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Antimicrobial Activity of Extracts from *In Vitro* Mycelia Culture-Derived Sclerotia of *Sclerotium rolfsii*

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Abstract

Sclerotium rolfsii is a soil-borne fungal pathogen that causes southern blight disease on a wide range of agricultural and horticultural crops. During colonization of host tissue, it produces a considerable mass of vigorous mycelia on the plant's surface leading to tissue decay and subsequent production of sclerotia. *S. rolfsii* is also known to produce sclerotial exudates which are thought to help the fungus survive. Analyzing the sclerotial extract (SCE) for anti-microbial activity potentially, may lead to the development of a biopesticide for disease control. To achieve this, we evaluated five different fungal culture media for sclerotia production in the laboratory and assayed for antifungal and antibacterial activities of the sclerotial extract on various plant pathogens. Sclerotia production (quantity and weight) was significantly greater in oatmeal agar (OMA) than in potato dextrose agar (PDA), V8 juice, cornmeal agar (CMA) and 2% water agar (WA). The mean diameter of sclerotia produced in PDA and OMA was similar but significantly greater than that of sclerotia produced in other media. Antimicrobial assays of the water-soluble sclerotial extracts showed that they reduced the growth of *Leptosphaeria maculans* and *Ralstonia solanacearum*, significantly inhibited the growth of *Xanthomonas campestris* pv *vesicatoria* and completely inhibited the growth of *Erwinia amylovora*in the laboratory. The minimum inhibitory concentrations (MIC) of the extracts were 10% and 25% for *E. amylovora* and *X. campestris* pv *vesicatoria* respectively. These results indicate that oatmeal agar is suitable for sclerotia production in the laboratory and that *S. rolfsii* SCE has considerable antimicrobial activity against some plant pathogenic fungi and bacteria.

Keywords: Antimicrobial activity, biopesticide, culture media, sclerotia, *Sclerotium rolfsii*

1. Introduction

Sclerotium rolfsii is a basidiomycetous soil borne plant pathogenic fungus that is known for causing southern blight disease on a wide range of crop and ornamental plants. This omnivorous species thrives under moist conditions and high temperatures, but has the ability to survive in cold conditions due to the hard shelled sclerotia produced. Sclerotia are reproductive fruiting structures produced by certain fungi as a mechanism for survival and propagation of the species. *S. rolfsii* usually attacks beneath the soil surface and the damage isn't visible above ground until the pathogen has spread and wiltinghas begun. Robust mycelia and small sclerotia can then be seen around the stem of the plant close to the soil surface. *S. rolfsii* can be managed by application of fungicides, plowing crop debris deep, crop rotation with grasses such as corn, soil solarization and use of antagonistic microorganisms (Rakh, 2011). *S. rolfsii* has been beneficially used for the production of the exopolysaccharide (EPS) scleroglucan which is a water soluble homopolymer with many industrial applications including food, cosmetics and medicine (Schmid et al., 2010). Christias (1980) observed that exudates in the form of small droplets appear on the sclerotial surface during sclerotial development and identified some chemical constituents. Other researchers have analyzed and determined the biochemical composition of the sclerotialexudates of *S. rolfsii* (Pandey et al., 2007) and of related fungus *Rhizoctonia solani* (Aliferis and Jabaji, 2010) and further demonstrated their bioactivity against microorganisms and plant species in vitro (Aliferis and Jabaji, 2010). The constituents were found to contain several phenolic compounds (Aliferis and Jabaji, 2010; Pandey et al., 2007; Sarma et al., 2002) including ferulic acid with antioxidant and antifungal properties (Pandey et al., 2007). However, it is not clear whether the antifungal compounds of the exudates are by-products of sclerotial formation or are a constituent of the sclerotia.

We hypothesize that the antimicrobial compounds in the exudates are contained in the sclerotia and can be derived from sclerotial extracts. The objectives of this study were 1) to evaluate the effects of different culture media on mycelia growth and production of *S. rolfsii* sclerotia in the laboratory;

2) todetermine the effects of *S. rolfsii*s clerotial extract on some plant pathogenic fungi and bacteria in vitro.

2. Materials and Methods

2.1. Sclerotia production

Sclerotium rolfsii was isolated from micro-sclerotia produced on the infected stem of a field-grown tomato plant at the Winfred Thomas Agricultural Research Station (WTARS) in North Alabama, and grown into pure mycelial cultures on PDA. The sclerotia produced subsequently, were used for further experiments.

2.2. Effect of fungal culture medium on sclerotia production

To determine a suitable culture medium for laboratory growth and propagation of *S. rolfsii* sclerotia, single pathogen microsclerotia were cultured in different fungal culture media including PDA, cornmeal agar (CMA), oatmeal agar (OMA), 6% V8 agar, and 2% water agar (WA).The various powdered media formulations were hydrated, autoclaved at 121 °C for 20 min and poured into 100×15 mm Petri dishes. After seeding each plate with a single sclerotium of *S. rolfsii*, plates were sealed with parafilm and incubated at 24 °C, 16 hrs light/8 hrs dark photoperiod for 21–30 days and subsequent sclerotia yield was quantified by determining the fresh weight, no. of sclerotia plate^{-1} and sclerotia diameter.

2.3. Preparation of extract

Sclerotia were harvested, weighed and placed in an autoclaved mortar. Sterile distilled deionized water was added at a ratio of 1:3 (w/v), and the mixture was ground with pestle to a smooth consistency. The crude extract was centrifuged at 7500 rpm for 20 min at 4 °C and the supernatant was collected and passed through a 0.2 µ sterile filter prior to conducting the antimicrobial activity assays.

2.4. Antibacterial activity assays

For bacterial growth inhibition assay, various dilutions of the extract were prepared by mixing a fixed volume of the extract with varying (increasing) volumes of aliquots of 3 bacterial species (*X. campestris* pv *vesicatoria*, *E. amylovora* and *Ralstonia solanacearum*) in log phase of growth in microfuge tubes to obtain six (0, 5, 10, 25, 50 and 100% [v/v]) concentrations (Table 1). The mixtures were incubated at 25 °C for 12 hrs and 100 µL was taken from each concentration and aseptically spread evenly on nutrient agar in Petri dishes. In another assay, 100 µL aliquots were taken from the culture broth of each bacterium and aseptically spread on solid nutrient agar to form a bacteria lawn. After the development of bacteria lawns, droplets of the sclerotial extracts of different dilutions were distributed evenly on the bacterial lawn to determine plaque-like inhibition zones (pliz).

2.5. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) gives an indication of the potency of the extract under consideration. To determine the MIC, dilutions of the extract were prepared and tested on *E. amylovora*-a bacterium whose growth was severely inhibited by the extract in preliminary assays. Four dilutions of the extract (100%, 50%, 25%, 10%, 5% and 0%) were prepared by adding specific volumes of bacterial culture in the log phase (OD=0.1) of growth to the extract. The diluted mixtures were spun on a vortex platform for 2 seconds, and incubated at 24 °C for 24 hrs after which the entire contents (200 µL) were taken and aseptically spread evenly on nutrient agar using a sterile triangular glass rod. The plates were incubated at 30 °C overnight and inspected for bacterial colony growth.

Table 1: Effect of concentration of sclerotial extract on growth inhibition in three plant pathogenic bacteria

Concentration of Sclerotial Extract (%)	Bacterial Growth Inhibition		
	E. amylovora	X. campestris pv vesicatoria	R. solanacearum
100			+
50	+	–	–
25	+	–	–
10	+	–	–
5	+	–	–
0 (Control	–	–	–

+ Growth inhibition; – No growth inhibition

2.6. Antifungal activity assay

The undiluted *S. rolfsii* extract was placed in two 3 mm wells made in PDA with the aid of the blunt end of a Pasteur pipet. The medium was inoculated with 3×3 mm^2 PDA agar blocks of 5-day old *A. solani* or *L. maculans* myceliacultures, incubated at 22 °C and monitored for mycelial growth.

2.7. Experimental design and data analyses

The experiments were laid out as completely randomized designs (CRD) with five or six replications. Numerical data including sclerotia fresh weight and diameter; fungal mycelia growth radii and no. of bacterial colonies were subjected to the analyses of variance and treatment means were separated using Tukey's honestly significant difference (HSD) test at p=0.05.

3. Results and Discussion

We observed that theproduction of fungal biomass and subsequently derived sclerotia, increased in the order WA, V8, CMA, PDA and OMA. In sclerotia-forming cultures, the fastest sclerotia production occurred at 7 days after culture (DAC) in V8 and CMAwhile the highest no. of sclerotia was formed in PDA and OMA where they appeared 14 DAC. The quantity of sclerotia produced per Petri plate increased in the order WA, V8, CMA, PDA and OMA with OMA yielding significantly the most sclerotia (Figure 1A). OMA also had

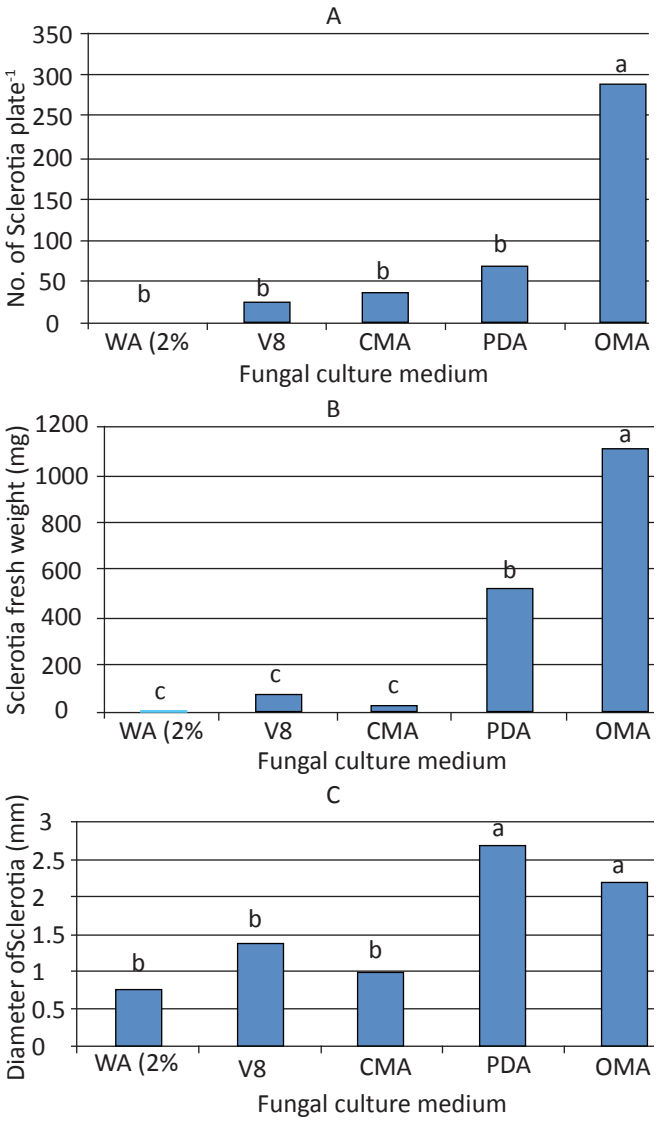

Figure 1: Laboratory production *S. rolfsiisclerotia*. Effect of fungal culture media on (A) the quantity, (B) fresh weight and (C) diameter of *S. rolfsii* sclerotia produced

significantly the highest sclerotia yield (fresh weight), and both OMA and PDA produced individual sclerotia yields that were significantly greater than those in all other media (Figure 1B). The average size (diameter) of sclerotia formed on CMA, WA and V8 was relatively small (< 1.5 mm) compared to that of sclerotia produced on PDA and OMA which was greater than 2 mm (Figure 1C). The largest sclerotia were produced on PDA. However, the rate of sclerotia production on PDA and OMA was slow and erratic such that some culture plates produced no sclerotia. A significant proportion of sclerotia produced in OMA released droplets of exudates during the maturing stage (Figure 2). The phenomenon of exudates production was not observed in other culture media. The large sclerotia on PDA were irregular in shape and tended to embed in the agar unlike those of other media which remained loosely attached to the mycelia on the surface of the agar.

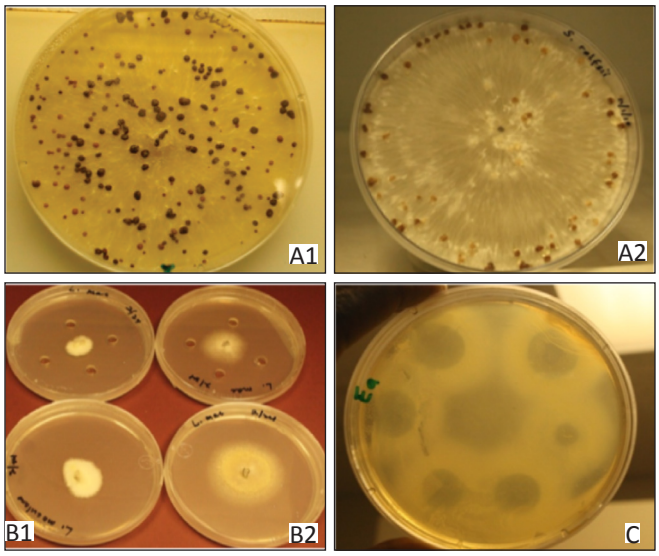

Figure 2: A1 & A2: Sclerotia of *S. rolfsii* on PDA; B1: Radial growth of *L.maculanson* PDAcontaining sclerotial extract (SCE); B2: Without sclerotial extract; C: Zones of *E. amylovora* growth inhibition (1.0-2.5 cm) on nutrient agartreated with SCE

Extracts of *S. rolfsii* inhibited the mycelial growth of *L. maculans* by about 50% whereas *A. solani*cultures showed little or no growth inhibition (Figure 3).

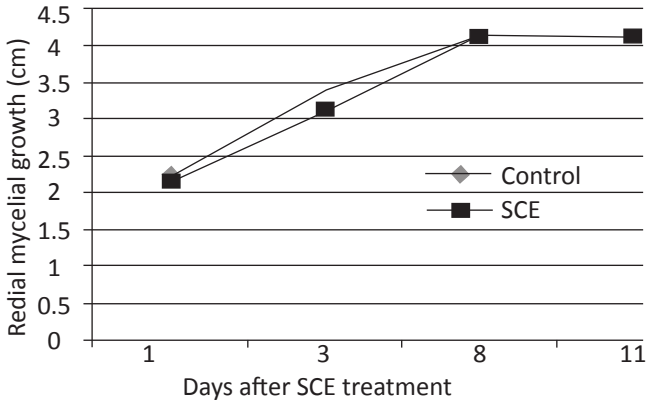

Figure 3: Inhibitory effect of *S. rolfsii* sclerotial extract on *Alternaria solani* mycelial growth

The inhibited bacterial cultures tended to produce a dark brown color. When a loop-full aliquot of bacteria taken from these cultures was streaked on nutrient agar, no bacterial growth was observed (Figure 4).

When SCE was inoculated with *A. solani*, no significant growth differences were observed betweenthe control and test plates. However, when inoculated with *L. maculans*, the growth of the fungus wassignificantly reduced compared to the control plates which were inoculated with distilled water. These results show that the soil-borne pathogen, *Sclerotium rolfsii* exhibits differential antifungal activity against some plant pathogenicfungi.

The potency and antimicrobial activity of the sclerotial extracts

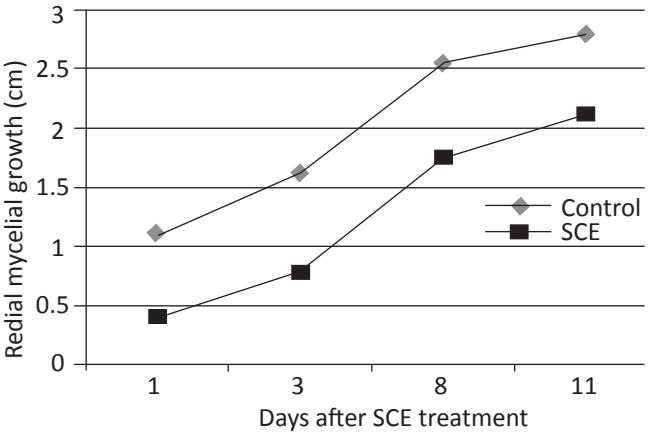

Figure 4: Inhibition of mycelial growth in *Leptosphaeria maculans* by *S. rolfsii* sclerotial extract

were greater if the extracts were used soon after preparation (24 hrs at room temperature). We observed that the activity of the extracts stored at room temperature or 4 °C for a few days reduced dramatically. Activity also progressively reduced for extracts stored at -20 °C. Extracts stored at -80 °C maintained activity.

4. Conclusion

Sclerotial extracts of the soil-borne plant pathogen, *Sclerotiumrolfsii*exhibit antifungal and antibacterial activity against some plant pathogenic fungi and bacteria respectively. The sclerotia can be produced abundantly on oatmeal agar in the laboratory within 14 days.The water soluble sclerotial extracts of *S*. *rolfsii*exhibited high antibacterial activity against *E*. *amylovora*– the cause of fireblight disease of pears and apples, but showed only moderate toxicity against X. *campestris*pv*vesicatoria* – cause of bacterial spot of tomatoand *L*. *maculans* –cause of blackleg disease of crucifers.This antimicrobial property can be harnessed into the production of a novelbiopesticide whose chemical composition could also be used as a template for the synthesis of pesticides for integrated bacterial plant disease management.

5. Acknowledgement

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