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Albocycline, the main bioactive compound from *Propionicimonas* sp. ENT-18 against *Sclerotinia sclerotiorum*

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ABSTRACT

The main aim of this study was to identify the active compound produced by the ant ectosymbiont *Propionicimonas* sp. ENT-18 against *Sclerotinia sclerotiorum*. An ethyl acetate whole extract from ENT-18 fermented broth was fractionated on Sephadex and Fluorisil columns prior to Q-TOF mass spectrometry analysis. The active fraction was found to contain the macrolide albocycline, which was deduced by interpreting the magnetic resonance data. Low concentration (1.6 μg/sclerotium) of the purified albocycline was sufficed to inhibit the sclerotia germination. Albocycline was considered as the main active molecule produced by ENT-18 against *S. sclerotiorum*. This is the first report of albocycline produced by a non-streptomycete and of its potential as an antimold molecule.

1. Introduction

Progress on agricultural production has been achieved upon several advances on the way crops are cultivated. However, crop yield is often affected by phytopathogens that lead to economic losses. For instance, the 'white mold' disease caused by *Sclerotinia sclerotiorum* can reach losses of US$ 200 million/year only in USA (Bolton et al., 2006). In the past, threats like the white mold were mitigated by using agrochemicals usually hazardous to human health and the environment. Moreover, the indiscriminate use of agrochemicals frequently leads to selection of resistant strains, effects on non-target organisms or cases of intoxication and environmental pollution (Gerhardson, 2002). Nowadays, it is increasingly clear the public perception against these compounds, which have made the search for more environmentally friendly methods to control phytopathogens a necessity (Walsh et al., 2001).

Biological control of phytopathogens using microorganisms has proved to be a feasible alternative for agrochemical replacement. In general, the use of these biocontrol agents is safe, cost-effective (Islam, 2011) and, in most cases, their byproducts present low toxicity to animals and humans (Bérdy, 2005). Thus, many bacterial genera (e.g. *Bacillus*, *Pseudomonas*, *Streptomyces*) have been tested and exploited as potential and effective biocontrol agents against different plant diseases (Weller, 1988; Haas and Defago, 2005; Ji et al., 2008; Zucchi et al., 2008). Among them, a specific bacterial group, *Actinomycetales*, became an important source of biocontrol agents, mainly due to their capacity to produce a large variety of biocompounds (Bérdy, 2005; Clárdy et al., 2006; Goodfellow and Fiedler, 2010).

In the case of sclerotia-producing phytopathogens, e.g. *S. sclerotiorum*, the biocontrol agents are of value to overcome common problems related to the recalcitrant feature of these structures. Furthermore, due to its saprophytic and aggressive behavior, lack of resistant cultivars and resistance to fungicides, this phytopathogen is extremely difficult to control and is often a great problem in temperate conditions (Coley-Smith, 1979; Steadman, 1983; Zhou and Boland, 1998; Bardin and Huang, 2001).

In our exploitation of the bacterial diversity in the insect associated actinobacterial symbionts as a source of new bioactive molecules (Zucchi et al., 2011), we identified a candidate strain, the isolate *Propionicimonas* sp. ENT-18, as a source of a bioactive compound to control *S. sclerotiorum*. Biological assays indicated toxicity was induced due to histological abnormalities in the sclerotium (Zucchi et al., 2010). Therefore, our main aim was to isolate and elucidate the structure of this active compound.

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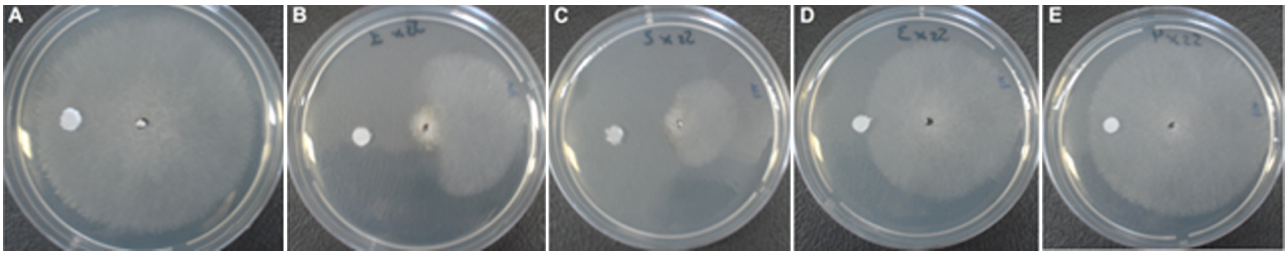

Fig. 1. Antibiosis test after Sephadex G-15 (GE) separation of the crude extract from isolate ENT-18. (A) Methanol control; (B) Fraction 1; (C) Fraction 2; (D) Fraction 3 and (E) Fraction 4.

2. Materials and methods

2.1. Isolate

The actinobacterium strain ENT-18 (GenBank accession number GQ304819) was maintained on ISP-2 (Shirling and Gottlieb, 1966) agar. The wild-type of *S. sclerotiorum* was kindly provided by Dr. Itamar S. de Melo (Embrapa, Brazil) and kept on Potato-Dextrose Agar (PDA). Both cultures were maintained at 4 °C until use. Sclerotia were obtained by aseptically transferring discs (0.5 cm diameter) from fresh colonies of *S. sclerotiorum* to Petri dishes containing PDA. Each disk was placed at the center of the plate which was incubated at 25 ± 1 °C for 20 days. The formed sclerotia were harvested with a bacterial loop and maintained at 4 °C.

2.2. Isolation and purification of the bioactive compound

The isolate ENT-18 was inoculated in conical flasks containing ISP-2 broth and incubated in a shaker (150 rpm) for seven days at 28 °C. The secondary metabolites were extracted using ethyl acetate and evaporated to dryness in a rotary evaporator (Canova et al., 2010). A total of 2 L of ISP-2 broth were fermented, which resulted in approximately 150 mg of crude extract. The residue obtained was diluted with methanol to a concentration of 25 μg μL^{-1}, and the solution was stored at 4 °C until use.

The ethyl acetate extract from *Propionicimonas* sp. ENT-18 (150 mg) was fractioned on a 20 cm × 0.5 cm Sephadex G-15 (GE) column with methanol as the eluent, and 32 fractions of 2.5 mL were obtained. Each fraction was assayed against the phytopathogen by antibiosis test in which filter discs (0.5 cm diameter) were placed at approximately 3 cm of the plate center and treated with 10 μL of the tested fraction. A sclerotium of *S. sclerotiorum* was placed in the center of the Petri dish and the plates were incubated for seven days at 28 °C. The active fractions (1–3) were pooled together and refractionated on a 20 cm × 0.5 cm Fluorisil® 60–100 (Mallinckrodt, Ireland) packed column, using methanol: ethyl acetate (6.5:3.5) as an eluent, and 20 fractions of 2.5 mL followed by 10 fractions of 5.0 mL were collected. An antibiosis test with each fraction was performed as described previously, and the most effective fraction was thereafter used as base material for the compound characterization.

2.3. Compound characterization

The active fraction was diluted in methanol/0.1% formic acid and introduced into the ion source at 5 μL min^{-1} with a syringe pump. Mass spectrometer analysis was performed using a hybrid quadrupole time-of-flight (Q-TOF) high resolution (7.000 FWHM) and high accuracy (5 ppm) Q-TOF mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ion source (ESI). The conditions for the positive electron spray ionization (ESI) were as follows: desolvation gas (nitrogen) was heated to 180 °C, the capillary potential was set to 3.5 kV, and the cone voltage was 25 kV. MS/MS tandem mass spectra were acquired by mass-selecting the target ion using the quadrupole mass analyzer followed by a 30 eV collision-induced dissociation (CID) using argon in the quadrupole collision cell and mass analysis by TOF.

The ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) and 2D heteronuclear single quantum coherence (gHSQC) and heteronuclear single bond correlation (gHSBC) spectra for albolcycline were recorded on a Bruker DRX 500 spectrometer (2.5 mm gradient probe) operating at 500.13 MHz (^{1}H) and 125.78 MHz (^{13}C). The 1D-NMR spectra were recorded under standard conditions. The 2D-NMR spectra were recorded with standard Bruker pulse programs. 2D (^{1}Hx^{13}C) gHSQC and gHSBC spectra were acquired applying 120 transients accumulated into 1-k data points with 256 experiments; the FID was zero filled to 2-k data. The active molecule (4.2 mg) was diluted in deuterated chloroform ($CDCl_3$) containing 0.03% (v/v) tetramethylsilane (TMS) for analysis.

2.4. Sclerotial survival and lethal concentration determination (LC_{50})

The same sample used for the compound identification was also assayed to evaluate its capacity to inhibit the sclerotia germination of *S. sclerotiorum*, following Zucchi et al. (2010). The sample was serially diluted (64–0.25 μg mL^{-1}). After obtaining the sclerotial survival pattern, the lethal concentration (LC_{50}) was calculated using the SAS software.

3. Results

3.1. Compound purification

The antibiosis test revealed that only the first three fractions obtained from the Sephadex separation were active against *S. sclerotiorum* (Fig. 1). These fractions were pooled and subjected to fractionation by size exclusion flash chromatography on a Fluorisil® 60–100 packed column. Again, only the three first fractions were active against the phytopathogen. Therefore, based on the inhibitory results with the Fluorisil fractions (data not shown), fraction 2 was chosen as base material for the elucidation of the compound structure as it demonstrated the highest fungal development inhibition.

3.2. Compound characterization

The active compound was characterized as the macrocyclic lactone albocycline. The spectroscopic data are in line with those reported in the literature (Nagahama et al., 1971; Thomas and Chidester, 1982).

Albocycline was obtained as a yellow viscous liquid and the ESI-TOF MS analysis showed a ion peak at *m/z* 309.20604 [M+H]$^{+}$. The molecular formula was determined as $C_{18}H_{28}O_4$ by high-resolution (HR) ESI-TOF MS.