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Direct effects of ulvan and a flour produced from the green alga *Ulva fasciata* Delile on the fungus *Stemphylium solani* Weber

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ABSTRACT

Alternative methods of fungal biocontrol are required, and this paper looks at natural sources of products which can be produced and used without significant risk to human health and the environment, thereby reducing the need for toxic pesticides. Even after the significant expenditures on agrochemical control agents, a significant fraction of agriculture production is regularly lost due to fungal diseases. Species of *Ulva* are known to produce bioactive substances that can induce plant resistance to diseases. Before committing to the extensive use of extracts from such algal sources as elicitors in agriculture, it is essential to confirm the specificity of their antifungal activities. This study, examined whether extracts and dried, milled flour of *Ulva fasciata* demonstrated antifungal activity against the commercially important pathogen, *Stemphylium solani*. Samples of *S. solani* were obtained from infected leaves of the tomato *Solanum lycopersicum* and were cultured on Potato-Dextrose-Agar medium, with three added concentrations of ulvan and flour obtained from *U. fasciata* (0.1; 0.5 and 1.0 $g{\cdot}L^{-1}$), in a BDO chamber, at 25 °C, for 10 days in the dark. The diameter of the mycelia was used as the parameter for fungal growth. Neither the flour nor ulvan from *U. fasciata* showed any direct anti-fungal activity, but the presence of compounds produced by *U. fasciata* showing antagonist physiological effects against *S. solani* should be investigated.

1. Introduction

Searches for alternative methods of fungal biocontrol in tomato cultivars have been stimulated by the need to reduce the use of agricultural chemical pesticides and to maximize food production thereby reducing risks to human health and the environment [1,2,3]. Many types of seaweed and their extracts have been used for such purposes in organic agriculture [4,5,6], and some of them are known to produce bioactive substances which are capable of inducing plant resistance [4,5,6,7,8,9] to foliar necrotic lesions caused by the fungus *Alternaria solani* Sorauer in the tomato *Solanum lycopersicum* L. [2].

Fungi are the main cause of foliar diseases, and 15% of the production costs of tomatoes involve investing in the serial applications of synthetic fungicides [10]. Leaf blight is a high-severity disease, with serious negative commercial implications which is caused by the fungus *Stemphylium solani Weber*; this fungus occurs worldwide in warm temperatures and high humidity [3]. It has a broad range of hosts and is known to contaminate > 20 cultivars, including tomatoes, garlic, onions, tobacco, maize, potatoes, spinach, cotton, peas, wheat, rice, and pepper. The most common symptom indicating an infection is the appearance of small lesions on leaves which soon increase in size to almost entirely cover them; this necrotic process is caused by one or more phytotoxic compounds [3,10,11]. Epidemic infections and reductions in tomato production have been reported recently in Brazil [3]. Interestingly, no fruit crops have been observed to be infected by this fungus [12].

A wide variety of macroalgal extracts have been used as biostimulants in conventional agriculture [4,5,9,13] and as such, alternatives to conventional, synthetic agrochemicals which are reported to be able to ameliorate selective biotic and abiotic stresses in crop plants [6], and additional studies for the use of many different types of seaweeds and their varied extracts in organic agriculture should be encouraged.

Ulva species are known to elicit plant defense mechanisms and reduce the severity of fungal attacks, and green seaweeds have been indicated as potential "eco-friendly" solutions to fungal infections that would otherwise require treatments with aggressive chemical applications [7,14,15,16,17,18,19]. One of the obstacles to the wide-scale use of bioactive compounds derived from macroalgae, however, is

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sustainable access to industrial-scale biomasses [20]. *Ulva* is a fast-growing, cosmopolitan genus of green alga, and it is considered suitable for mass production through aquaculture [21], and blooms ("green tides") have been reported around the world [22] that might also serve as sources of raw material.

Ulva spp. are known to produces a suite of polysaccharides called ulvans that have been shown to trigger elicitor responses in treated plants by elicitation priming of their defense mechanisms [2,7,14,23,24,25]. Ulvans are sulphated polysaccharides, rich in glucuronic acid, rhamnose xylose and other sugars. In general, ulvan yields range from 15 to 40% of the dry algal mass [15,28]. Additionally, *Ulva* spp. are rich in proteins and minerals [18,23,26,27].

Bioactive compounds from different seaweeds vary in composition in accordance with the taxon, and chemical and structural diversities [20]. *Ulva fasciata* Delile represents a potential source of bioactive compounds [29], and extracts of *Ulva lactuca* Linnaeus are known to promote the development of *S. lycopersicum* and stimulate seed germination and seedling growth [2]. Additionally, activity as a defense elicitor against plant pathogens has been observed [6,15,16]. Despite the defense elicitation activity of ulvans in tomato cultivars, direct anti-fungal activity has not been reported [2,7,14,16], and their protective effects may reflect the priming or activation of onset-related defense enzymes and metabolites, instead of any direct anti-fungal activity [30]. On the other hand, as the growth of some fungi can be stimulated by sugars (polysaccharides) [31], ulvans of themselves could act as a catalyst for un-wanted and possibly detrimental fungal growth in plants instead acting to reduce potential fungal-related damage. A case in point is that red and green algal extracts have been observed to enhance the *in vitro* growth of arbuscular symbiotic fungi which have beneficial effects on plant growth [32]. Hence it is incumbent that ulvans and *Ulva*-derived flour is tested to determine suitability for large-scale application.

Little is currently known about the effects of crude seaweed flour on fungal growth. Similarly, extraction costs could be avoided if a crude flour made from dried and milled biomass acted as efficiently as the purified ulvan in agricultural situations. This study is an initial exploratory approach to investigate the effectiveness of the direct action of *U. fasciata* products (ulvan and flour) on the growth of *S. solani* and to test the hypothesis that the fungus was directly inhibited in the presence of algal flour or ulvan.

2. Materials and methods

2.1. Sample collection, algal identification and preparation of ulvan and U. fasciata flour

Samples were collected at Arpoador Beach, Rio de Janeiro State, Brazil (22°59′23.91″S × 43° 11′29.69″W). According to the main criteria used to identify species of *Ulva* (Koeman 1985), identification was based on the macroscopic morphology of the blade its size, form, number of pyrenoids, arrangement and size of the cells in surface view, and measurement of the thallus-thickness and cells in basal and apical cross sections, and also the cell height-to-width ratio in both regions of the thalli. Measurements were made on 20 specimens. Voucher material was registered at the Herbarium of the Botanical Garden of Rio de Janeiro (RB 753.462).

The samples were washed in seawater and dried (60 ± 2 °C) for 48 h to a constant dry mass. The thalli were then crushed in a ball mill (SOLAB-SL38) for three minutes to obtain the crude seaweed flour. The extraction procedure for ulvan was adapted from the protocol of Paulert et al. [15]. Dried alga flour was rehydrated in the ratio of 10 g of dry seaweed to 100 mL of distilled water and autoclaved for 40 min at 120 °C (Bio Eng, autoclave A75, 4900w). The aqueous slurry was then centrifuged (3500 g) for 5 min (Eppendorf, centrifuge 5804 R); the polysaccharides liberated in to the supernatant were precipitated with three volumes of ethanol (98 °GL) and subsequently held at − 20 °C for 48 h before re-centrifugation for 5 min. The pelleted material (ulvan) was dried (60 ± 2 °C) to constant mass.

2.2. Isolation of S. solani

Samples of *S. solani* were obtained from infected leaves of *S. lycopersicum* to be cultured on Potato-Dextrose-Agar medium (PDA). To that end, tomato leaves were washed with a 0.2% Tween 20 solution, followed with 1% NaClO for 2 min, rinsed three times with sterile distilled water and surface dried on filter paper for 5 min. Small samples were cut from the peripheral regions of the lesions on the leaves (including part of the healthy and chlorotic region) and sown onto Petri dishes containing PDA. Upon observing mycelial development, samples were scraped in to Petri dishes containing PDA and cultivated in a BOD incubator at 25 °C with a 12 h photoperiod. The identification of *S. solani* was based on the predominant etiological agents in the collection area, the symptoms of the infected tomato leaves, the characteristics of isolates cultivated in V8 medium and evaluations of the characteristics of the conidiophores and conidia according to Ellis [33].

2.3. Culture conditions used for S. solani

In order to ensure optimum *S. solani* culture conditions in the experiment and to avoid external interferences in the results, the ideal culture temperature of the strain was determined. Therefore, five temperature regimes (20.0, 22.5, 25.5, 27.5, 30.0 °C) were tested to determine the ideal conditions for cultivating *S. solani*, using 10 replicates of each treatment. The samples were maintained for 10 days in the BOD under a 12 h photoperiod. The growth of the mycelia was measured every two days.

2.4. Testing effects of Ulva fasciata ulvan and flour against S. solani

Three concentrations of ulvan and *U. fasciata* flour (0.1; 0.5 and 1.0 $g \cdot L^{-1}$) were tested. *S. solani* samples were cultivated in the following treatments: Control; *Ulva* flour (0.1, 0.5 and 1.0 g seaweed $flour \cdot L^{-1}$); and ulvan (0.1, 0.5 and 1.0 g $ulvan \cdot L^{-1}$) in a BDO chamber at 25 °C, for a period of 10 d. Cultivation was made in the dark so as to stimulate fungal growth following the protocols of Miranda et al. [34]; Shahbazi et al. [35] and Delgado et al. [36]. The concentrations of ulvan and *U. fasciata* flour used were within the concentration ranges employed in the experiments of Araújo et al. [14]; Paulert et al. [15] and Hernández-Herrera et al. [2]. Five replicates were used. The diameters of the circular colonies were measured using a digital pachymeter (Mitutoyo Sul Americana Ltda.).

2.5. Data analysis

The assumptions of normality (Shapiro-Wilk test) and homogeneity (Cochran test) of the data were verified. Differences between the temperature treatments for *S. solani* cultivation and among the *U. fasciata* treatments were tested using one-way ANOVA. Comparisons of means were performed using the Fisher LSD test.

The data were presented as mean ± standard deviation values and the confidence interval chosen for significance tests was 95% ($p < 0.05$), using *Statistica* software (Stat Soft, Version 6.0).

3. Results and discussion

The identity of the alga was confirmed as *U. fasciata* as its thallus was light to dark-green, foliaceous and ribbon shaped, affixed by a basal disc. The thallus was 11.88 ± 2.64 cm long and 2.66 ± 0.54 cm wide. In superficial view, cells were irregular, polygonal, containing 1–4 pyrenoids and in the basal region measured 15.71 ± 0.79 μm × 10.57 ± 0.49 μm and in apical region with 14.86 ± 2.29 μm × 9.71 ± 1.28 μm. In cross section in the basal

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