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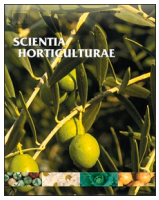

Evaluating *Ecklonia maxima* water-soluble polysaccharides as a growth promoter of tomato seedlings and resistance inducer to Fusarium wilt

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ABSTRACT

Alternatives to chemicals for plant management are increasingly used to reduce environmental pollution. Seed treatment with natural products may act as a priming effect by stimulating seedling growth and plant defence responses against fungal pathogens. In this framework, algae produce a wide variety of bioactive metabolites, which can be used in agriculture as biofertilizers or biostimulants. The purpose of this study was to investigate the possible role of water-soluble polysaccharides (WSPs) from the brown alga *Ecklonia maxima* applied on tomato seed in enhancing plant growth and inducing resistance to *Fusarium oxysporum* via modulation of multiple physiological parameters and metabolic pathways. Here, we first characterized the *E. maxima* WSPs by FT-IR spectroscopy, and then we tested the WSPs as growth promoters on tomato seedlings, and the physiological and defence responses of plants during pathogen infection. We found that WSP seed treatment without pathogen challenge stimulated seedling height and root growth by 24.5 and 62.9%, respectively. Under pathogen infection, plants exhibited long-lasting resistance against *F. oxysporum* until 46 days after seed treatment. The metabolic changes associated with resistance to Fusarium wilt in plant roots were related to an increase in phenols, flavonoids and protein contents as well as a higher chitinase and β-1,3-D-glucanase enzyme activity. Moreover, *PR1a, PR3* and other defence gene expressions were significantly increased. Resistance to *F. oxysporum* as a result of WSP seed treatment was also supported by FT-IR analysis of tomato roots. Infected roots showed a decrease in the relative intensity of the bands due to the syringyl ring and amide I and amide II in proteins. In contrast, WSP treatment alone and in the presence of the pathogen exhibited a spectral profile similar to that of the control. This research emphasizes the potential role of algal polysaccharides applied by seed treatment in promoting seedling growth and priming plant resistance against soil-borne pathogens.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the world's most widely cultivated solanaceous plants with 187 million tons produced worldwide in 2020 on a cultivated area of almost 5 million hectares. China is the world's largest producer, while Italy and Spain are the first and second countries, respectively, in Europe. In terms of plant production, tomato is second only to potato and first amongst vegetables (FAOSTAT, https://www.fao.org/faostat/en/#data, accessed on 19 October 2022).

Several pathogenic fungi can infect tomato plants, from seeding to harvest, both in the greenhouse and in the field (Jones, 1991; Pandey et al., 2022). Amongst fungal pathogens, *Fusarium oxysporum* f. sp. *lycopersici* causes yield losses in a range between 20 and 80% as reviewed by Pandey et al. (2022). This fungus is a soil-borne pathogen that can survive for many years as latent mycelium in plant debris or as resting spores in the soil. The pathogen penetrates the roots of young plants and colonizes the xylem vessels, interfering with the rough sap flow and causing the leaves to fall and yellowing. Infection is favoured by root damage which may occur during the transplanting process or be caused by soil organisms. A serious infection can lead to permanent wilting and plant death (Srinivas et al., 2019).

The management of Fusarium wilt disease covers several strategies, including resistance, crop rotation, grafting, cultural practices, and physical soil sanitation, but none of these is fully effective (Chellemi

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et al., 2016). Although chemical pesticides to treat soils as fumigants are highly effective, their continued use was problematic for environmental contamination and residual toxicity or adverse effects on non-target organisms. Nowadays, the use of these chemicals is not allowed in Europe, because they are not approved under Reg. (EC) No 1107/2009 (EU pesticides database, https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/start/screen/active-substances, accessed on 8 November 2022). Furthermore, within the Green Deal policy, soil health will benefit from a 50% and 20% reduction in pesticides and fertilizers respectively by 2030 (Montanarella and Panagos, 2021). In this framework, special focus has been given to soil, a "non-renewable resource" which requires sustainable management programs to control pathogens to preserve its quality, productivity and food security (Abawi and Widmer, 2000; FAO, https://www.fao.org/3/i4373e/i4373e.pdf; accessed on 19 October 2022). Accordingly, alternative crop protection measures are being explored, seeking to reduce the use of synthetic fungicides.

Several studies have shown the potential properties of bioactive compounds from marine algae in various fields from pharmaceuticals, cosmeceuticals and functional food (Hakim and Patel, 2020; La Bella et al., 2021). In agriculture, algae are mostly known for their beneficial effects on crops due to their ability to induce tolerance to biotic or abiotic stresses when applied as fertilizers or biostimulants (Fleurence, 2021; Khan et al., 2009; Poveda and Díez-Méndez, 2022; Righini et al., 2018). The presence of bioactive molecules, such as polysaccharides, betaines, macro- and micronutrients, and hormone-mimetic compounds, makes algae highly indicated for improving plant performance and biological control of fungal pathogens. (La Bella et al. 2021; Lee and Ryu 2021; Shukla et al., 2021). Brown algae are the most commonly used in agriculture and, amongst them, *Ascophyllum nodosum* is well-established and has long been accepted by growers because it improves productivity in stressed crops (Hines et al., 2021; Khan et al., 2009; Righini et al., 2018). Besides *A. nodosum*, another brown alga applied in agriculture is *Ecklonia maxima* (ECK), contained in several commercial products such as Kelpstar®, Kelpack, and Eckol (Di Mola et al., 2019; Khan et al., 2009; La Bella 2021; Rengasamy et al., 2015; Rouphael et al., 2018). In particular, foliar ECK application boosts the growth and yield of baby lettuce and spinach in addition to improving plant quality (Di Mola et al., 2019; Rouphael et al., 2018). *Ecklonia maxima* (Osbeck) Papenfuss is a perennial species belonging to the Laminariales order, Chromista Kingdom, typically living along the southern Atlantic coast of Africa (Algabase). A feature of the cell wall of ECK is the presence of sulfated ramified polysaccharides such as fucoidans, one of its major constituents representing 5 to 20% of the algal dry weight (Deniaud-Bouët et al., 2017; Vera et al., 2011).

Overall, polysaccharides and derived oligosaccharides from algae have shown elicitor activities in plants by triggering defence responses against pathogens (Righini et al., 2022; Stadnik and de Freitas, 2014; Vera et al., 2011). Some studies have been addressed to extend the knowledge of how polysaccharides from brown algae help the plant counteract stresses. For example, fucoidans from *Bifurcaria bifurcata* and *Fucus spiralis* increased the phenylalanine ammonia-lyase (PAL) activity and phenols and lignin content in the date palm roots (Bouissil et al., 2020), while sulfated polysaccharides from *Cystoseira myriophylloides* showed to increase PAL and tyrosine ammonia-lyase activities and polyphenols, and lignin contents in olive leaves (Aitouguinane et al., 2022). On wheat plants, polysaccharides from *Lessonia nigrescens* promoted growth, decreased membrane lipid peroxidation, increased chlorophyll content, improved antioxidant activities, and plant resistance to salt stress (Zou et al., 2019). Thus, algal polysaccharides have become a target of study for their ecological properties and ability to enhance plant defence and growth-enhancing mechanisms. Recently, Righini et al. (2019) have examined the activity of ECK polysaccharides applied on strawberry fruits in pre-harvest against *Botrytis cinerea* showing a reduction of both fruit infected area and pathogen sporulation. These outcomes suggested that polysaccharides may be an eco-friendly solution to prevent pathogen attacks.

In the present study, we have explored the seed priming role with water-soluble polysaccharides of ECK as tomato growth promoters and resistance inducers against *Fusarium oxysporum* f. sp. *lycopersici*. More specifically, we have examined the seed-priming effect of polysaccharides on seedlings and the long-term effect on plants after transplanting in the presence and absence of pathogen infection and the main defence pathways used by plants to counteract *F. oxysporum*.

2. Materials and methods

2.1. Algal material and WSP extraction

All experiments were carried out with water-soluble polysaccharides (WSPs) from the brown alga ECK provided by the Spanish Bank of Algae, Marine Biotechnology Centre, University of Las Palmas de Gran Canaria. It was collected from the coast of South Africa, harvesting only the fronds from the kelp plant and leaving the beds to regenerate the kelp forests.

WSPs were extracted by suspending a fine powder of ECK dry thallus in 200 mL of sterile distilled water (10 mg/mL) under continuous stirring for 12 h at 50 °C (Righini et al., 2021). The suspension was then filtered through sterile filter paper and lyophilized for 4 days until use. The yield related to the WSPs was calculated according to Álvarez-Gómez et al. (2016) with modifications: (WSPs g/dry thallus g) × 100. Lyophilized WSPs were then dissolved in water and immediately used to prime tomato seeds as described in 2.4 paragraph.

2.2. Spectroscopic characterization

The ATR-FTIR spectra of freeze-dried WSPs and roots were analysed using a Bruker Tensor FT-IR instrument (Bruker Optics, Ettlingen, Germany) with an accessory for analysis in micro Attenuated Total Reflectance (ATR). The sampling device contained a microdiamond crystal, a single reflection with an angle of incidence of 45 ° and a penetration of around 2 μm (Specac Quest ATR, Specac Ltd., Orpington, Kent, UK). Spectra were recorded by accumulating 64 scans at a spectral resolution of 4 cm^{-1}. Background spectra were also taken against air under the same conditions before each sample. The Savitzky-Golay smoothing and baseline correction were applied to the spectral ranges of 4000 – 400 cm^{-1}. Additionally, for root samples the region from 1800 to 1500 cm^{-1} was resolved by peak-fitting analysis using Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH, USA). The peak fitting was performed using the Gaussian functions. Baseline, peak centre, and peak width parameters were set and released during fitting to facilitate parameter initialization. The iteration procedure was stopped when the best fit was achieved (reduced chi-square $< 1 \times 10^{-3}$, coefficients of determination by 0.999, standard error (SE) from 0.0007–0.0001). All these values denote the accuracy and effectiveness of the fitting process. An integration was performed on the identified peaks to calculate the peak area. The integration of the peak area of the individual peaks was processed and converted into the percentage of single peak area for each sample. This percentage was used as relative amount of different functional groups. The percentages were used for comparing different treatments using the *t*-test ($p < 0.05$).

2.3. Plant material and pathogen

Untreated tomato seeds of cv. Marmande (L'Ortolano, Savini Vivai, Italy) were used for the experiments. The plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici* DISTAL2019 (FOX) belongs to the collection of the Department of Agricultural and Food Sciences, University of Bologna, Italy. The fungus is stored on a medium containing doses of potato dextrose broth (PDB 12%, Biolife S.r.l., MI, Italy) supplemented with glycerol solution 10% (vol/vol) in tubes at −80 °C and transferred and cultivated on potato dextrose agar (PDA 3.9%, Biolife S.r.l., MI,

Italy) in Petri dishes (Ø 9 cm) at the time of use.

2.4. Seed priming with WSPs

Tomato seeds were firstly surface disinfected with ethanol (70%) for 5 min and with sodium hypochlorite solution (1.5% active Cl) for 3 min (modified from Mbega et al., 2012). The seeds were thrice rinsed with sterile distilled water and then treated with lyophilised WSPs dissolved in distilled water to obtain the three concentrations of 0.3, 0.6 and 1.2 mg/mL which were previously used for WSPs of the red alga *Jania adhaerens* (Righini et al., 2022). For treatment, 300 seeds were immersed overnight in 6 mL aliquot of each WSPs concentration and for control 300 seeds were immersed in 6 mL of sterile distilled water, in Falcon tubes. After treatment, seeds were rinsed two times in sterile distilled water and then all seeds (treated and control) were sown in alveolate polystyrene trays (tray, 54 × 31 × 5.6 cm; hole 2.7 × 5 cm), 1 seed per hole, 100 seeds per tray (replicate). Trays were filled with potting soil (Gesal-Compo, Fabrik GmbH, Mannheim, Germany). They were incubated under greenhouse conditions at 24–26 °C (day), 20–22 °C (night), 70% relative humidity, 14-h photoperiod and regularly irrigated.

2.5. Seed priming effect with WSPs on seedling growth parameters

Seedling emergence percentage (EP), emergence index (EI), mean emergence time (MET) and time to 50% emergence (E_{50}) were calculated with the following modified formulas:

AUEC = area under emergence curve, the integration of the fitted emergence curve between $t = 1$ and the endpoint 16 days (calculated with GraphPad Prism software, version 5.01)

EP = (number of emerged seedlings/total number of sowed seeds) × 100 (Hernández-Herrera et al., 2014);

EI = Σ (Gt/Tt), where Gt is the number of seeds that emerged on day t and Tt is the number of days (Aosa, 1983);

MET = $\Sigma (D \times n)/\Sigma n$ where n is the number of seeds that emerged on day D and D is the number of days counted from the beginning of the test (Ellis and Roberts, 1981, modified);

$E_{50} = t_i + [((N + 1)/2 - n_i)/(n_j - n_i)] * (t_j - t_i)$ where N is the final number of seeds germinating and n_i, n_j, total number of seeds germinated by adjacent counts at time t_i, t_j, where $n_i < (N + 1)/2 < n_j$ (Coolbear et al., 1984).

Seedling emergence percentage (EP) was assessed in each tray every 3 days for sixteen days. Seedlings were regularly irrigated until the stage of 2nd true leaf.

Ten seedlings were gently removed from each tray and washed with tap water. The total seedling length was measured and the seedling vigour index (SVI= seedling length (cm) × emergence percentage) was calculated according to Orchard (1977). Seedlings were then dried in a hoven at 60 °C for 72 h for dry weight determination. The experiment was repeated three times ($n = 3$).

2.6. Seed priming effect with WSPs against Fusarium oxysporum f. sp. lycopersici and on plant growth

Seedlings at the stage of 2nd true leaf (16-day-old) obtained from 0.6 mg WSPs/mL-treated and control seeds, were divided into four groups: 1) 60 seedlings from 0.6 mg WSPs/mL-treated seeds (20 seedlings from each tray) transplanted in 20 pots (3 seedlings per pot; pot: Ø 16 cm, h 15 cm) containing potted soil artificially infected with FOX (WSPs+FOX); 2) 60 seedlings from control seeds (20 seedlings from each tray) transplanted in 20 pots (3 seedlings per pot) containing potted soil artificially infected with FOX (FOX); 3) 60 seedlings from 0.6 mg WSPs/mL-treated seeds (20 seedlings from each tray) transplanted in 20 pots (3 seedlings per pot) containing potting soil not infected with FOX (WSPs); 4) 60 seedlings from control seeds (20 seedlings from each tray) transplanted in 20 pots (3 seedlings per pot) containing potting soil not infected with FOX (CTRL). Pots were incubated under greenhouse conditions at 24–26 °C (day), 20–22 °C (night), 70% relative humidity, 14-h photoperiod and regularly irrigated. The experiment was repeated three times ($n = 3$).

For pathogen inoculation, 15-day-old FOX colonies grown on a PDA medium were used. Fungal colonies and medium were harvested from Petri dishes, weighted, added with sterile distilled water (10 mL water/10 g fungal colony+medium), homogenized and then mixed with potting soil at the rate of 2% (w/w, fungal colony+medium/soil). The inoculated potting soil was covered with a black plastic film and incubated at 25 °C for two days in a growth chamber before the transplant.

Thirty days after transplant, plants from 5 pots (groups 1 and 2) were gently removed, plant height and root length were measured, and disease incidence and severity and plant mortality were evaluated. For disease severity, plant height, yellowing and wilt symptoms were considered by using a 6-point scale based where: 0, plant without disease symptoms; 1, very slight wilt (mild chlorosis on lowest leaves only); 2, lower leaves dead, some upper leaves wilt slight chlorosis and up to 30% plant length reduction; 3, lower leaves dead, some upper leaves wilted and 31–60% plant length reduction; 4, lower leaves dead, severe wilt of upper leaves and plant length reduction higher than 60%; 5, dead plant (modified from Righini et al., 2022). On the same date, plants from 5 pots (groups 3 and 4) were removed and plant height and root length were measured.

2.7. Seed priming effect with WSPs on the protein content, chitinase and β−1,3-glucanase activities, phenol and flavonoid content, expression of PR proteins and polyphenol pathway genes, and determination of functional groups in roots

For each of the following determinations, roots from plants (5 pots from each group) were washed with distilled water, pulled together snap frozen in liquid nitrogen and stored at −80 °C. For the determination of protein content, chitinase and β−1,3-glucanase activities, phenol and flavonoid content, and functional groups, root samples were also freeze-dried (FDR) and maintained at ambient temperature in the dark for 2 weeks until use.

2.7.1. Protein content, and chitinase and β−1,3-glucanase activities

For root protein extraction and measurement, and for the detection of enzyme activity, 30 mg of freeze-dried tissue were ground to a fine powder in liquid nitrogen using prechilled mortars. Two mL of chilled 20 mM sodium acetate buffer (pH 5.5) containing 1% (w/v) polyvinylpolypyrrolidone (Sigma–Aldrich Co.) was added to the powder. After incubation for 60 min at 4 °C under continuous gentle stirring, the extract was centrifuged twice for 20 min at 12,000 rpm and 4 °C and the supernatant was filtered with GV Millex® Syringe Filter Unit (Millipore Corporation, USA).

The protein-dye binding method of Bradford (1976) was used to determine protein concentration using bovine serum albumin (Bio-Rad Laboratories, Inc.) as standard in a 96 wells microplate (Greiner CELLSTAR®) incubated on an ice bath in the dark for 10 min and the absorbence was measured at λ = 595 nm. The root protein content was expressed as μg protein per mg of freeze-dried roots (μg protein mg^{-1} FDR).

Protein extracts were used in a glass Petri plate (14 cm diam.) assay on agarose gel for determination of both chitinase and glucanase activities with modification (Bargabus et al., 2004).

For chitinase activity, 200 μL (15 μg of proteins) of each extract was added in triplicate to 7 mm-diameter wells cut in agarose gel (1.5%) containing 0.01% glycol chitin. The same volume of chitinase standard from *Streptomyces griseus* (Sigma-Aldrich St Louis, MO, USA) was used. After incubation at 37 °C for 24 h, plates were irrigated with 50 mL of 500 mM Tris–HCl (pH 8.9) containing 0.01% (w/v) fluorescent brightener. Two h later, the plates were rinsed with distilled water, flooded with distilled water overnight in the dark, and then observed at λ = 302

nm under a UV light source. Images of non-fluorescent lytic zones corresponding to the enzyme activity were taken with a digital camera, to visualize non-fluorescent lytic zones corresponding to the enzyme activity and then the area (mm^2) of the chitinase activity lytic zone was calculated with the Quantity One 1-D analysis software v. 4.6.6 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The specific chitinase activity was expressed as units. One unit corresponds to the mg of N-acetyl-D-glucosamine released/h/mg of protein in comparison to the standard.

For the detection of β−1,3-glucanase activity, 0.5 mg/mL of laminarin (from *Laminaria digitata*, Sigma Aldrich St Louis, MO, USA) were added to agarose gel (1.5%) in glass Petri plates. Two hundred μL (15 μg of proteins) for each sample, in 3 repetitions, were added to 7 mm-diameter wells cut in the gel. As a standard, β−1,3-D-glucanase (from *Helix pomatia*, Sigma Aldrich) was used. Each plate was incubated for 24 h at 37 °C, and then coloured with 50 mL of 2,3,5-triphenyl tetrazolium chloride (0.15% in NaOH 1 M) for 30 min at 37 °C until the pink lytic zones on a white background corresponding to the glucanase activity were visible. Images of gels were taken and processed by the Quantity One 1-D analysis software as above described for chitinase activity. Glucanase activity was expressed as units. One unit corresponds to the release of 1 μmol of glucose from laminarin/min in comparison to the lytic zone of the standard.

2.7.2. Phenol and flavonoid content and determination of functional groups

Phenols were extracted from freeze-dried roots ground with 0.1 mL absolute methanol per mg root and incubated for 30 min on ice. The samples were then centrifuged for 30 min at 4 °C and 12,000 rpm, and the supernatants were used for phenols measurement by the Folin method (López Arnaldos et al. 2001). For the standard curve, gallic acid (Sigma Aldrich, Merk, Darmstadt, Germany) was used (Meenakshi et al., 2009). One mL of Na_2CO_3 (2%) and 75 μL of Folin Ciocalteau reagent (Sigma Aldrich, Merk) were poured in 50 μL of phenolic extract. After 15 min incubation in the dark at 25 °C, the absorbence was measured at λ = 725 nm. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per mg freeze-dried roots (mg GAE g^{-1} FDR).

Flavonoids were extracted from 1 mg of freeze-dried roots crushed with 50 μL acidified methanol solution (HCl 1%). The absorbence was measured at λ = at 300 nm and flavonoid content was evaluated using the quercetin standard calibration curve. Flavonoid content was expressed as mg quercetin equivalents (QE) per mg freeze-dried roots (mg QE g^{-1} FDR).

The ATR-FTIR spectra of plant FDR from the four groups were analysed using the same instrument and procedure for WSPs characterization as above in Materials and Methods 2.2.

2.7.3. Expression of PR proteins and polyphenol pathway genes

The gene expression was investigated by real-time PCR, after extraction of RNA and reverse-transcription, as described in Righini et al. (2022). Briefly, ground frozen tissues were extracted with RNAeasy Plant Mini kit (Qiagen, Hilden, Germany) and RNA was quantified by using a spectrophotometer (Nanovue, GE Healthcare Life Sciences, Buckinghamshire, UK). PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan) was used to reverse-transcribe equal amounts of RNA (250 ng) and obtain cDNA from each sample. TB Green Premix Ex Taq II (Takara) was used for Real-time PCR analysis (LightCycler instrument, Roche Molecular Biochemicals, Basel, Swiss) with gene-specific primers from Merck (Table 1). The abundance of mRNAs was calculated by the comparative Ct method, with Actin-7 as the normalization control, and represented as the number of molecules per 100,000 Actin-7 molecules.

2.8. Statistical analysis

All experiments were conducted using a completely randomized design. The effect of tomato seed treatment with WSPs on seedling parameters, and on the expression of PR protein and polyphenol pathway genes, protein content, chitinase and β−1,3-glucanase activities, phenol and flavonoid content, and determination of functional groups in roots was analysed by one-way ANOVA ($p < 0.05$). Duncan's test ($p < 0.05$) was performed for multiple comparisons in the case of significant effects detected by ANOVA. The comparison between the plant height and root length against the respective control both in the presence (+FOX) and absence (-FOX) of the pathogen and between the disease index values in treated plants in comparison to the untreated ones was carried out by *t*-test ($p < 0.05$). All analyses were performed with GraphPad Prism software, version 5.01. The Person multiple correlation has been carried out to estimate the linear relationship amongst disease index and root length and plant height, and amongst protein, phenol and flavonoid content and enzyme activities ($p < 0.05$).

3. Results

3.1. WSP determination and characterization

The yield of WSPs extracted from the dry thallus of ECK was 28.9% ± 0.7 (w/w).

The WSP spectrum (Fig. 1) attributions were given in accordance with the literature (Matsuhiro, 1996; Gómez-Ordóñez and Rupérez, 2011; Yin et al., 2021). The spectrum was characterized by a broad band at 3306 cm^{-1} (OH– stretching vibrations of different oxygenate compounds) and a shoulder absorption band at 2929 cm^{-1} (CH_2 stretching vibration) in fucose. The strong bands at 1609 cm^{-1} and 1410 cm^{-1} (asymmetric and symmetric stretching vibrations of the carboxylate group, respectively) might be attributed to uronic acid. The appearance of the band at 1245 cm^{-1} (C—O and S=O stretching vibration) can be either associated with uronic acid and also with fucoidan and sulfated polysaccharides. The polysaccharide region between 1100 and 1000 cm^{-1} is typical of a pyranose ring (glucose and galactose). The bands at 896 cm^{-1} and 818 cm^{-1} (C1-H deformation vibration) are characteristic of β-mannuronic acid residues. Lastly, the band at 619 cm^{-1} is assigned to the skeleton bending of the pyranose ring.

Table 1
Primer sequences used in PCR amplification.

Gene names[a]	Forward	Reverse	Annealing T (°C)
Actin-7	GGGATGGAGAAGTTTGGTGGTGG	CTTCGACCAAGGGATGGTGTAGC	61
PAL5	CACTGTAAGCCAAGTAGCCAAA	CTGCAGGGGTCATCAGCATA	59
HCT	CGGACGTTACCATCACTGGA	AAGGAGGACTCAGTAGCTTTG	59
HQT	GGTGTTTTGTTTGTTGAGGCTG	GACTCCGCCACACTTGAAAC	59
FLS	GATTTGGCCTCCTCCTGCTA	TCCAAACCAAGCCCAAGTGA	59
PR1a	AGGATGCAACACTCTGGTGG	GCACAACCAAGACGTACCGA	60
PR3	AGAGTTCCAGGGTACGGTGT	CCAATTCGACTTTCCGCTGC	59
PR4	GATGCTGACAAGCCTCTGGA	CCCTCAAGCATCTACCGCAT	59
DFR	GATGGTGCGACAGAAATGGC	CTGCAGTGCTTCGGGTAGAA	59

[a] PAL5, phenylalanine ammonia-lyase; HCT, hydroxycinnamoyl CoA shikimate hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl CoA quinate hydroxycinnamoyl transferase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; PR1a, PR3 and PR4, pathogenesis-related proteins.

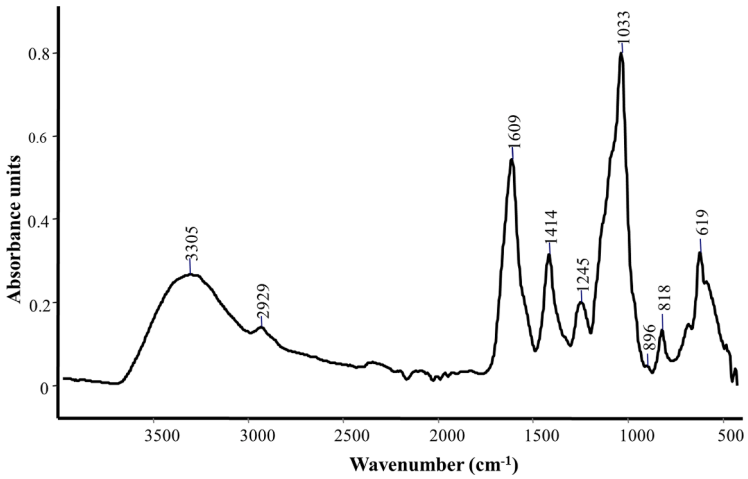

Fig. 1. ATR-FTIR spectrum of water-soluble polysaccharides from *Ecklonia maxima*.

3.2. WSP priming effect on seedling growth parameters

Table 2 shows that all WSP concentrations applied to seeds significantly increased emergence over time with respect to the control as indicated by the AUEC. The EI was lower in the control than in all WSPs-treated seeds. The highest EI values were obtained with 0.6 and 1.2 mg WSPs mL^{-1}. Data indicate that the same concentrations mostly decreased MET significantly. Indeed, seedlings took 13.10 and 13.04 days at 0.6 and 1.2 mg WSPs mL^{-1}, respectively, in comparison to 14.10 days in the control. The control also showed the highest TE_{50} value (12.76 days), whereas all the WSP concentrations significantly reduced this parameter from 16.0 to 9.7%. The concentration of 0.3 mg mL^{-1} was the most effective in reducing the TE_{50} value. All concentrations significantly enhanced SVI in comparison to the control and the concentration of 0.6 mg mL^{-1} gave the highest increase (51.9%).

Based on these results, we have chosen the concentration of 0.6 mg mL^{-1} to continue the experiments discussed below, because it showed the highest SVI and increased most growth parameters as much as 1.2 mg mL^{-1} .

3.3. WSP priming effect against Fusarium oxysporum f. sp. lycopersici and on plant growth

Seed priming with WSPs reduced the disease incidence, plant mortality and disease index by 58.3, 70.1% and 54.0%, respectively compared to the infected control (FOX) (Fig. 2A and B). Additionally, stem height and root length were both significantly increased (Fig. 2C and D) with respect to FOX. The disease index was negatively correlated with root length ($r = -0.74$; $p = 0.09$) and plant height ($r = -0.90$; $p = 0.01$), while the correlation between root length and plant height was positive ($r = 0.87$; $p = 0.02$).

In pot soil not infected with the pathogen, seed priming with WSPs significantly promoted plant growth, resulting in 24.5 and 62.9% increases in plant height and root length, respectively, in comparison to the CTRL (Fig. 3).

Table 2
Effects of seeds treatment with water-soluble polysaccharides from *Ecklonia maxima* at 0.3, 0.6 and 1.2 mg mL^{-1} on seedling growth parameters.

Treatments	Parameters[a]				
	AUEC	EI	MET (days)	SVI	TE_{50} (days)
Control[b]	353.8 ± 13.1 a	11.8 ± 0.2 a	14.10 ± 0.03 c	904.5 ± 17.0 a	12.76 ± 0.17 d
0.3 mg/ mL^{-1}	533.2 ± 7.1 b	18.1 ± 0.1 b	13.45 ± 0.01 b	1238.2 ± 18.1 b	10.73 ± 0.15 a
0.6 mg/ mL^{-1}	543.4 ± 16.7 b	19.2 ± 0.3 c	13.10 ± 0.04 a	1373.8 ± 39.7 c	11.19 ± 0.14 b
1.2 mg/ mL^{-1}	530.0 ± 7.6 b	19.6 ± 0.3 c	13.04 ± 0.06 a	1242.3 ± 32.5 b	11.52 ± 0.13 c

[a] AUEC = area under emergence curve, EI = emergence index, MET = mean emergence time, SVI = seedling vigour index, TE_{50} = time to reach 50% emergence. Data are the mean of 3 experiments ($n = 3$) ± SD.
[b] Control = seed immersed in water.

3.4. WSP priming effect on roots

All the following determinations were carried out in the root of plants grown from seeds primed with WSPs at 0.6 mg mL^{-1} or untreated, transplanted at day 16th in potting soil not-infected or infected with FOX and analysed after further 30 days.

3.4.1. Protein content and enzymatic activities

Multiple comparison analyses amongst different samples showed that seed priming with WSPs significantly augmented the protein content in the roots in plants transplanted in pathogen-infected soil (WSPs+FOX) (Fig. 4).

Chitinase activity was significantly enhanced by WSP+FOX up to 27-fold compared to the CTRL (Fig. 5A), while WSP+FOX increased β−1,3-D-glucanase activity by 4-fold in comparison with the CTRL (Fig. 5B). WSPs and FOX displayed values of both enzymatic activities higher than CTRL (Fig. 5A and B).

3.4.2. Phenol and flavonoid content

A significant increase in phenol and flavonoid content was observed in infected plants after pre-treatment with WSPs (WSPs+FOX) compared to CTRL (Fig. 6A and B). Moreover, WSPs and FOX treatments were able to promote a significant increase of flavonoids in comparison to CTRL (Fig. 6B).

3.4.3. Expression of PR proteins and polyphenol pathway genes

The expression of several genes involved in the mechanisms of resistance to pathogens was investigated. Most of these genes are highly expressed in the roots of tomato plants, based on data reported on http://bar.utoronto.ca/eplant_tomato/. Amongst analysed genes, seed priming with WSPs was able to increase significantly the expression of PR1a, a marker of SAR, and PR3, a chitinase involved in the ISR pathway (Fig. 7A and B).

However, the expression of the gene encoding PR4, a different chitinase, was not found significantly modified in the samples submitted to WSPs treatment compared to the control (data not shown). Concerning genes involved in the pathways of phenylpropanoids (PAL, catalysing the first and committed step) and flavonoids (FLS, DFR), the highest

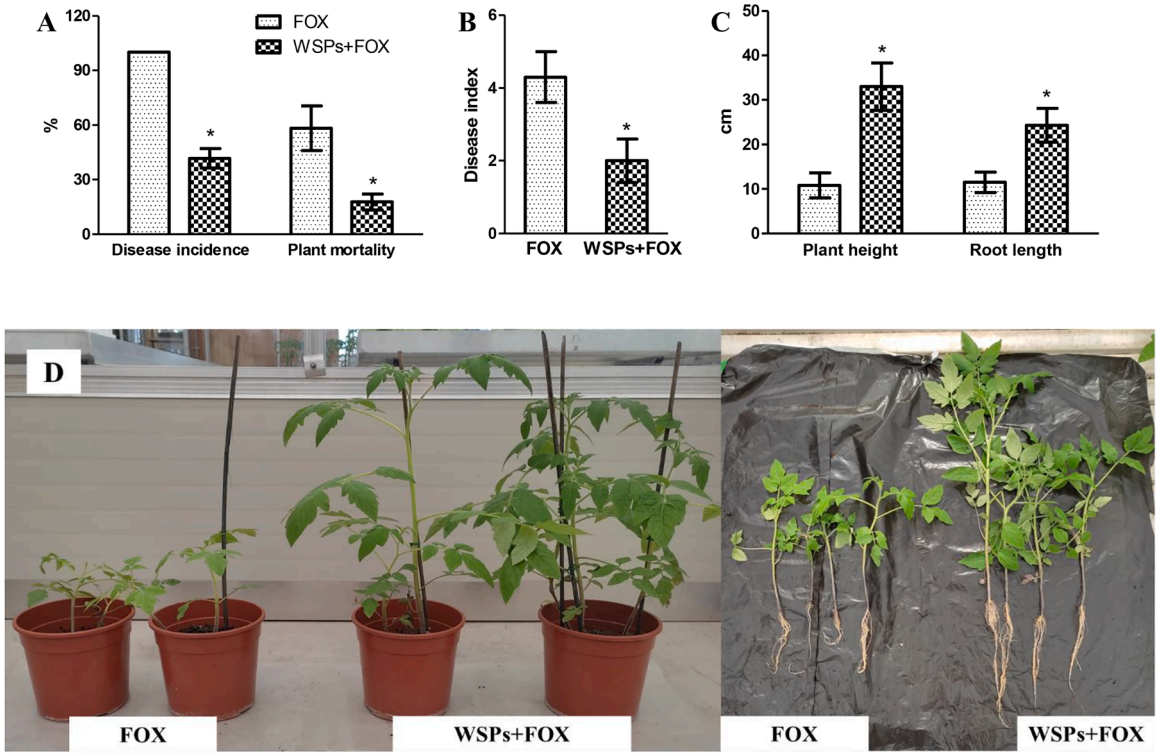

Fig. 2. Effect of seed treatment with 0.6 mg mL^{-1} of water-soluble polysaccharides from *Ecklonia maxima* (WSPs) on disease incidence and plant mortality (A), disease index (B) and plant height and root length (C) after transplant in pot soil infected with *Fusarium oxysporum* f. sp. *lycopersici* (FOX). The results are means of three experiments ($n = 3$) ± SD. The asterisk indicates a significant difference according to the *t*-test ($p < 0.05$). *D* = on the left, untreated plants transplanted in pots with FOX and plants from seed treated with WSPs and transplanted in soil infected with FOX (WSPs+FOX); on the right, plants withdrawn from the soil for the measurements.

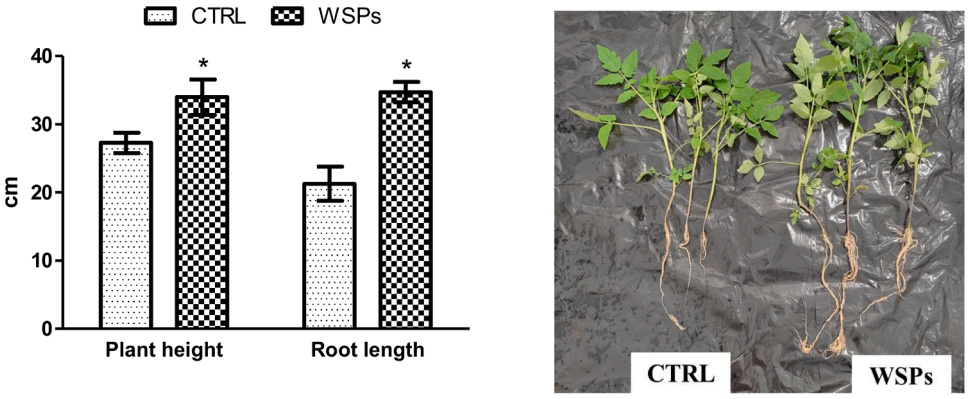

Fig. 3. Effect of seed treatment with 0.6 mg mL^{-1} of water-soluble polysaccharides from *Ecklonia maxima* (WSPs) on plant height and root length after transplant in pot soil with respect to plants from untreated seeds (CTRL). The results are means of three experiments ($n = 3$) ± SD. The asterisk indicates a significant difference according to the *t*-test ($p < 0.05$). On the right, plants withdrawn from the soil for the measurements.

level of the messengers for PAL and FLS (Fig. 7C and D) was detected in infected samples (FOX). PAL expression was also significantly increased in WSPs+FOX samples with respect to the CTRL, while in WSPs-primed samples it was similar to the CTRL. An increase in DFR transcript was found only in the WSPs+FOX (Fig. 7F). Concerning the chlorogenic pathway (for phenol biosynthesis), HCT expression was significantly increased in FOX samples, both with (WSPs+FOX) and without WSPs (FOX) (Fig. 7E), while HQT did not show differences (not shown).

3.4.4. *Effect on roots structure*

All spectra in Fig. 8 are characterized by typical bands of protein, lignin and polysaccharide (Calone et al., 2021; Ertani et al., 2018; Kubovský et al., 2020; Nikalje et al., 2019; Schulz and Baranska 2007). The regions between 3400 (O—H stretching in alcohols, phenols, acids and intramolecular H bonds) and 2900 cm^{-1} (C–H stretching in CH_3 and CH_2 groups) and between 1100 (C–O–C stretching of a primary alcohol in cellulose and hemicelluloses) and 897 cm^{-1} (C1–H deformation of glucose ring in cellulose and hemicellulose) showed no relevant structural changes (region of the spectra not shown). Conversely, the most important structural changes were observed in the region between 1800 −1200 cm^{-1} (Fig. 8).

The band at 1735 cm^{-1} is typical of an unconjugated carbonyl group in xylan and hemicelluloses. The protein bands at 1640 cm^{-1} (amide I) and 1540 cm^{-1} (amide II) overlapped with those of lignin. As for lignin, the bands are centred at 1600 cm^{-1}(C=C stretching mode of the aromatic ring) and 1515 cm^{-1} (breathing vibration of benzene ring). The

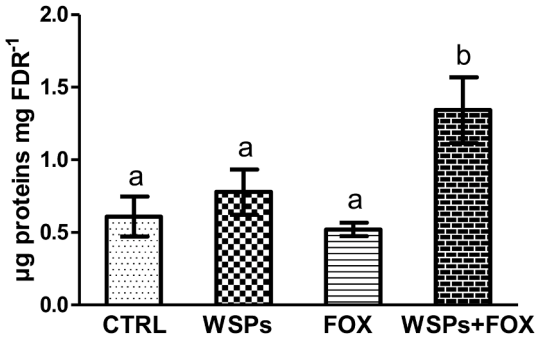

Fig. 4. Protein content in roots of untreated plants transplanted in pots without FOX (CTRL), plants from seed treated with WSPs transplanted in pots without FOX (WSPs), untreated plants transplanted in pots with FOX (FOX) and plants from seed treated with WSPs transplanted in pots with FOX (WSPs+FOX). WSPs were applied at 0.6 mg mL^{-1}. Columns are mean values of 3 independent experiments ($n = 3$) ± SD. ANOVA analysis: $F_{(3,8)} = 16.88$, $p = 0.008$. Different letters indicate significant differences amongst treatments and the control according to the LSD test ($p < 0.05$).

latter band is coupled with guaiacyl and syringyl units and progressively decreased in WSPs+FOX (grey line), WSPs (green line) and FOX (black dashed line) roots. Other bands associated with lignin appeared at 1453 cm^{-1} (O—CH_3 bending), 1263 cm^{-1} (C–O stretching of guaiacyl ring, C–O stretching vibration in xyloglucan and also amide III) and 1234 cm^{-1} (C–O stretching of syringyl ring). Lastly, the characteristic bands attributed to polysaccharides are located at 1416 cm^{-1} and 1371 cm^{-1} (C–H and CH_2 bending in cellulose and hemicelluloses, respectively), at 1316 cm^{-1} (CH_2 wagging). Variations in the absorbence values and shapes of the bands and their location in the region under consideration can be detected. Structural changes were observed in FOX (Fig. 8, black dashed line). Specifically, there is a decrease in the relative intensity of the bands at 1540 cm^{-1} (amide II), 1515 cm^{-1} (syringyl units), 1453 cm^{-1} (O—CH_3 groups) and 1234 cm^{-1} (syringyl ring), showing a reduction of proteins, syringyl compounds and the breakdown of aliphatic side chains. Treatments with WSPs (Fig. 8, green line) and WSPs+FOX (grey line) exhibited a spectral profile very similar to that of the CTRL (red line) albeit minor changes in relative intensity of the investigated bands can be observed.

The contribution of the different bands in the region from 1800 to 1500 cm^{-1} was semi-quantitatively estimated using a curve peak fitting (Fig. 9). Amide I and Amide II accounted for 52% and 11% respectively in CTRL, they did not change in WSPs and WSPs+FOX (51 and 12%). Conversely, Amide I and Amide II in the infected roots (FOX) were 27% and 8%, respectively. The aromatic ring in lignin (1600 cm^{-1}) increased in FOX to 50%, while it was 25% in WSPs, 23% in WSPs+FOX and 18% in CTRL. Syringyl unit accounted for 6% (FOX and WSPs+FOX), 5% (WSPs) and 9% (CTRL). Esters in hemicellulose consisted of 8% and 6% in FOX and WSPs+FOX roots, respectively and 9% in CTRL. In WSPs treatment, it accounted for 5%.

4. Discussion

In recent years, alternative solutions for plant management are highly recommended to avoid soil pollution caused by the application of chemical fertilizers and pesticides. Brown algae may represent an innovative solution because they are a source of several bioactive compounds showing many biological activities in the agricultural field (Craigie, 2011; Khan et al., 2009; Righini et al., 2018). Algal polysaccharides are storage compounds and amongst the major components of brown algae cell walls and have received considerable study for their biological activity (Khan et al., 2009; Vera et al., 2011). Polysaccharides interact with plants acting as elicitors triggering plant defence responses useful to withstand phytopathogen infection (Vera et al., 2011). As the polysaccharides of *Ecklonia maxima* are poorly investigated, this study focused on their role in protecting tomato plants from Fusarium wilt and promoting seedling and plant growth by seed treatment.

FT-IR spectrum analysis of WSPs confirms the existence of mannuronate residues (896 and 818 cm^{-1}) in alginate (Rupérez et al., 2002). Additionally, the presence of oxygenated functional groups such as carboxylates (1600 and 1400 cm^{-1}) in alginates is of great interest as

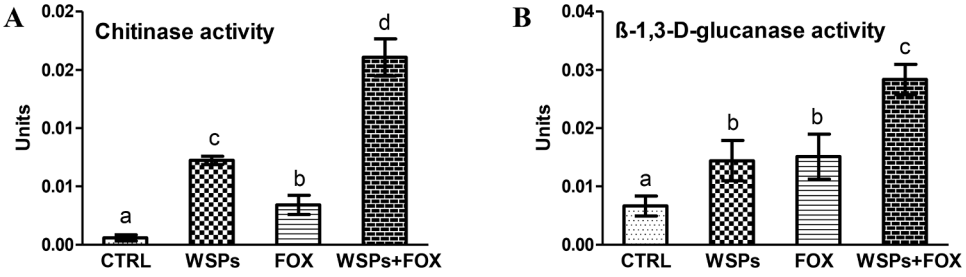

Fig. 5. Chitinase (A) and β–1,3-*D*-glucanase (B) activities in roots of untreated plants transplanted in pots without FOX (CTRL), plants from seed treated with WSPs transplanted in pots without FOX (WSPs), untreated plants transplanted in pots with FOX (FOX) and plants from seed treated with WSPs transplanted in pots with FOX (WSPs+FOX). WSPs were applied at 0.6 mg mL^{-1}. Columns are mean values of 3 independent experiments ($n = 3$) ± SD. ANOVA analysis: chitinase activity, $F_{(3,8)} = 159.57$, $p = 0$; glucanase activity, $F_{(3,8)} = 26.65$, $p = 0.0002$;. Different letters indicate significant differences amongst treatments and the control according to the LSD test ($p < 0.05$).

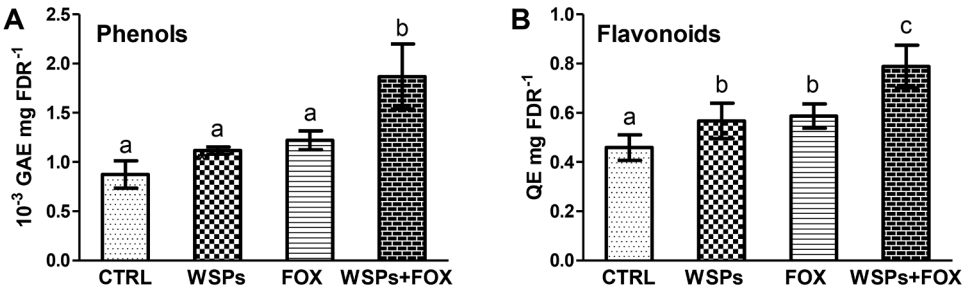

Fig. 6. Phenol (A) and flavonoid (B) content in roots of untreated plants transplanted in pots without FOX (CTRL), plants from seed treated with WSPs transplanted in pots without FOX (WSPs), untreated plants transplanted in pots with FOX (FOX) and plants from seed treated with WSPs transplanted in pots with FOX (WSPs+FOX). WSPs were applied at 0.6 mg mL^{-1}. Columns are mean values of 3 independent experiments ($n = 3$) ± SD. ANOVA analysis: phenols, $F_{(3,8)} = 15.67$, $p = 0.001$; flavonoids, $F_{(3,8)} = 12.84$, $p = 0.002$. Different letters indicate significant differences amongst treatments and the control according to the LSD test ($p < 0.05$).

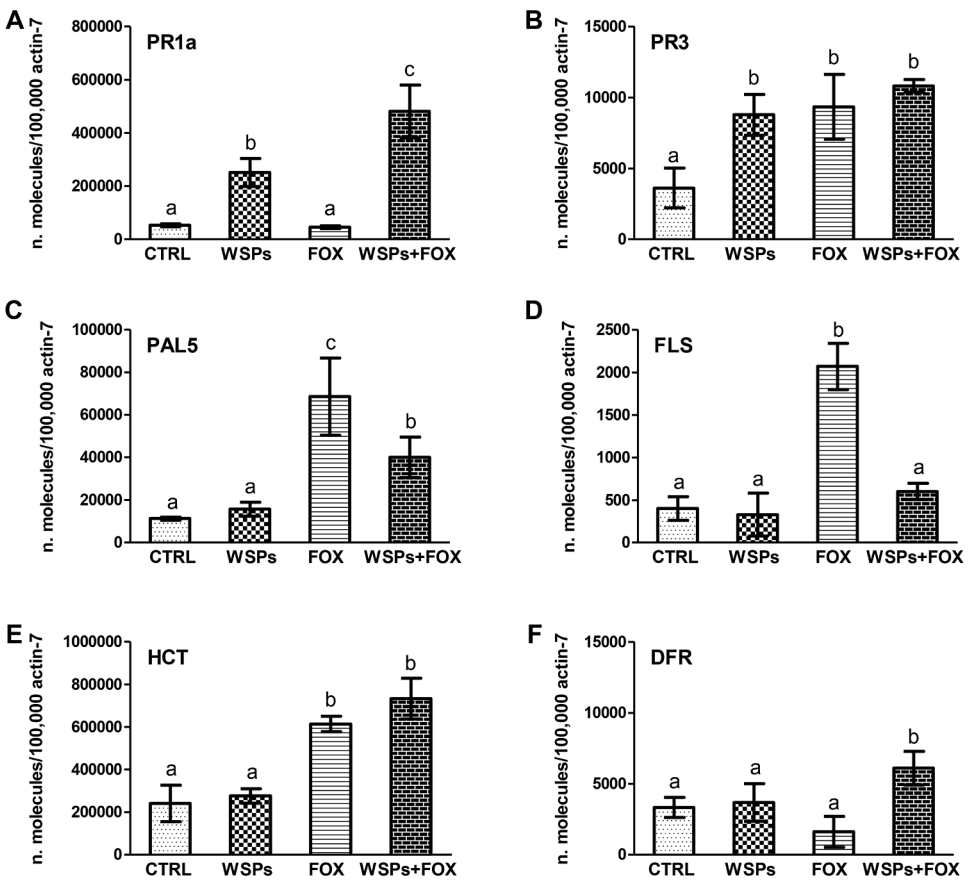

Fig. 7. Transcriptional expression levels of genes in tomato plants as a response to seed treatment with 0.6 mg mL^{-1} of *Ecklonia maxima* water-soluble polysaccharides (WSPs) in presence of *Fusarium oxysporum* f. sp. *lycopersici* (FOX) or absence. CTRL, untreated plants transplanted in pots without FOX; WSPs, plants from seed treated with WSPs transplanted in pots without FOX; FOX, untreated plants transplanted in pots with FOX; WSPs+FOX, plants from seed treated with WSPs transplanted in pots with FOX. PR1a (A) and PR3 (B), pathogenesis-related proteins; PAL5 (C), phenylalanine ammonia-lyase; FLS (D), flavonol synthase; HCT (E), hydroxycinnamoyl CoA shikimate hydroxycinnamoyl transferase; DFR (F), dihydroflavonol 4-reductase. Columns are mean values of 3 independent experiments ($n = 3$) ± SD. Different letters indicate significant differences amongst treatments and the control according to the LSD test ($p < 0.05$).

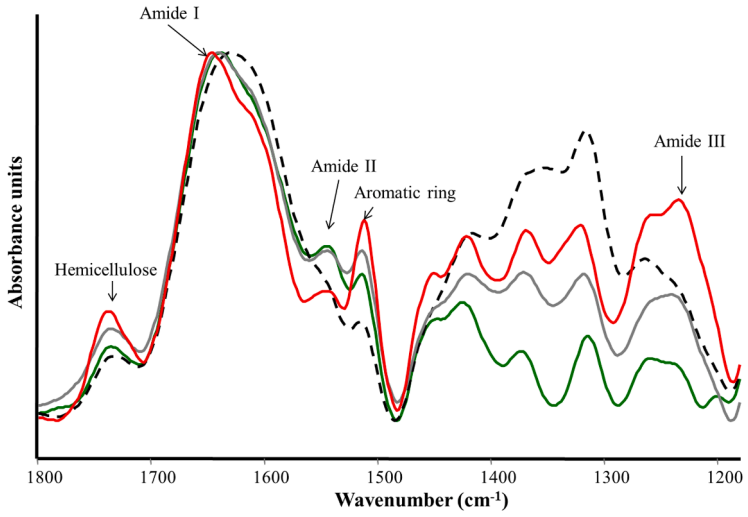

Fig. 8. ATR FT-IR root spectra. CTRL (red line), untreated plants transplanted in pots without FOX; WSPs (green line), plants from seed treated with WSPs transplanted in pots without FOX; FOX (black dashed line), untreated plants transplanted in pots with FOX; WSPs+FOX (grey line), plants from seed treated with WSPs transplanted in pots with FOX. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

they promote the synchronization of biological signals (Saberi Riseh et al., 2022) and are endowed with antioxidant power. As a result, they improve the rate of seed germination (Hu et al., 2004), stimulate plant growth and promote resistance to biotic and abiotic stresses (González et al., 2012; Saberi Riseh et al., 2022). Furthermore, the presence of sulfate groups (1245 cm^{-1}) makes alginate biologically active in protecting against oxidative stress agents, although these groups are affected by the degree of sulfation, concentration, and oxidation (Zhong et al., 2020).

Seed priming is an extensively used treatment involving not only the seed but the plant as a whole and can be termed an induced phase in which the plant reacts more quickly and effectively to stress (Lechowska et al., 2019; McDonald, 2000; Paparella et al., 2015; Parera and Cantliffe, 1994). In this view, plants exposed to primary stress trigger a series of short-term metabolic changes that provoke stress memory, resulting in more effective responses to subsequent stresses. Although

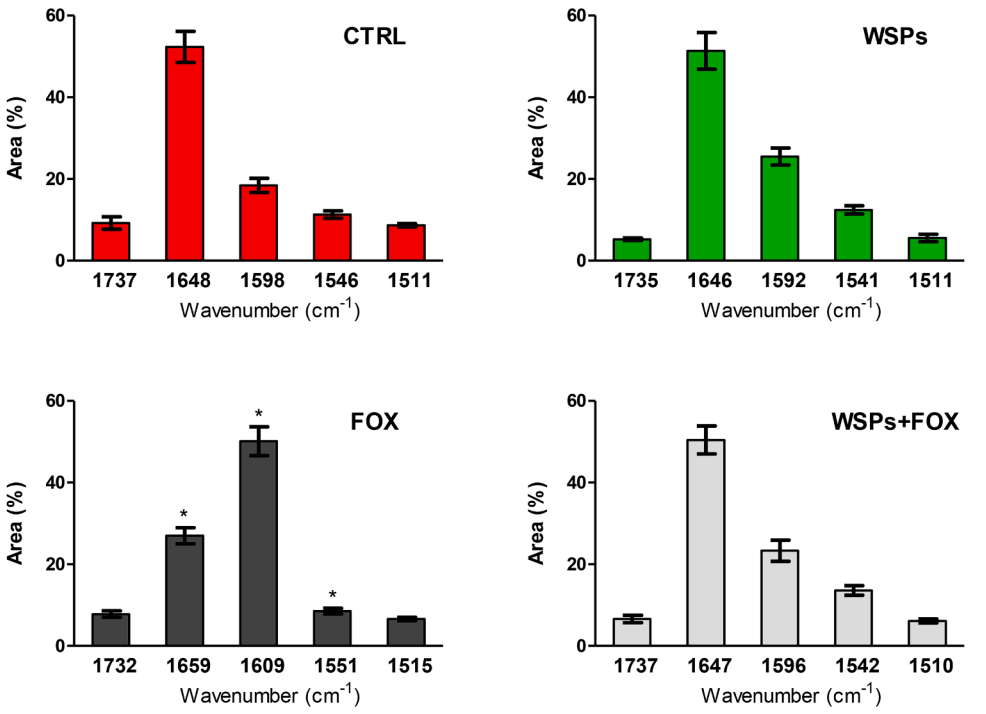

Fig. 9. Histograms of peak areas (%) were processed by using a Gaussian curve fitting in the spectral region from 1800 to 1500 cm^{-1}. CTRL, untreated plants transplanted in pots without FOX; WSPs, plants from seed treated with WSPs transplanted in pots without FOX; FOX, untreated plants transplanted in pots with FOX; WSPs+FOX, plants from seed treated with WSPs transplanted in pots with FOX. Columns are mean values of 3 independent experiments ($n = 3$) ± SD. *, significant differences between FOX and CTRL, WSPs and WSPs+FOX, *t*-test ($p < 0.05$).

the importance of seed priming has been proven for a long time, the physiological and biochemical basis of this handling has not been well elucidated, and that is the focus of this paper. Moreover, seed priming supplemented by algal WSPs as bioactive molecules has been poorly researched. *Jania adhaerens* and *Ulva* spp. WSPs primed tomato and maize seeds, respectively by increasing seedling germination and plant growth (Hamouda et al., 2022; Righini et al., 2022). In addition, WSPs from *J. adhaerens* reduced the disease severity caused by *F. oxysporum* over a period of 20 days after transplantation under greenhouse conditions (Righini et al., 2022). Important breakthroughs have been made in improving seedlings and enhancing plant growth, however, the real novelty of applying ECK WSPs on seeds lies in the boosting of the plant's defence system against *F. oxysporum* f. sp. *lycopersici*, thereby counteracting the disease event. In more detail, seed treatment with WSPs increased seedling growth parameters such as AUEC, SVI and EI. These results are supported by Mzibra et al. (2018) that applied polysaccharide-enriched extracts of the two brown algae *Fucus spiralis* and *Bifurcaria bifurcate* with different methods in agarized medium, and under greenhouse conditions by peat moss irrigation. Similarly, Hernández-Herrera et al. (2016) obtained the promotion of tomato seed germination and plant growth with polysaccharide-enriched extracts from *Padina gymnospora*, in vitro conditions. Overall, these results provide evidence that brown algal polysaccharides act as effective growth-promoting molecules of seedlings. Furthermore, on adult plants at the end of the trials, we detected persistent plant growth stimulation in morphometric parameters, height and length, following transplanting seedlings obtained from WSP-treated seeds. We highlight that this long-term effect also led to a protective boost to plants against Fusarium wilt, with a reduction in the disease index and an augmentation of plant resistance responses detected 46 days after seed treatment. Thus, the key role of brown algal polysaccharides, as drivers triggering the plant's defence system against pathogens (Aziz et al., 2004; Klarzynski et al., 2000, 2003; Paulert et al., 2010) is also corroborated by our findings.

The positive impact of seed priming with WSPs has also been proven at the level of plant primary metabolism from the point of view of protein content and enzyme activities of chitinase and β−1,3-D-glucanase in the roots of plants transplanted and grown in soil infected by *F. oxysporum* f. sp. *lycopersici*. The higher protein content may imply that the assimilation of C and N, as a result of optimized use of nutrients, is actually enhanced by WSP-seed treatment when plants were grown in infected soil. At the molecular structure level, the beneficial effect of WSP seed priming is confirmed by the high presence of amide I and amide II in proteins and of aromatic content in lignin of roots with and without Fusarium challenge. Conversely, in the roots of plants infected with Fusarium, amide I and amide II decreased, presumably by the production of peptidases by the pathogen able to degrade host proteins for nutrient acquisition or catabolic activities (Qian et al., 2022). Chitinases and β−1,3-D-glucanases are pathogenesis-related (PR) proteins involved in plant defence responses against stresses and, specifically, they are capable to degrade fungal cell walls (Morohashi and Matsushima 2000; Witmer et al., 2003). Our results showed that polysaccharides from *E. maxima* increased plant enzymatic activities such as chitinases and β−1,3-D-glucanases, particularly following pathogen infection. Similar results were obtained for the expression of the PR1a gene, involved in SAR response, and the PR3 gene, which codes for an endochitinase highly expressed in tomato roots (http://bar.utoronto.ca/eplant_tomato/). The persistent increase of PR1 and PR3 following seed priming with WSPs may enhance the ability of tomato plants to respond to and cope with Fusarium infection. Without any pathogen challenge on tomato, Righini et al. (2022) obtained values of β−1,3-D-glucanase activity as well as PR expression higher than the control in 20-day-tomato plants from seeds treated with *J. adhaerens* polysaccharides, while Rachidi et al. (2021) demonstrated an enhancement of chitinase and glucanase enzymatic activities on 30-day-transplanted plants by injection of microalgal polysaccharides on leaves.

To our best knowledge, the present study is the first to exhibit the effect of seed priming with ECK WSPs on the accumulation of secondary metabolites such as soluble phenols and flavonoids in plants affected by *F. oxysporum f.* sp. *lycopersici.* The mentioned metabolites, which are involved in defence against stresses (e.g., cell wall thickening and antimicrobial activity) (Dixon, 2001), are generally synthesized through the phenylpropanoid pathway, followed by the chlorogenic and flavonoids pathways (Aseel et al., 2019; Heldt and Piechulla, 2021). The literature only reports an increase of phenolic compounds in plants treated with algae but without a pathogen attack. For example, polysaccharides from the brown algae *Fucus spiralis* and *Bifurcaria bifurcate*

increased total polyphenol content in roots of date palm seedlings (Bouissil et al., 2020) and a commercial product based on extracts from other brown algae, *Laminaria digitata* and *Ascophyllum nodosum*, applied by irrigation increased leaf phenol content in olive trees (Graziani et al., 2022). In broccoli, a spray treatment with a commercial product containing an extract from *Ascophyllum nodosum* enhanced both phenols and flavonoids (Lola-Luz et al., 2014).

In the present study, we also report an increased expression of some key enzymes of the main phenylpropanoid pathway (PAL) and successive pathways for phenol and flavonoid biosynthesis (FLS, HCT and DFR) in infected plants and/or infected plants pretreated with WSPs, suggesting their role in the plant defence responses (Aseel et al., 2019). For some of these genes, the level of expression was augmented in infected plants that did not receive the seed priming. However, it should be considered that the determination has been carried out 30 days after pathogen infection and 46 days after the treatment with WSPs, therefore a transient induction of these enzymes may have occurred after seed priming, allowing overall an early increase of phenol and flavonoid content.

5. Conclusion

Algae products are increasingly used in crop management for their plant growth promotion effect together with their involvement in the elicitation of defence mechanisms against stresses. Algal polysaccharides are natural polymers that, due to their potential properties, have been considered a sustainable choice for the induction of plant resistance against pathogens. These compounds applied by seed priming showed a role by stimulating the plant's immune system as an effective way to promote disease resistance. Seed treatment with polysaccharides leads to the activation of the main primary and secondary metabolic pathways involved in plant protection against Fusarium wilt. Thus, these compounds are promising candidates to be studied for further application in field-scale experiments.

Credit author statement

Conceptualization, H.R., R.R. and O.F.; methodology, V.Z. and I.B., formal analysis, H.R. and R.R.; investigation, H.R., R.R., V.Z., S.C. and A. M.Q.; resources, R.R.; data curation, H.R., R.R., O.F., F.F. and S.C.; writing—original draft preparation, H.R., R.R., S.C., O.F. and A.M.Q.; writing—review and editing, H.R., R.R., O.F. and F.F..; supervision, F.F.; funding acquisition, R.R. and A.M.Q. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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