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Ulvans induce resistance against plant pathogenic fungi independently of their sulfation degree

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ABSTRACT

The present work aimed to evaluate the defense responses induced by chemically sulfated ulvans in *Arabidopsis thaliana* plants against the phytopathogenic fungi *Alternaria brassicicola* and *Colletotrichum higginsianum*. Derivatives with growing sulfate content (from 20.9 to 36.6%) were prepared with SO_3-pyridine complex in formamide. NMR and FTIR spectroscopic analyses confirmed the increase of sulfate groups after the chemical sulfation process. The native sulfated polysaccharide (18.9% of sulfate) and its chemically sulfated derivatives similarly reduced the severity of both pathogenic fungi infections. Collectively, our results suggest that ulvans induce resistance against both fungal pathogens independently of its sulfation degree.

1. Introduction

Seaweeds represent a rich but still underexploited source of bioactive compounds. Among them, carbohydrates are probably the most abundant organic compounds in the oceans with a great molecular diversity (Alves, Sousa, & Reis, 2013; Khan et al., 2009; Sharma, Fleming, Selby, Rao, & Martin, 2014; Stadnik & de Freitas, 2014). Ulvans are water-soluble sulfated heteropolysaccharides extracted from the cell walls of the green macroalgae *Ulva* spp. They have been one of the most studied algal carbohydrates with potential application in several areas, including agriculture (Alves et al., 2013; Stadnik & de Freitas, 2014).

Ulvans are composed by rhamnose (16–45%), xylose (2–12%), glucose (0.5–6.5%), uronic acid (6.5–19%) and sulfate (16–23%). These sulfated polysaccharides are structurally constituted by two main repeating disaccharides, the ulvanobiuronic acid type A [→4)-β-D-GlcA-(1 → 4)-α-L-Rha 3S-(1 →] and type B [→4)-α-L-IdoA-(1 → 4)-α-L-Rha 3S-(1 →] (Alves et al., 2013; Lahaye & Robic, 2007; Stadnik & de Freitas, 2014). Ulvans are known to exhibit different biological activities in the pharmaceutical and biomedical context (Alves et al., 2013). In crop sciences, studies have revealed that they can be applied to stimulate growth and defense of plants (Alves et al., 2013; Stadnik & de Freitas, 2014).

The current worldwide concern on toxic residues in the environment and public health has fostered the development of clean technologies. In this scenario, the induction of resistance using algal polysaccharides arises as an eco-friendly strategy to control plant diseases. Studies have shown that ulvans are able to protect bean plants against a broad range of fungal diseases such as rust (*Uromyces appendiculatus*) (Delgado, de Freitas, & Stadnik, 2013), anthracnose (*Colletotrichum lindemuthianum*) (de Freitas & Stadnik, 2012) and powdery mildew (*Erysiphe poligoni*) (Jaulneau et al., 2011). Moreover, it has shown promising results against powdery mildew (*Blumeria graminis*) on wheat and barley (Paulert, Ebbinghaus, Urlass, & Moerschbacher, 2010) and *Glomerella* leaf spot (*C. gloeosporioides*) on apple (Araújo & Stadnik, 2013).

The biological activity of sulfated polysaccharides from marine algae has been frequently associated with several structural factors including molecular weight, chemical composition, chain conformation, and content and positioning of sulfate groups (Alves et al., 2013; Cassolato et al., 2008; Duarte et al., 2004; Faria-Tischer et al., 2006). As consequence, knowing how such factors can affect the activity of polysaccharides has provided opportunities for obtaining molecules with new, or even, enhanced elicitor activity (Qi,

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Zhao, Zhang, Li, & Zhao, 2005; Qi et al., 2012). For instance, the pre-treatment with a highly sulfated red seaweed polysaccharide λ-carrageenan (35%) induces resistance to *Sclerotinia sclerotiorum* resulting in less foliar damage while the ι-carrageenan, with a lower degree of sulfation (30%), increases disease severity (Sangha, Ravichandran, Prithiviraj, Critchley, & Prithiviraj, 2010). Laminarin, an unsulfated β-1,3 linear glucan induces the expression of ethylene-dependent proteins while its sulfated derivative can activate the expression of both ethylene- and salicylic acid-dependent proteins (Ménard et al., 2004). Accordingly, sulfated laminarin is more efficient to reduce tobacco mosaic virus-symptoms in tobacco plants than the unsulfated natural polysaccharide (Ménard, Ruffray, Fritig, Yvin, & Kauffmann, 2005).

Changes in biological activity due to chemical modifications have also been reported for the algal polysaccharide ulvan. In comparison with native ulvan (19.5% of sulfate), chemically sulfated ulvans (30.8 and 32.8% of sulfate) exhibit enhanced scavenging activity on hydroxyl radical, reducing power and chelating ability on ferrous ion (Qi, Zhang, Zhao, Chen, & Niu, 2005). The degree of sulfation of ulvans seems to affect its elicitor activity on plants as the complete desulfation suppresses its ability to enhance the activity of PAL in tomato seedlings infected by *Fusarium oxysporum* f.sp. *lycopersici* (El Modafar et al., 2012).

Although some information relating the effect of chemical modification of ulvans on biological activity is already available for animals (Qi, Zhang, et al., 2005, Qi, Zhao, et al., 2005; Qi et al., 2012), there is a lack of studies regarding plant-pathogen models. Therefore, this work aimed to evaluate the efficiency of chemically sulfated ulvans to control *Alternaria brassicicola* and *Colletotrichum higginsianum* in *Arabidopsis thaliana*.

2. Material and methods

2.1. Materials

Ulva fasciata Delile samples were harvested in December 2011 at Armação beach (27.4454°S; 48.2956°W) in Florianópolis-SC, Brazil. All the other chemicals and reagents used were of analytical grade.

2.2. Isolation of ulvan

Ulvan was obtained as previously described by Paulert et al. (2009). Briefly, the ground dried alga (100 g) was autoclaved for 2 h at 110 °C in distilled water (1 L). The resulting aqueous solution was filtered and the polysaccharide precipitated with ethanol (3 v) for 48 h at −20 °C. The precipitated was filtered under vacuum (Whatman #40 filter paper), washed three times with ethanol and resuspended in distilled water. The remaining ethanol adsorbed to the precipitated polysaccharide was removed during dialysis against tap water for 48 h and against distilled water for another 48 h in a 3600 Da Mw cutoff dialysis membrane. The resulting products were concentrated under vacuum, lyophilized, kept at −20 °C until use and named Native Ulvan (NU).

2.3. Sulfation of ulvan

Ulvan derivatives with five different degrees of sulfation were produced as described by Ménard et al. (2004), with some modifications. Dry native ulvan NU (2 g; 18.9% of sulfate) was added to 80 mL of formamide and the mixture was stirred at 60 °C for 30 min in order to disperse it into solvent. Sulfation was performed by continuous addition of SO_3-pyridine complex (5.9 g) for 2 h at 60 °C. Stirring was continued for another 2 h. Derivatives with growing sulfate content were obtained by increasing the amount of SO_3-pyridine complex added during the reaction. After cooling to room temperature, the mixture was neutralized with a 2 M NaOH solution and precipitated with ethanol (3 v) for 24 h at −20 °C. The precipitated compound was filtered under vacuum (Whatman #40 filter paper), washed three times with ethanol and resuspended in distilled water. The remaining ethanol adsorbed to the precipitated was removed during dialysis against tap water for 48 h and against distilled water for another 48 h in a 3600 Da Mw cutoff dialysis membrane. The retained material was concentrated under vacuum, lyophilized and kept at −20 °C until use.

2.4. Analytical methods

Sulfate was estimated according to the turbidimetric method of Dodgson and Price (1962), using potassium sulfate as standard. Uronic acid was determined according to Filisetti-Cozzi and Carpita (1991), using glucuronic acid as standard. For monosaccharide composition, the polysaccharide samples were hydrolyzed using M TFA at 100 °C for 4 h. Hydrolysis products were reduced with $NaBH_4$, and after acetylation (1:1 acetic anhydride-pyridine for 12 h at 25 °C), the resulting alditol acetates derivatives were analyzed by gas chromatography–mass spectrometry (GC–MS). GC–MS analyses of the alditol acetates derivatives were performed with a Varian 3800 chromatograph, equipped with a fused silica capillary column (30 m × 0.25 mm) coated with DB-225MS (Durabond), and a Varian Saturn 2000R ITD spectrometer and were identified by their typical electron-impact fragmentation profiles and GC retention times (Jansson, Kenne, Liedgren, Lindberg, & Lonngren, 1976). The chromatograph was programmed to run at 50 °C for 1 min, then 50–215 °C at 40 °C min^{-1}, using helium as carrier gas (1 mL min^{-1}).

2.5. High-pressure size-exclusion chromatography (HPSEC) analysis

HPSEC was carried out with a 1 mg mL^{-1} solution of the polysaccharide, using a multidetection equipment with a Waters 2410 differential refractometer (RI) and a Wyatt Technology Dawn-F multi-angle laser light scattering (MALLS) detector adapted online. Four Waters Ultrahydrogel columns (2000, 500, 250 and 120) were connected in series and coupled to the multi-detection equipment. The eluent was a solution of 0.1 M $NaNO_2$ containing NaN_3 (0.2 g L^{-1}). All experiments were carried out at 25 °C.

2.6. Nuclear magnetic resonance (NMR)

NMR analyses were performed on a Bruker Advance DRX400 NMR spectrometer equipped with a 5-mm multinuclear inverse detection probe. The base frequency was 100.63 and 400.13 MHz for ^{13}C and ^{1}H nuclei, respectively. Analyses were recorded at 70 °C. For ^{13}C NMR spectra, the samples were dissolved in D_2O (40 mg mL^{-1}). For ^{1}H and 2D NMR experiments, samples were deuterium exchanged with D_2O 99.9% (3×) and then dissolved in D_2O (20–25 mg mL^{-1}). ^{13}C and ^{1}H NMR acquisition parameters were previously reported (Ascêncio, Orsato, França, Duarte, & Noseda, 2006). Chemical shifts are expressed relative to an internal acetone standard at 31.45 and 2.225 ppm for ^{13}C and ^{1}H NMR spectra, respectively.

2.7. Fourier transform infrared spectroscopy (FTIR)

FTIR analyses were performed on a Bruker Optics, Alpha model spectrometer equipped with an attenuated total reflectance (ATR) probe. Samples spectra were collected between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} and a scan rate of 16. The software used was Opus 6.5.