA), and then it was incubated for 10 min at 95°C in a PCR thermal cycler (T3 DNA thermal-cycler, Biometra GmbH, Gottingen, Germany). Finally, 200 µL aliquots of each sample were transferred to a 96-well flat-bottom polystyrene microplate (Costar 3595, Corning, NY, USA) and the absorbance at 620 nm was measured by a spectrophotometer (Infinite 200 PRO series, Tecan, Männedorf, Switzerland). Please note that the adhesive aluminum cover was also heated at 95°C in the thermal cycler to avoid acid vapor condensation. Visible alterations of the aluminum cover were not observed probably thanks to the protection offered by the adhesive layer on the acid-exposed face. The analysis was performed in triplicates on multiple samples (n = 4).

Total soluble reducing sugars were quantified via the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) adapted for 96-well microplates (Cianchetta et al. 2010). Pure glucose dilutions were included as standards. The assay was performed in citrate buffer 50 mM, pH 4.8, for 5 min at 95°C. The microplates were analyzed using a spectrophotometer (Infinite 200 PRO series, Tecan, Männedorf, Switzerland) at a 660 nm wavelength. The analysis was performed in triplicates on multiple samples (n = 4).

Glucose in the aqueous extract was quantified by an enzymatic colorimetric assay (D-Glucose HK Assay Kit, Megazyme, Astori Tecnica, Poncarale, Italy) according to the producer’s instructions. The analysis was performed in triplicates on multiple samples (n = 4).

Phycobiliproteins were determined according to Righini et al. (Righini et al. 2023) by resuspending the freeze-dried aqueous extract in phosphate buffer (0.2 M, pH 7) and then stirring the suspension at room temperature in the dark. After 4 h, the suspension was centrifuged for 20 min at 13°C, 5000 rpm, and the phycocyanin, allophycocyanin, and phycoerythrin in the supernatant were quantified at 652, 615, and 562 nm spectrophotometrically (Bennett and Bogorad 1973; Bryant 1982) by using the following equations:

\[
\text{Phycocyanin (PC)} \left( mg \text{ g}^{-1} \right) = \frac{A_{615} - (0.474 \times A_{652})}{5.34} \\
\text{Allophycocyanin (APC)} \left( mg \text{ g}^{-1} \right) = \frac{A_{652} - (0.208 \times A_{615})}{5.09} \\
\text{Phycoerythrin } \left( mg \text{ g}^{-1} \right) = \frac{A_{562} - (2.41 \times \text{PC}) - (0.849 \times \text{APC})}{9.62}
\]

Values expressed as %TS of total carbohydrates, glucose, reducing sugars, phycoerythrin, phycocyanin, and allophycocyanin were calculated by dividing the measured concentration (in mg L\(^{-1}\)) by the concentration of total solids in the aqueous extract (in mg L\(^{-1}\)). Errors were calculated by adding errors in quadrature assuming errors in the measurements are governed by the normal distribution and that the measured quantities are independent from each other.
Lipids were extracted from 70–75 mg of freeze-dried extract in duplicates with hexane. Briefly, 40 mL of solvent g\(^{-1}\) of dry material were utilized, the mixture was vortexed for 3 min with glass beads then the liquid fraction was recovered after centrifugation, for a total of three extractions on the same pellet. The recovered liquid fractions were pooled and warmed at 75°C in a water bath to eliminate most of the solvent and finally dried to a constant weight under a vacuum (centrifugal evaporator Jouan RC10-10, Thermo Electron Industries SAS, Château-Gontier, France). Finally, the lipid fraction was determined gravimetrically.

FT-IR spectra of lyophilized AME were recorded by using a Tensor FT-IR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with an accessory for analysis in micro-ATR (Specac Quest ATR, Specac Ltd., Orpington, Kent, UK). The spectra were acquired (64 scans per sample or background) in the range of 4000–400 cm\(^{-1}\) at a resolution of 4 cm\(^{-1}\) and processed using the Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH, USA). The analysis was performed three times.

**Tomato seeds and treatment**

Tomato (*L. esculentum* L.) seeds, 'Marmande' cultivar, were purchased from L'ortolano s.r.l. (Cesena, Italy). Before treatment, the seeds were sterilized in 70% ethanol for 2 min, then 2.5% sodium hypochlorite for 2 min, rinsed thrice in sterilized distilled water, and blotted on sterile filter paper.

For the seed treatment, AME was opportunistly diluted to obtain 2.5, 5, and 10 mg mL\(^{-1}\) doses, into which the sterilized seeds were immersed at room temperature in the dark for 12 h. Sterile distilled water was used as a control. Then the seeds were collected and rinsed in sterile distilled water to eliminate any extract residual and left to dry on filter paper under a laminar air flow hood for 10 min before use.

**Pathogens and substrate inoculation**

The strain of *P. ultimum* 22 (PU) was made available by CREA-Research Centre for Agriculture and Environment, Bologna, Italy, while the strain of *R. solani* DAFS3001 (RS) was made available by the Department of Agricultural and Food Sciences, Alma Mater Studiorum, University of Bologna, Italy.

Both pathogens were maintained as mycelium on potato dextrose agar 3.9% (PDA, Difco) slant at 4°C and transferred on PDA plates at room temperature for short-term use.

For the greenhouse assay, a growth substrate (7/3 weight/weight, sterile peat/sand mix) was inoculated with the single pathogens (PU or RS) as follows: mycelium disks from actively growing colonies were transferred on PDA plates and incubated at room temperature for 5 days, then the entire plate content (colony + PDA) was homogenized in sterile distilled water with a kitchen blender and mixed with the substrate (2% weight/weight, pathogen/substrate). After inoculation, the substrate was covered with a black plastic film and incubated at 24–26°C for 2 days. Not inoculated substrate (control) was prepared in the same way.

**Direct activity of the extract toward the pathogens**
The poisoned substrate technique was used to test the effect of the extract on the *in vitro* growth of the pathogens. PDA was amended with dilutions of AME to obtain the following final concentrations: 0, 1, 2.5, and 5 mg mL\(^{-1}\), then 10 mL poisoned PDA was poured into Petri plates (9 cm diam.) which were inoculated with one mycelial disk from PU or RS actively growing colonies, then incubated at 22°C for 42 h, in triplicates. The averaged radial growth of the colonies was calculated based on three measurements per plate.

**Seed treatment effect on the plantlet growth in microcosm and chemical changes**

Tomato seeds were treated with dilutions of AME at 0, 2.5, 5, and 10 mg mL\(^{-1}\) doses as described above and seeded in 400 mL glass jars containing 200 g perlite and 100 mL of diluted Hoagland's solution, prepared according to (Maurer et al. 2021) (3 replicates × 2 jars × 6–10 seeds each). After 2 weeks of incubation in a growth chamber (Percival® AR-36LC8, Percival Scientific, Inc. Perry, IA, USA), at 24°C and 16h/8h light/darkness photoperiod, the plantlets were collected, and the fresh weight of the entire plantlets and rootlets was recorded (3 replicates), after exudate collection (see below).

The lyophilized root samples were ground with a ball mill before FT-IR analysis. The section on chemical and Spectroscopic Analyses describes the procedure. The spectra of the roots from seeds non-treated and treated with AME at 5 mL\(^{-1}\) were shown. The region between 1800 and 1500 cm\(^{-1}\) corresponding to the main functional groups was studied in more detail using curve-fitting analysis by Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH, USA). The best-fit parameters were determined using the Gaussian model. This was achieved by minimizing the reduced Chi-square (\(\chi^2\)), resulting in coefficients of determination (R\(^2\)) ranging from 0.999 to 0.988 and standard error (SE) ranging from 0.0005 to 0.001. The identified peaks were integrated to calculate the peak area.

**Effect of root exudates from treated plantlets on pathogen growth**

The root exudates were collected from the plantlets of the experiment described above. About 20 two-week-old plantlets per each tested AME dose (0, 2.5, 5, and 10 mg mL\(^{-1}\)) were collected and pooled. The root apparatus was washed in distilled water then immersed in 12 mL sterilized distilled water into 20 mL glass vials and incubated in the growth chamber at the above-described conditions for 20 h. After plantlet removal, the exudate solution was sterile filtered, normalized per seedling or root weight, obtaining 10 mL exudates per dose, then stored at -20°C until analysis. To assess the effect of the exudates on the mycelial growth of the pathogens, 96-well flat-bottom polystyrene microplates (Costar®3595, Corning, NY, USA) were prepared, each well containing 200 µL normalized root exudate, 15 µL potato dextrose broth, and 15 µL of diluted inoculum obtained from 24 h (PU) or 7 d (RS) old, colonized PDA plates, after milling and syringe extrusion, with 10 replicates. Microplates were incubated for 16 h at 24°C, then the mycelial growth was followed through spectrophotometric analysis (200 PRO series, Tecan, Kawasaki, Japan) at a
660 nm wavelength from 16 until 80 h after pathogen inoculation. Blanks without inoculum were included.

**Greenhouse assay**

The experiment was conducted at the fully air-conditioned and automated glass greenhouse complex of DISTAL, University of Bologna. Plastic pots (13.5 × 11.5 × 7.5 cm) containing the artificially inoculated substrate, as already described above, were seeded with a total of 50 seeds per pot, with 3 pots per treatment (0, 2.5, 5, and 10 mg mL\(^{-1}\) AME doses). Pots with non-inoculated substrate served as healthy controls. Pots were then disposed over a bench according to a completely randomized design, at the following greenhouse conditions: 24–26°C (day), 20–22°C (night), with a 12 h/12 h photoperiod, and 70% relative humidity.

The percentage of standing plants was recorded 21 days after the sowing. Then, all plants were gently removed from the substrate, their height was measured, and the root apparatus was washed under tap water to record disease symptoms. Disease incidence was calculated as the percentage of plants showing disease symptoms over the total of examined plants.

For PU, the disease severity was evaluated on the whole plant using a visual disease assessment based on a 0–4 scale (Jabiri et al. 2021) with modifications, as follows: 0 = no visible disease symptoms; 1 = ≤ 20% moderate level of general decay; 2 = extensive general decay, and with an obvious reduction in overall plant development (but < 50% of root system missing); 3 = very severe levels of general decay associated with an extensive reduction of the root apparatus (> 50% root system missing); 4 = dead plant.

The disease severity caused by RS was evaluated by scoring the root apparatus based on a 0–5 scale (Righini et al. 2022b), where: 0 = absence of necrosis (0% of symptoms); 1 = very slight necrosis (up to 5% of root with symptoms); 2 = slight necrosis (6–20% of root with symptoms); 3 = moderate necrosis (20–50% of root with symptoms); 4 = severe necrosis (51–70% of root with symptoms); 5 = severe crown and root necrosis (> 70% of root with symptoms)

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

**Biochemical assays**

To verify the possible involvement of induced resistance mechanisms in the plantlets due to seed treatment with AME, some enzymatic activities considered markers were determined on 14-day-old seedlings. For this purpose, seeds were treated with AME at the dose of 5 mg mL\(^{-1}\) in sterile water and were seeded on sterile filter paper in sterile polystyrene Petri plates (diam. 14 cm), 60 seeds per plate, with 3 replicates, including non-treated control. Plates were incubated in a growth chamber (Percival®AR-36LC8, Percival scientific, Inc. Perry, IA, USA) at 24°C, 16 h light, and the experiment was repeated thrice (n = 3). The dose of 5 mg mL\(^{-1}\) was chosen because no significant differences among treatments were observed in the greenhouse experiment.
To determine enzymatic activities, total proteins were extracted from 0.5 g fresh roots randomly collected from each plate of each experiment. The samples were immediately snap-frozen in liquid nitrogen and then ground to a fine powder using a pre-chilled mortar and pestle, and total proteins were extracted by 20 mM sodium acetate buffer pH 5.2 (1 mL g\(^{-1}\) of fresh weight) containing 1% polyvinylpolypyrrolidone (Sigma–Aldrich Co.) (Roberti et al. 2015). After incubation at 4°C for 90 min under continuous gentle stirring, the samples were centrifuged twice at 12,000 rpm for 20 min at 4°C and the supernatant was harvested and filtered using a GV Millex® Syringe Filter Unit (Millipore Corporation, USA) to remove solid particles. Protein concentrations were determined by the protein-dye binding method of (Bradford 1976), using bovine serum albumin (BioRad Laboratories, Inc.) as the standard.

The activity of three chitinases, β-N-acetylhexosaminidase (EC 3.2.1.52), chitin 1,4-β-chitobiosidase, and endochitinase (EC 3.2.1.14), was assayed in triplicate for each experiment (n = 3) following a modified procedure by (Tronsmo and Harman 1993) in 96-well microplates. Chitinase assays were based on colorimetric determination of p-nitrophenyl cleaved from the chitin-analogous substrates, p-nitrophenyl-β-D-N, N', N''-triacetylchitotriose, p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-β-D-N, N'-diacetylchitobiose, respectively (all from Sigma–Aldrich Co). Fifty microliters of each substrate in 50 mM acetate buffer, pH 5.0 (2 mg mL\(^{-1}\)) were added to 90 µL of the protein extract (15 µg total protein) from each sample. After a 15-minute incubation in a water bath at 50°C, 50 µl of 0.2 M Na\(_2\)Ca\(_3\) were added to stop the reaction, and the absorbance was measured at 405 nm. Each chitinase activity was calculated using the p-nitrophenyl absorption coefficient of 18.5 mM\(^{-1}\) cm\(^{-1}\).

The activity of β-1,3-glucanase (EC 3.2.1.39) was evaluated in triplicate for each experiment by measuring the production of reducing sugars, using laminarin (Sigma, USA) as the substrate and following a modified protocol of (Abeles and Forrence 1970). The reaction mixture consisted of 60 µL of the protein extract (15 µg proteins) and 60 µL laminarin (2%). After incubation for 2 h at 37°C, 70 µL of 3.5 dinitrosalicylic acid were added and the reaction mixture was heated at 100°C for 20 min. After cooling in an ice bath, the absorbance was measured at 492 nm. Glucose was used as a standard. Enzyme activities were expressed as U (µmol min\(^{-1}\)) g\(^{-1}\) protein.

In addition, the epicotyl and hypocotyl content of total phenols, the epicotyl content of chlorophyll a and b, and carotenoids were determined as follows: freeze-dried seedlings (for each determination, 5 seedlings per plate, in triplicates, for n = 3 experiment) were first ground with 0.1 mL of absolute methanol per mg of root and incubated for 30 min on ice. The Folin method (López Arnaldos et al. 2001) was used for phenol measurement. The samples were centrifuged for 30 minutes at 4°C and 12,000 rpm, and 50 µL of supernatant was added with 1 mL of Na2CO3 (2%) and 75 µL of Folin Ciocalteau reagent (Sigma Aldrich, Merck). After 15 min of incubation in the dark at 25°C, the absorbance was measured at \(\lambda = 725\) nm. Gallic acid (Sigma Aldrich, Merck, Darmstadt, Germany) was used as the standard curve (Meenakshi et al. 2009). The total phenolic content was expressed as µg gallic acid equivalents per g of fresh roots.

The determination of chlorophylls was carried out in fresh epicotyls (FE). Twenty-five mg of samples were crushed with MeOH (100%, 1 mL), vortex mixed 3 times for 1 min each time and then incubated
overnight in the dark at 20°C. The absorbance at 665, 652, and 470 nm was measured in supernatants after centrifugation at 12000 rpm for 30 min. The chlorophyll content was calculated using the following equations (Wellburn 1994; Lichtenthaler and Buschmann 2001):

\[
Clorophyll_{a} (\text{mg FE} \mu g)^{-1} = 16.72 \times A_{665} - 9.16 \times A_{652}
\]

\[
Clorophyll_{b} (\text{mg FE} \mu g)^{-1} = 34.09 \times A_{652} - 15.28 \times A_{665}
\]

\[
Carotenoids \left( \frac{\text{mg FE}}{\mu g} \right) = \left( 1000 \times A_{470} - 1.63 \times Cla - 104.9 \times Clb \right) / 221
\]

Before performing the biochemical assays, the percentage of germinated seeds per plate was recorded, and the root length, seedling fresh, and dry weight (70°C for 3 days) were determined on 10 seedlings per plate, for each experiment.

**Statistical analysis**

All experiments were arranged according to a completely randomized design. All data were analyzed by ANOVA, after checking the homogeneity of variance, and, if the p-value was less than 0.05, the means were separated by Tukey’s test (p < 0.05). Percentage data were arcsin transformed before analysis. All analyses were performed with GraphPad Prism software, San Diego, CA, USA, version 5.01.

**RESULTS**

**Extract characterization**

Table 1 reports the composition parameters of AME. The total solids content (7.8 mg mL\(^{-1}\)) indicates that about one-third of the raw algal biomass (20 mg mL\(^{-1}\)) was solubilized in water by the autoclave-assisted extraction, with proteins representing the main fraction (56%), followed by carbohydrates and ashes. Among phycobiliproteins, phycoerythrin is the most prevalent (31.2 ± 0.4 mg L\(^{-1}\)) followed by phycocyanin and allophycocyanin (28.6 ± 0.1 and 23.3 ± 0.9 mg L\(^{-1}\), respectively). The extract is also characterized by a C:N ratio of 3.8, and by the presence of a small amount of reducing sugars, glucose, and lipids.
Table 1
Characterization of the *Anabaena minutissima* aqueous extract (obtained after centrifugation by an autoclave-assisted method, from 20 mg mL$^{-1}$ of freeze-dried biomass in water, at 100°C, 20 min, 1 bar). The means and standard deviations (sd) of the composition parameters are reported ($n = 3$).

<table>
<thead>
<tr>
<th>Composition parameters</th>
<th>Measurement unit</th>
<th>Mean values ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS)</td>
<td>mg mL$^{-1}$</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Moisture</td>
<td>% TS</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Ashes</td>
<td>% TS</td>
<td>24.5 ± 0.5</td>
</tr>
<tr>
<td>C</td>
<td>% TS</td>
<td>34.5 ± 0.1</td>
</tr>
<tr>
<td>N</td>
<td>% TS</td>
<td>9.05 ± 0.03</td>
</tr>
<tr>
<td>C:N ratio</td>
<td></td>
<td>3.82 ± 0.03</td>
</tr>
<tr>
<td>Total proteins (N x 6.25)</td>
<td>% TS</td>
<td>56.6</td>
</tr>
<tr>
<td>Phycoerythrin</td>
<td>% TS</td>
<td>0.400 ± 0.007</td>
</tr>
<tr>
<td>Phycocyanin</td>
<td>% TS</td>
<td>0.367 ± 0.005</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>% TS</td>
<td>0.299 ± 0.012</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>% TS</td>
<td>26.9 ± 0.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>% TS</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Total soluble reducing sugars</td>
<td>% TS</td>
<td>3.8 ± 1.3</td>
</tr>
<tr>
<td>Total lipids</td>
<td>% TS</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>pH in water</td>
<td></td>
<td>7.18 ± 0.01</td>
</tr>
</tbody>
</table>

Figure 1 displays the FT-IR spectrum of AME. In general, the spectrum is dominated by the typical bands of proteins (Giordano et al. 2001; Righini et al. 2020a) and carbohydrates. In particular, a broad band appearing between 3600 and 3400 cm$^{-1}$ is assigned to the O-H stretching vibration. The shoulder at 3070 cm$^{-1}$, known as amide A, is typical of secondary amides associated with the N-H—O = C hydrogen bond. (Rao 1963). The bands at 2931–2879 cm$^{-1}$ are attributed to the asymmetric and symmetric stretching vibration of $-\text{CH}_2$ functional groups, respectively. The strong peaks at 1640 cm$^{-1}$ and 1540 cm$^{-1}$ are due to Amide I and Amide II (Rao 1963) as well as the bands between 1300 and 1240 cm$^{-1}$ are assigned to the C–N stretching and N–H bending vibrations in Amide III (Rao 1963).

The band at 1390 cm$^{-1}$ is assigned to C–OH bending vibration with the contribution of symmetric stretching vibration of the carboxylate group. The intense band at 1030 cm$^{-1}$ is attributed to C–O, O–C–
O and C–C stretching vibrations of the pyranose ring. The presence of Amide in protein and carbohydrate groups is also supported by the high content in protein and total carbohydrates (Table 1).

**Preliminary tests**

At first, the direct activity of AME on the colony growth of the two soilborne pathogens, PU and RS, was tested *in vitro* on PDA amended with 3 extract doses. Depending on the pathogen, a moderate inhibitory or stimulatory effect on the colony growth was found. The radial growth of PU was reduced by 9.1% with the maximum dose tested (Fig. 2A), whereas RS growth was stimulated by 8.2% already with the lowest one (1 mg mL⁻¹), compared to unamended control (Fig. 2B).

Subsequently, the effect of the seed treatment with AME on tomato seedling growth was tested in a growth chamber. The lowest AME dose (2.5 mg mL⁻¹) showed a stimulatory effect (30.0%; 37.2%), while the highest one (10 mg mL⁻¹) reduced both the seedling (Fig. 3A) and the rootlet (Fig. 3B) fresh weight compared to the non-treated control. The root exudates collected from these plantlets were tested on the mycelial growth of both pathogens in microplates, without showing any effect (data not shown).

The spectra of two-week-old tomato roots after seed treatment with AME at 2.5 mg mL⁻¹ in comparison with water treatment (control) are shown in Fig. 4A. The spectra of tomato roots treated with other doses are not shown, as the best biological response was obtained at the dose indicated above. The main peaks occur: 1735 cm⁻¹ due to C–O stretching motion of ester in lipids and a cell wall pectin (Schulz and Baranska 2007); 1640 cm⁻¹ (amide I) and 1540 cm⁻¹ (amide II) in proteins (Naumann et al. 2010) 1517 cm⁻¹ assigned to breathing vibration of benzene ring in lignin; 1454 and 1421 cm⁻¹ due to methyl and methylene bending motion, respectively; 1371 cm⁻¹ was due to O–CH₃ and C–H symmetric deformation; 1322 cm⁻¹ was assigned to aromatic ring breathing with C–O stretching in lignin; 1241 cm⁻¹ was attributed to amide III plus C–O stretching coupled with C–C vibrations (Rao 1963). Generally, the spectra showed a similar profile. However, due to the treatment, some bands corresponding to amides (1640 cm⁻¹, 1544 cm⁻¹, and 1241 cm⁻¹) displayed an increase in relative intensity. A curve peak fitting was used to semi-quantitatively estimate the contribution of the various bands in the region from 1800 to 1500 cm⁻¹ in terms of area percentage (Fig. 4B). In the control, amide I accounted for 40% and amide II for 8.5%. With AME treatment the contribution of amide I and amide II increased, accounting for 58% and 11%, respectively (Fig. 4B). The lignin component represented by the area percentage of the bands at 1600 cm⁻¹ (C = C stretching mode of the aromatic ring) and 1510 cm⁻¹ (breathing vibration of the benzene ring) accounted for 34% and 6%, respectively in the control. By contrast, with AME treatment their contribution decreased, accounting for 17% and 5%, respectively. Esters consisted of 11.5% and 9.5% in control and in the case of AME treatment, respectively (Fig. 4B).

**Greenhouse assay**
In the greenhouse, the seed treatment with AME was tested against the two pathogens artificially inoculated into a growing substrate.

For PU, the lowest AME dose (2.5 mg mL\(^{-1}\)) increased by 18.1% the percentage of standing plants compared to the infected control (C + PU) (Fig. 5A, C). Plant development, measured as height, was restored to the level of the healthy control (C-PU) at all doses tested (Fig. 5B, C).

The disease incidence and the disease severity recorded 21 days after sowing (Fig. 6A, B), were significantly reduced by AME at all doses compared to the control (C + PU). All doses reduced the disease incidence similarly (36.9% on average). In the case of disease severity, a dose-depending effect was observed \([F(1, 10) = 84.31, p = < 0.001, R^2 = 0.894]\) and the highest reduction (45.6%) was obtained with 10 mg ml\(^{-1}\).

Regarding RS, seed treatment with AME increased the percentage of standing plants compared to the infected control (C + RS) by 46.2% on average (Fig. 7A). The two highest doses (5 and 10 mg mL\(^{-1}\)) even increased by 16.8% this percentage compared to the non-infected control (C-RS). The plant height was restored to the level of healthy control, regardless of the doses tested (Fig. 7B, C).

The disease incidence (Fig. 8A) and severity (Fig. 8B) recorded at the end of the experiment by evaluating the degree of root necrosis, were significantly reduced at all the AME doses tested, more than halving the values of the infected control (C + RS). Particularly, the disease was reduced by 50.9% and 70.5% for incidence and severity, respectively.

**Effect of the seed treatment on seedling biochemical and growth parameters**

The seed treatment with AME at 5 mg ml\(^{-1}\) induced a significant enhancement in β-1,3-glucanase, β-N-acetylhexosaminidase, and endochitinase, activity in the root apparatus of 14-day-old seedlings, compared to non-treated controls, while the increase in chitin 1,4-β-chitobiosidase activity was not statistically significant. (Table 2). The treatment also increased the content of chlorophyll a and b, and carotenoids, in the epicotyls, while the content of total phenols was not influenced either in the epicotyls or in the hypocotyls.

Regarding the growth parameters, the treatment increased the length and fresh weight of the hypocotyls, and the dry weight of the seedlings, besides enhancing the percentage of germinated seeds, compared to the non-treated control (Table 2).
Table 2
Biochemical and growth parameters of 14-day-old tomato seedlings after seed treatment with *Anabaena minutissima* aqueous extract at 5 mg mL\(^{-1}\) dose in comparison to non-treated control. The means and standard deviations are reported (n = 3). The asterisk (*) indicates a statistically significant difference between treatment and control, ns: not significant.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plant part</th>
<th>Measurement unit</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>hypocotyl</td>
<td>µg g(^{-1}) FW(^a)</td>
<td>1461 ± 31</td>
<td>1576 ± 119 ns</td>
</tr>
<tr>
<td>β-1,3-glucanase</td>
<td>hypocotyl</td>
<td>µmol min(^{-1})</td>
<td>197 ± 167</td>
<td>577 ± 137*</td>
</tr>
<tr>
<td>β-N-acetylhexosaminidase</td>
<td>hypocotyl</td>
<td>U g(^{-1}) protein</td>
<td>59 ± 5</td>
<td>77 ± 10*</td>
</tr>
<tr>
<td>Endochitinase</td>
<td>hypocotyl</td>
<td>U g(^{-1}) protein</td>
<td>11 ± 1</td>
<td>13.8 ± 0.2*</td>
</tr>
<tr>
<td>Chitin 1,4-β-chitobiosidase</td>
<td>hypocotyl</td>
<td>U g(^{-1}) protein</td>
<td>10.1 ± 1</td>
<td>11.8 ± 1.5 ns</td>
</tr>
<tr>
<td>Total phenols</td>
<td>hypocotyl</td>
<td>µg eq. gallic acid g(^{-1}) FW</td>
<td>23.1 ± 0.7</td>
<td>16.2 ± 0.4 ns</td>
</tr>
<tr>
<td>Total phenols</td>
<td>epicotyl</td>
<td>µg eq. gallic acid g(^{-1}) FW</td>
<td>29.5 ± 0.6</td>
<td>30.8 ± 1.7 ns</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>epicotyl</td>
<td>µg g(^{-1}) FW</td>
<td>143.3 ± 9.8</td>
<td>217.1 ± 3.9*</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>epicotyl</td>
<td>µg g(^{-1}) FW</td>
<td>114.8 ± 5.4</td>
<td>134.3 ± 9.6*</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>epicotyl</td>
<td>mg g(^{-1}) FW</td>
<td>17.9 ± 1.1</td>
<td>23.8 ± 0.9*</td>
</tr>
<tr>
<td><strong>Growth parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germination</td>
<td>seed</td>
<td>%</td>
<td>85.1 ± 4.4</td>
<td>93.5 ± 1.3*</td>
</tr>
<tr>
<td>Dry weight</td>
<td>seedling</td>
<td>mg DW(^c) per plant</td>
<td>1.8 ± 0.2</td>
<td>2.6 ± 0.4*</td>
</tr>
<tr>
<td>Fresh weight</td>
<td>epicotyl</td>
<td>mg FW per plant</td>
<td>11 ± 1</td>
<td>12 ± 1 ns</td>
</tr>
<tr>
<td>Fresh weight</td>
<td>hypocotyl</td>
<td>mg FW per plant</td>
<td>12 ± 1</td>
<td>19 ± 5*</td>
</tr>
<tr>
<td>Length</td>
<td>hypocotyl</td>
<td>mm</td>
<td>36.5 ± 5.4</td>
<td>48.8 ± 5.3*</td>
</tr>
</tbody>
</table>

\(^a\) FW = Fresh weight; \(^b\) U = µmol min\(^{-1}\); \(^c\) DW = Dry weight.

*Student’s t-test (p = 0.05): β-1,3-glucanase, t(5) = -3.05, p = 0.04; β-N-acetylhexosaminidase, t(5) = -2.88, p = 0.04; endochitinase, t(5) = -4.25, p = 0.01; chlorophyll a, t(5) = -12.08, p = 0.0003; chlorophyll b, t(5) = -3.06, p = 0.04; carotenoids, t(5) = -7.35, p = 0.0002; seed germination, t(5) = -3.17, p = 0.03; hypocotyl length, t(5) = -2.83, p = 0.05; seedling dry weight, t(5) = -3.30, p = 0.03; hypocotyl fresh weight, t(5) = -2.42, p = 0.07.
DISCUSSION

This study assessed the effectiveness of AME obtained by an autoclave-assisted method, in priming tomato seeds toward two soil-borne pathogens, PU and RS, which are responsible of remarkable yield losses. This method permitted to enrich the extract in both proteins and carbohydrates, which are enhancers of primary (photosynthesis, proteins) and secondary (polyphenols, flavonoids and carotenoids) metabolic pathways and intracellular transport when used as biostimulants (Colla et al. 2015; Barone et al. 2018; Ertani et al. 2019; Baghdadi et al. 2022; Borella et al. 2023).

The protein content of the extract was much higher than that obtained in a previous work at a lower temperature (6% at 50°C vs 57% at 100°C) (Righini et al. 2020b). Nonetheless, partial protein denaturation cannot be excluded, with a possible reduction in the seed priming effectiveness. For instance, phycoerythrin resulted unstable at 80°C (Nath et al. 2023). The relatively low level of phycobiliproteins found in AME compared to the raw algal biomass (Righini et al. 2023; Nath et al. 2023) suggests that these compounds were poorly extracted or more probably denatured by the extraction method used. The carbohydrate content of AME at 100°C resulted in 27%, lower than in the extract at 50°C by using cetavlon detergent (Righini et al. 2019). The extraction at 50°C was probably more efficient because cetavlon reduced impurities consisting of proteins and nucleic acids (Kothari et al., 2013).

A preliminary experiment was performed in vitro to assess the potential effect of AME on the pathogens’ growth. Although no dose effect was observed, PU mycelium showed a slight growth reduction, while that of RS was promoted. The high protein content of AME may have stimulated the secretion of proteolytic enzymes, leading to the release of amino acids and ammonium ions (Yike 2011; Kudryavtseva et al. 2013). Ammonium ions can be toxic, especially during the mycelium growth phase, as they can disrupt cell membranes (Yike 2011). Our findings were also corroborated by Howell et al. (1988) who observed that the biological control agent Enterobacter cloacae inhibited PU growth more than RS by producing ammonium.

In this study, the effect of seed priming with AME on tomato seedling growth was initially studied in the microcosm, then in the greenhouse under pathogen challenge.

In the microcosm, the observed stimulation of the seedling growth after seed treatment with AME confirms the priming effect already reported with the extract obtained at 50°C (Righini et al. 2021b), thus the biological activity has been maintained, despite the high temperature of extraction, maybe also due to a combined effect of carbohydrates and proteins.

Notably, rootlets were more developed because of AME treatment than untreated rootlets. Structural changes in the rootlets were also noted: AME treatment led to an increase in protein content, estimated as amide I and amide II. In contrast, no increase in lignin (bands at 1600 and 1510 cm$^{-1}$) was observed as a result of AME treatment. Conversely, the amount of lignin was more consistent in the spectra of roots treated with AME extract at 50°C (Righini et al. 2021b).
Considering that the high temperature of extraction could have partially denatured the proteins, AME could contain protein fragments and aminoacids that could have been absorbed by the seed during the treatment. These compounds could have caused gene transcription in the embryo leading to rootlet stimulation, as observed in maize seedlings exposed to protein hydrolysates (Santi et al. 2017). Seed priming with protein hydrolysates was recently reported to improve seedling growth and stress tolerance in Arabidopsis (Sorrentino et al. 2021) and tomato, through regulation of reserve mobilization, osmotic adjustment, and antioxidant mechanism (Wang et al., 2022).

Under greenhouse conditions, the seed priming with AME resulted in a reduction of disease incidence and severity, consistent with what was reported about RS root rot in a previous work about tomato seed priming with the aqueous extract of A. minutissima obtained at 50°C (Righini et al. 2021b), confirming again that the extract obtained at high temperature maintained its bioactivity. Another outcome from greenhouse experiments with both pathogens concerns the restoration of plant development to the level of healthy control due to AME-seed treatment at all doses in addition to the disease reduction.

In this study, the protective effect against RS and PU lasted up to the end of the experiments, 21 days after the treatment. Considering that AME was applied by seed immersion followed by water-washing to remove the extract, we suppose the involvement of elicitation of plant defense mechanisms underlying the prolonged observed reduction in disease symptoms. This hypothesis was confirmed by the increase of activities of defense-related enzymes such as chitinases and β-1,3-glucanases and by the increase in carotenoids observed in seedlings from primed seeds with AME.

Plant chitinases and glucanases are crucial in defense against phytopathogens such as fungi and oomycetes. These enzymes work synergistically together to weaken and disrupt the structural integrity of cell wall structural components, damaging pathogen growth and proliferation (Bishop et al 2000; Vaghela et al., 2022; Perrot et al. 2022). Chitinases can degrade the chitin of the fungal cell wall. When plants are under pathogen stress, they strongly express chitinases as part of their defense response. Glucanases, on the other hand, target the beta-1,3-glucan polymers that are also abundant in oomycetes and fungal cell walls.

During plant growth, these enzymes are continuously expressed and are even essential for several physiological processes such as embryogenesis and synthesis of cell wall components (Vaghela et al., 2022; Perrot et al. 2022). Kabir et al. (2016) found that chitinases are produced during the seed stage and are effective against fungi. The genes that encode these enzymes are expressed in the first few days of the germination process. Additionally, new chitinase genes are transcribed during this time (Gomez et al. 2002). Hirano et al. (1990) found that treating radish seeds with depolymerized chitosan and its oligosaccharides led to increased levels of chitinases in seedlings. Similarly, Samarah et al. (2020) demonstrated that treating pepper seeds with chitosan enhances chitinase and glucanase activity, leading to increased seed germination.

In the elicitation of plant defense responses, proteins and carbohydrates which are the main component of AME may be perceived as non-self-molecules through specific pattern recognition receptors in the
plant cell surface triggering all the immune cascade events that lead to the production of defense enzymes such as chitinases and glucanases (Pastor-Fernández et al. 2023). An increase in defense enzyme activity has been observed in tomato seedlings from seeds primed with phycobiliproteins from _A. minutissima_ (Righini et al. 2023). As regards polysaccharides Righini et al. (2022b) demonstrated that those of a red alga, _Jania adhaerens_, showed a seed-priming effect against PU and RS.

Because of AME treatment, other seedling parameters were improved such as fresh and dry weight and hypocotyl length. Moreover, in epicotyl, an enhancement of chlorophyll a and b and carotenoids was observed. These molecules have a global effect on plant fitness since they are involved in the photosynthesis process. In particular, carotenoids are a group of isoprenoid metabolites that play an essential role in photosynthesis, photoprotection, and pigmentation. Furthermore, carotenoids also play a regulatory role in phytohormone synthesis thereby on plant growth (Sun et al. 2022) and carotenoid derivatives possess the capability to function as signaling molecules, in response to environmental stresses (Morelli and Conception 2023; Uarrota et al. 2018).

**CONCLUSIONS**

Overall, seed priming with AME can be considered a viable strategy for biofortifying seedlings in the nursery stage when seedlings are more vulnerable to pathogenic attack. We have demonstrated that AME treatment has great potential as a biostimulant for triggering seedling growth and enzyme activities as well as secondary metabolism correlated to plant defenses. An important finding is the maintaining of the plant's immune memory until the end of the greenhouse experiments. The use of AME for seed priming has the potential to innovate plant disease management by using eco-friendly substances in compliance with current regulations aimed at reducing the environmental impact of synthetic pesticides. Although encouraging outcomes have been observed, research on priming with cyanobacteria extracts is still in the early stage of development and more studies are needed to verify the long-term protective effect of AME in a large-scale application.

**Declarations**

**Funding**

This research was funded by the Italian Ministry of Agricultural, Food, Forestry and Tourism Policies (MiPAAFT) under the DIBIO-BIOPRIME project, (DM N. 3400, 20 December 2018) and by the Interreg MAC Program 2014–2020, grant number MAC2/1.1b/269: REBECA-CCT.

**Competing interests**

Not applicable

**Availability of data and material**
The data and material presented in this study are available from the corresponding author upon request.

Authors' contributions


References


serovar Typhi. Vaccine, 31(42), 4714-4719.


Figures
Figure 1

FT-IR spectrum of the *Anabaena minutissima* aqueous extract
Figure 2

Effect of *Anabaena minutissima* aqueous extract at different doses on the *in vitro* radial growth of (A) *Pythium ultimum* and (B) *Rhizoctonia solani* at 42 h. Histograms represent mean values of 3 independent experiments (n = 3); bars are the standard deviations of the means; different letters indicate significant differences among extract doses according to the Tukey test (*p* < 0.05). ANOVA results: (A) $F_{(3,8)} = 7.80$, $p = 0.0093$; (B) $F_{(3,8)} = 11.16$, $p = 0.0031$
Figure 3

Effect of tomato seed treatment with the aqueous extract of *Anabaena minutissima* at different doses on the fresh weight of two-week-old seedlings, shown for (A) entire seedlings and (B) rootlets. Histograms represent the mean values of 3 independent experiments (n = 3); bars represent the standard deviations of the means; different letters indicate significant differences among the treatments, according to Tukey’s test (p < 0.05) ANOVA results: (A) $F_{(3,8)} = 429.12, p < 0.0001$; (B) $F_{(3,8)} = 163.52, p < 0.0001$
Figure 4

(A) FT-IR spectra of two-week-old tomato seedling rootlets after seed treatment with *Anabaena minutissima* aqueous extract (black line) at a concentration of 5 mg mL$^{-1}$ and water as control (red line).

(B) Area percentage of more significant deconvolute bands in the region from 1800 to 1500 cm$^{-1}$ (red=water treatment; black = treated; bars= standard error)
Effect of tomato seed treatment with different doses of *Anabaena minutissima* aqueous extract on (A) the standing plant percentage, (B) plant height, and (C) plant development at 21 days after sowing in a substrate artificially infected with *Pythium ultimum* (PU) in the greenhouse assay. C-PU = non-infected control, C+PU = infected control. Histograms are the mean values of 3 independent experiments (n = 3); bars represent the standard deviations of the means; different letters indicate significant differences among the treatments, according to Tukey’s test ($p < 0.05$). ANOVA results: (A), $F_{(4,10)} = 4.24, p = 0.0291$; (B), $F_{(4,10)} = 14.45, p = 0.0004$
Figure 6

Effect of tomato seed treatment with different doses of *Anabaena minutissima* aqueous extract on (A) the disease incidence and (B) disease severity, based on plant decay degree, at 21 days after sowing in a substrate artificially infected with *Pythium ultimum* (PU) in the greenhouse assay. C+PU = infected control. Histograms are mean values of 3 independent experiments (*n* = 3); bars represent the standard deviations of the means; different letters indicate significant differences among the treatments according to Tukey's test (*p* < 0.05). ANOVA results: (A), $F_{(3,8)} = 14.58$, *p* = 0.0013; (B), $F_{(3,8)} = 37.37$, *p* < 0.0001.
Figure 7

Effect of tomato seed treatment with different doses of *Anabaena minutissima* aqueous extract on (A) the standing plant percentage, (B) plant height, and (C) plant development at 21 days after sowing in a substrate artificially infected with *Rhizoctonia solani* (RS) in the greenhouse assay. C-RS = non-infected control, C+RS = infected control. Histograms are the mean values of 3 independent experiments (n = 3); bars represent the standard deviations of the means; different letters indicate significant differences among the treatments according to Tukey’s test ($p < 0.05$). ANOVA results: (A), $F_{(4,10)} = 19.91$, $p = 0.0001$; (B) $F_{(4,10)} = 12.14$, $p < 0.0007$
Figure 8

Effect of tomato seed treatment with different doses of *Anabaena minutissima* aqueous extract on (A) the disease incidence and (B) disease severity, based on root necrosis degree at 21 days after sowing, in a substrate artificially infected with *Rhizoctonia solani* (RS) in the greenhouse assay. C+RS = infected control. Histograms are mean values of 3 independent experiments (n = 3); bars represent the standard deviations of the means; different letters indicate significant differences among the treatments, according to Tukey's test ($p < 0.05$). ANOVA results: (A), $F_{(3,8)} = 27.15$, $p = 0.0002$; (B), $F_{(3,8)} = 13.24$, $p < 0.0018$