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Sulfated polysaccharide from *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva primes defense responses against anthracnose disease of *Capsicum annuum* Linn.

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ABSTRACT

Organic approaches for controlling plant diseases with biopolymers have garnered increasing attention in recent years because of their eco-friendliness. In the present study, the biopolymer carrageenan was extracted from the red seaweed *Kappaphycus alvarezii* and used as a potent elicitor of plant resistance against anthracnose disease in chili plants. Biophysical analysis by FT-IR confirmed that the isolated polysaccharide is κ-carrageenan. Foliar applications of κ-carrageenan induced the defense-related antioxidant enzyme peroxidase in chili leaves. Furthermore, two-dimensional gel electrophoresis (2D-PAGE) and liquid chromatography mass spectrometry (LC-MS/MS) analyses of chili leaves pretreated with κ-carrageenan showed up-regulation of 13 known proteins and induction of 4 new proteins, including dehydroascorbate reductase I, dehydroascorbate reductase II, NAD(P)H Quinone oxidoreductase, and Eukaryotic Translation Initiation Factor 5A. An analysis of gene expression using quantitative real time polymerase chain reaction (qRT-PCR) also confirmed the expression of the pathogenesis-related proteins *PR1*, *PR5*, *PDF1.2*, and *NPR1* following treatment with carrageenan. *In vitro* experiments using κ-carrageenan support its fungistatic potential through alteration of the membrane permeability of *Colletotrichum gloeosporioides* (Penz.) Sacc. Pathogenesis tests yielded a lower disease score for chili plants pretreated with κ-carrageenan. Hence, κ-carrageenan could be a potent natural fungicide and elicitor of disease resistance in plants.

1. Introduction

Seaweeds are considered as an important source of unique polysaccharides such as agar-agar, alginates, and carrageenan [1]. Among these products, carrageenan has been shown to trigger plant defense mechanisms against viral plant pathogens [2]. Carrageenan is a natural biopolymer containing a sulfate group (anion) and is obtained from different species of red seaweed belonging to the phylum Rhodophyta. Among the different species of red seaweed, *Kappaphycus alvarezii* is known to be a good source of carrageenan. In addition to carrageenan *K. alvarezii* also contains the two hemicelluloses, β-D-glucan sulfate and β-3-glucomannan, which control the gelation of κ-carrageenan by cross-linking [3]. The various methods for extracting carrageenan from seaweed have been well-documented, and studies showed that the extraction temperature and extraction time are critical factors influencing the molecular weight and gelation of carrageenan [4,5]. However, the quality of carrageenan extracted also depends on the drying temperature of the sea weed [6]. Carrageenan possesses antioxidant activity that can scavenge hydrogen peroxide. Carrageenan has also been implicated in promoting plant growth and inducing plant defenses [7,8]. Carrageenans are composed of linear units of D-galactose residues linked with alternating β-(1,3) and β-(1,4) linkages that are substituted by one (κ-carrageenan), two (ι-carrageenan), or three (λ-carrageenan) ester-sulfonic groups per digalactose unit [9]. A previous report indicates that the number of sulfate groups bound to carrageenan determines the strength of the induced plant defense response despite the is not clearly understood [10]. However, the antifungal activity and influence of κ–carrageenan on plant immunity induction against phytopathogens, especially fungal pathogens are yet to be explored.

Chili is susceptible to many fungal diseases that can reduce its commercial value. Among these diseases, anthracnose caused by *Colletotrichum gloeosporioides*, results in drastic damage to chili plants [11,12]. Worldwide, attempts are being made to control pathogens and plant diseases by deploying a variety of systemic fungicides. Spraying of fungicide carbendazim onto chili plants is the effective strategy to control anthracnose disease. However, the chemicals used have such limitations as environmental toxicity, effects from fungicide residues, pathogen resurgence, and development of resistance by pathogens. On

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the other hand, algal based polysaccharide elicitors are biopolymers and eco-friendly biostimulants which are absent in land plants [13]. Considering all of the drawbacks in using synthetic chemicals to eradicate pathogen *C. gloeosporioides*, the focus of the present study was to investigate the potency of the biopolymer κ-carrageenan, isolated from the marine red sea weed *Kappaphycus alvarezii*, on enhancing the induction of plant defense arsenal against anthracnose disease of chili.

2. Materials and methods

2.1. Biological materials

Chili seeds (*Capsicum annuum*, cv CO1) were procured from Tamil Nadu Agricultural University (TNAU) in Coimbatore, Tamil Nadu, India. The chili plants were grown in pots containing commercial manure mixed with vermiculite under a 16/8 h, day/night light cycle at 25 °C. The plants were grown under similar conditions for 40 days and used for the elicitor treatment and pathogen infection. The red seaweed *K. alvarezii* was cultivated with the floating raft method for 45 days. Next, 5 kg was collected when it was a reddish brown color and at the tetrasporic stage during November 2015 at Pudukkottai (Kottaipattinam, 9°56′24.87″N 79°09′ 55.38″E) on the East Coast of Tamil Nadu, India. *K. alvarezii* originated in the Philippines and is cultivated commercially.

2.2. Preparation and characterization of carrageenan

Kappaphycus alvarezii was brought to the laboratory. Salt, sand, and foreign matter were removed by washing with tap water. Next, 100 g of biomass was then air dried at 50 °C until it reached a constant weight, and 1 g each of both dry and wet material was chopped separately, and heated with 100 mL of distilled water for 1 h followed by constant stirring at room temperature for 12 h. The resulting residues were referred to as dry aqueous warm and wet aqueous warm, respectively. Similarly, carrageenan was extracted from both dry and wet chopped materials by cold water with constant stirring at room temperature for 12 h. The resulting residues were referred to as dry aqueous cold and wet aqueous cold, respectively. Carrageenan was separated from the above residues using filter cloth and was filtered through Whatman filter paper No. 2. The filtrate was treated with various solvents, including ethanol, methanol and propanol (1:3 (V/V)), and stored at 4–6 °C for 4 days to promote the precipitation of polysaccharides. After polysaccharide precipitation, samples were centrifuged at 4000 ×*g* for 30 min, the precipitates were collected and dissolved in a minimum quantity of water, and high-molecular-weight crude polysaccharide preparations were obtained by lyophilization and stored at −4 °C. The infrared (IR) spectra of the total extracted carrageenan from *K. alvarezii*, was determined using FT-IR (Fourier Transform Infrared Spectroscopy). One gram of the lyophilized extract was ground with potassium bromide (KBr) powder, dispersed in a KBr disk, and pressed into 1-mm pellets for FT-IR measurements (wavenumber range of 500 and 4000 cm^{-1}) using 16 scans [14]. For comparison, an authentic carrageenan standard (Aquagiri, Chennai, India) was used.

2.3. Elicitor treatment and pathogen inoculation

Carrageenan extracted using dried material with warm water (aqueous warm) yielded quality κ-carrageenan (based on FT-IR analysis) and also yielded maximum quantity, hence was used as an elicitor throughout this study. The healthy leaves of 40-day-old plants from the greenhouse were used for elicitor treatment and pathogen infection. Elicitor (κ-carrageenan (0.3%)) dissolved in 0.1% Tween 20 was applied with a hand sprayer until it ran off both upper and lower leaves. A fungicide (Carbendazim (0.5%)) was used as a positive control and was also applied by spraying onto the leaves. Control plants were sprayed with 0.1% Tween 20. After 24 h of elicitor treatment, the leaves were inoculated with the pathogen *Colletotrichum gloeosporioides* (2×10^6 conidia·mL^{-1}). Leaves were harvested 48 h after each treatment and collected in liquid nitrogen.

Table 1
Primers used for qRT-PCR analysis. Primers are shown in the 5′-3′ orientation. (F): Forward; (R): reverse primer.

Gene	Primer name	Primer sequence (5′-3′)
PR1	PR1-F	CACAATGCAGCTCGTAGACA
	PR1-R	GCTAGGTTTTCCCCGTAAGG
PR5	PR-F	CTCATGCTGCCACTTTTGACA
	PR-R	CGGCCACTACCATCAAAGTT
NPR1	NPR1-F	AGGAGCACTTGAATCGGCT
	NPR1-R	CAGACAAGTCATCAGCATCC
Def 1.2	Def-F	CAGGTAATATGGGCAACTGA
	Def-R	CACAATTGCTGATCCCAGTC
EF	EF-F	GAAGCTCTCCAGGAGGCACT
	EF-R	CCAGGATGGTTCATGATGATG

2.4. Effect of κ-carrageenan and fungicide on the mycelial growth of Colletotrichum gloeosporioides

A potato dextrose agar (PDA (Potato - 200; Dextrose - 20; Agar - 20 g/L)) medium amended with different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5%) of κ-carrageenan or the fungicide carbendazim (0.1, 0.2, 0.3, 0.4 and 0.5%) was inoculated with 6-mm-diameter mycelia agar discs from 6-day-old cultures of the pathogen *C. gloeosporioides* that had been grown on PDA medium. The 6-mm-diameter mycelial agar discs were taken with a cork borer. The plates were examined for fungal growth after 120 h. To analyze the effect of κ-carrageenan on mycelial dry weight, potato dextrose broth (PDB (Potato - 200; Dextrose - 20 g/L)) was amended with different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5%) of κ-carrageenan or fungicide (0.1, 0.2, 0.3, 0.4 and 0.5%) and filter sterilized. The PDB was inoculated with 6-mm diameter mycelia agar discs from 6-day-old cultures of the pathogen *C. gloeosporioides*. The flasks were maintained at 25 °C with gentle shaking and after 96 h the mycelial growth was assessed by measuring the dry weight [15].

2.5. Plasma membrane integrity assay

To analyze the potency of κ-carrageenan in altering the plasma membrane permeability of the test pathogen, mycelia of *C. gloeosporioides* were incubated in the presence of the elicitor (κ-carrageenan) at a concentration of 0.3% or the fungicide carbendazim (0.5%). After 15, 30, 60, 120, and 240 min of incubation, mycelia were harvested by centrifugation at 10,000*g* for 15 min, washed in 50 mM phosphate buffer with a pH of 7.0 and stained with 10 μg/mL propidium iodide for 15 min [16]. Mycelia were observed under a fluorescence microscope (with excitation and emission wavelengths of 493 and 636 nm) (Leica Microscope DM 2500, Germany).

2.6. Preparation of enzyme extracts

Frozen leaf samples (500 mg) were homogenized in 1.5 mL of 100 mM potassium phosphate buffer with a pH of 7.5 and containing 10 μL of 0.1 M Na-EDTA (sodium-ethylenediamine tetraacetic acid) and 1% PVPP (polyvinylpolypyrrolidone) at 4 °C. After filtration by four layers of cheese cloth and centrifugation at 9000 ×*g* for 10 min at 4 °C, the cell free supernatant was collected and protein content was quantified using the dye binding method [17] with BSA (Bovine Serum Albumin) (fraction V) as a standard.

2.7. Guaiacol peroxidase (GPX) activity

Treated leaves were harvested after 48 h from controls (without any treatment), pathogen-infected plants, elicitor-treated plants and plants that treated with both the elicitor and pathogen *C. gloeosporioides*