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# Anti-*Xanthomonas* activity of Antarctic fungi crude extracts

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Agriculture suffers considerable losses in its production caused by plant diseases. In the citrus culture some problems were associated with citrus canker caused by *Xanthomonas citri* subsp. *citri.* That same way, sugarcane plantations are impaired to leaf scald disease caused by *Xanthomonas albilineans*. One of the measures used to contain citrus canker is the spray of cupric bactericides; however for scalding leaves there are no satisfactory control. Due to the phytosanitary problem caused by *Xanthomonas* (*X. citri* and *X. albilineans*) and by the difficulty of its control, the search for new forms of defense, less harmful to the environment, has become increasingly required. Thus, the aim of this work was to obtain crude organic extracts from filamentous fungi isolated from Antarctic soil samples and assess its bioactivity potential against *X. citri* and *X. albilineans*. One hundred and twenty-two extracts were tested, seven extracts inhibited the cell growth of *X. citri*, one was bioactive only against *X. albilineans* and one extract inhibited the cell growth of both bacteria. The bioactive extracts had a mean inhibition value of 96% against both bacteria. The values of MIC90 and MBC of bioactive extracts were also determined; for *X. citri* the isolate 3.1 Fe presented the lowest values of MIC90 (0.28 mg/mL) and MBC (1.0 mg/mL) and the two bioactive isolates for *X. albilineans* (1.1-Fe and B-Fe) presented the same values of MIC90 (1.4 mg/mL) and MBC (1.5 mg/mL). The filamentous fungi that produced positive extracts were identified as belonging to the genus *Pseudogymnoascus* (n=8) and *Cladosporium* (n=1). The filamentous fungi isolated from Antarctic soil produced compounds with bioactivity against phytopathogens from the *Xanthomonas* genus.

**Key words:** Antarctic fungi; antibacterial action; citrus canker; leaf scald; natural extracts.

## INTRODUCTION

*Xanthomonas* are rod-shaped Gram-negative and obligate aerobe bacteria, which have a polar flagellum (Tessmann, 2002). This genus comprises 27 species that cause serious disease in about 400 plants hosts (Ryan et al., 2011) and affect at least 124 monocotyledonous and 268 dicotyledonous plant species (Leyns et al., 1984).

All citrus species and varieties of commercial importance are affected by citrus canker caused by

*Xanthomonas citri* subsp. *citri* (Schaad et al., 2006). Every year orchards around the world suffer losses due to citrus canker, mainly countries with favorable climates for the development of *X. citri*. The control of this disease is accomplished by uses of less susceptible varieties, eradication of contaminated plants, decontamination of seeds and equipment, use of windbreakers and mostly use of antibiotics and copper sprays. This causes damage to the environment due to its accumulative potential in soil and trophic chain (Rocha and Azevedo, 2017).

The leaf scald in sugarcane is a disease caused by *X. albilineans*. This infection can be characterized in three ways: Asymptomatic or latent form (plants can tolerate the pathogen for a few weeks without exhibiting symptoms); chronic form (characterized by white or yellow streaks parallel to the main vein on leaves); acute form (presents attacks on xylem reducing sucrose production, and when in advanced stages can kill the plant) (Tokeshi and Rago, 1980). The leaf scald control is still scarce; it is only advisable to clean equipment and seedlings to reduce the propagation of the pathogen.

Considering the phytosanitary problems caused by *Xanthomonas* (*X. citri* and *X. albilineans*), it becomes necessary to research new forms of control, more efficient and less damaging to the environment.

Substances obtained from natural sources are important given the variability of such products. In general, natural molecules show greater structural diversity and several bioactive functions (Feher and Schmidt, 2003; Dayan et al., 2009; Newman and Cragg, 2016).

New microorganisms and natural products can be isolated from extreme environments, such as Antarctica (Pikuta et al., 2007). Many studies highlight the potential of the Antarctic environment in the production of bioactive metabolites (Furbino et al., 2014), antifungal substances (Svahn et al., 2015) and active compounds against *Xanthomonas* species, including *X. citri* (Moncheva et al., 2002; Encheva-Malinova et al., 2015; Puric et al., 2018; Vieira et al., 2018).

Thus, the present work is aimed to discover new fungi isolated from Antartic soils with potential for the production of bioactive compounds against *X. citri* and *X. albilineans.*

## MATERIALS AND METHODS

### Strain of filamentous fungi

Sixty-one filamentous fungi were used in this study. Among them, twenty-three were isolated from soil under an iron bar in Deception Island (South Shetland Islands, Antarctica) (60º41'459''W), twenty-three from soil at 4.0ºC (62º05'092''S) and fifteen from soil at 1.6ºC (57º56'763''W), in King George Island (South Shetland Islands, Maritime Antarctica). All of filamentous fungi are being maintained by cryopreservation at -80ºC and Castellani at 4ºC at Central of Microbial Resources (CRM-UNESP).

### Crude extracts production

The fungi were reactivated on agar malt broth petri dishes (2% malt and 1.5% agar) at 15ºC for 10 days. After their growth, disks of 5 mm of agar plus mycelium were transfered to 150 mL of 2% liquid malt broth in erlenmeyer flasks. This culture was shaken at 15ºC for 20 days at 150 rpm. After the period of incubation, the biomass and metabolic medium (supernatant) were separated using vacuum filtration.

### *Intracellular extracts*

The biomass was extracted 3 times with 40 mL methanol by maceration.

### *Extracellular extracts*

The supernatant was extracted 3 times with 60 mL ethyl acetate by liquid-liquid extraction. The extracts were concentrated in a low pressure rotary evaporator and dissolved in dimethylsulfoxide (DMSO) 10% (v/v) until the concentration of 30 mg/mL.

### Cultivation of *Xanthomonas*

*X. citri* strain 306 (IBSBF 1594) was activated on agar NYG (3 g yeast extract, 5 g peptone and 20 mL glycerol) broth at 29ºC for 2 days. Then, bacterial cells were cultured in 30 mL of liquid NYG broth medium, shaken at 200 rpm and 29ºC until $OD_{600} = 0.8$.

*X. albilineans* (IBSBF 1387) was activated on agar YSG medium (5 g yeast extract, 5 g glucose, 0.5 g monobasic ammonium phosphate and 0.2 g anyhidrous dibasic potassium phosphate) broth at 29ºC for 5 days. Then, bacterial cells were cultured in 30 mL of liquid YSG broth medium, shaken at 200 rpm and 29ºC. The bacterial inoculum was standardized at $10^7$ CFU/mL ($OD_{600} = 0.9$).

### Anti-*Xanthomonas* activity assessment

The bioassay was accomplished in 96-well plates with triplicate of events and experiments, based in the Resazurin Microtiter Assay (REMA) method (Silva et al., 2013). For the bioassay against *X. citri* the maximum concentration tested of extracts was 2.1 mg/mL followed by microdilution until 0.016 mg/mL and for *X. albilineans* the maximum concentration tested of extracts was 3 mg/mL. Kanamycin (0.02 mg/mL) was used as positive control (PC), DMSO 1% (v/v) was used as vehicle control (VC) and negative control (NC) was made with medium (NYG) and inoculum. The inoculum was diluted (1:10) in NYG and aliquote of 10 μL was added to all wells used and standardized at $10^5$ CFU/well. After a 16 h incubation at 29ºC 15 μL of resazurin 0.01% (w/v) was added and submitted to another incubation for about 1 h at the same temperature. The REMA was made in triplicate.

A Biotek Synergy H1MFD microplate reader was used to measure cell viability. It scans the fluorescence of the resorufin (resazurin reduced by microbial metabolism) at 530 nm (excitation) and 590 nm (emission). The fluorescence units (FU) detected by the reader were used to calculate growth inhibition in percentage, according to the formula:

$$\frac{[(average\ FU\ negative\ control) - (average\ FU\ test)]}{(average\ FU\ negative\ control)} \times 100$$

Inhibition rate above 90% was considered as positive.

The minimal extract concentration needed for inhibition of 90% of cells (MIC90) was calculated based on the equation obtained from the regression curve constructed with the Rema bioassay data.

The bactericidal assay was performed as follows: A small aliquot from each well of the REMA plates was inoculated in petri dishes (150 x 15 mm, NYG medium) with 96-pin microplate replicator. After incubation for 48 h at 29°C, it was possible to visually analyze the bacterial growth. The minimal concentration in which there was no bacterial growth was considered the minimal bactericidal concentration (MBC) of the extract.

### Identification of the Antarctic fungi

DNA extraction was performed directly from the mycelium after the fungal growth in PDA medium for 7 to 14 days at 15°C, following a modified version of the CTAB method (Möller et al., 1992; Gerardo et al., 2004). ITS amplification was performed using primers ITS4 (5'TCCTCCGCTTATTGATATGC3') and ITS5 (5'GGAAGTAAAAGTCGTAACAAGG3') (Schoch et al., 2012).

PCRs were performed in a final volume of 25 µL (4 µL of dNTPs [1.25 mm each]; 5 µL of 5x buffer; 1 µL of BSA [1 mg/mL]; 2 µL of MgCl2 [25 mm]; 1 µL of each primer [10 µm]; 0.2 µL of Taq polymerase [5 U/µL], 2 µL of diluted genomic DNA [1:100] and 8.8 µL of sterile ultrapure water). PCR conditions for ITS were 94°C/3 min followed by 35 cycles at 94°C/1 min, 55°C/1 min and 72°C/2 min. Amplicons were purified using the Exonuclease I and Alkaline phosphatase (Thermo Scientific, Massachusetts, USA), according to the manufacturer's protocol. Samples were then quantified in NanoDrop® (Thermo Scientific) and sequenced using BigDye Terminator® v.3.1 kit (Applied Biosystems, California, USA), following the manufacturer's instructions. Sequences obtained were compared with sequences deposited in the NCBI – GenBank database. Alignments were performed separately for each molecular marker using MAFFT v.7 (Katoh and Standley, 2013). The phylogenetic analyses were done using the program MEGA v. 7 (Tamura et al., 2013). The Kimura 2-parameter model (Kimura, 1980) was used to estimate evolutionary distance for the Neighbor-joining analysis. Branch support was calculated by bootstrap with 1,000 replicate runs.

### RESULTS

A total of 122 extracts was produced. All of them were tested against *X. citri*. Eight extracellular extracts presented, in the maximum concentration, averages of fluorescence measurements (Table 1) which when applied in the formula to calculate growth inhibition in percentage showed inhibition rates above 90% (Table 1) and were considered positive. The intracellular extracts showed no antibacterial activity against the tested *Xanthomonas* species.

The MIC90 values (Table 1) for the extracts of 2-Fe, 6-Fe, B-Fe and G-Fe were around 2.0 to 1.7 mg/mL, three extracts (11-Fe, C-Fe and D-Fe) presented values between 0.80 and 0.60 mg/mL, and the extract of 3.1-Fe attained the lowest MIC90 (0.28 mg/mL).

In the bactericidal assay, four extracts (2-Fe, 6-Fe, B-Fe and G-Fe) presented bacterial growth in the highest concentration tested (2.1 mg/mL), therefore they were considered bacteriostatic. The extracts of C-Fe, D-Fe, 3.1-Fe and 11-Fe were bactericidal, highlighting 3.1-Fe and 11-Fe, which had the lowest MBC value (1.0 mg/mL).

In the experiments with *X. albilineans,* two of the 122 extracts were considered positive, the extracts of 1.1-Fe and B-Fe presented a percentage of 97% inhibition of bacterial growth in the concentration 1.5 mg/mL. The MIC90 values for both extracts (1.1-Fe and B-Fe) were about 1.4 mg/mL. In the bactericidal bioassay the both extracts were bactericidal up to the concentration of 1.5 mg/mL (MBC).

In this study, eight bioactive fungi (1.1- Fe, 2-Fe, 3.1-Fe, 6-Fe, 11-Fe, B-Fe, C-Fe and G-Fe) were identified as belonging to the genus *Pseudogymnoascus* based on molecular taxonomy and phylogentic analysis (Figure 1).

The bioactive fungus D-Fe was identified as belonging to the genus *Cladosporium* (Figure 2). The taxonomic marker used (ITS) showed to be very conservative, since different species of the genus *Cladosporium* grouped in the same cluster of the Antarctic-derived fungus D-Fe.

### DISCUSSION

Positive results of extracellular extracts may be associated with defense mechanism, since some microorganisms can produce substances that inhibit potential competitors (Smid and Lacroix, 2013; García et al., 2017). The samples tested are crude extracts, a reduction in MIC90 value is expected after purification and isolation of the bioactive molecules.

Based on this work, *X. albilineans* may be considered more resistant than *X. citri*, since only two extracts were inhibitory against *X. albilineans*. This also can be noted by the lower inhibition capacity of the antibiotic used as positive control (kanamicin) for this bacterium. In plantations, this resistance can be observed, since leaf scalding has no effective chemical combat and it is more difficult to contain than citrus canker.

The extract produced by the fungus B-Fe (*Pseudogymnoascus*) showed satisfactory bioactivity against both bacteria, and it can be considered as a promising microbial genetic resource in the fight against *Xanthomonas*.

The bioactive extracts (Table 1) were also tested, in another study, against *X. euvesicatoria* (bacterial spot on pepper and tomato) and *X. axonopodis* pv. *manihotis* (cassava bacteriosis) and only the fungus D-Fe showed activity against *X. axonopodis* pv. *manihotis*.

About identification, the *Pseudogymnoascus* ITS-rDNA sequences were highly similar, suggesting that it could be representatives of the same species. Amplicons were compared to homologous sequences deposited in the NCBI-GenBank database. The isolates showed 100% similarity with different strains of *Pseudogymnoascus* sp. Data from phylogenetic analysis showed that the isolates were placed in a separate cluster in relation to the other *Pseudogymnoascus* species (bootstrap value of 71%). These results suggest that *Pseudogymnoascus* strains could represent a new species. However, as reported by Chaturvedi et al. (2010), others taxonomic markers have

**Table 1.** Extracellular bioactive extracts against *X. citri.*

| Extract | Fluorescence unit* | Inhibition (%)* | MIC 90 (mg/mL) | R² |
|---------|-------------------|-----------------|----------------|-----|
| 2-FE | 1621.2 | 96.02 ± 0.27 | 2.00 | 0.9997 |
| 3.1-FE | 3671 | 91.00 ± 0.55 | 0.28 | 0.9806 |
| 6-FE | 880 | 97.84 ± 0.17 | 1.99 | 0.9989 |
| 11-FE | 1096.2 | 97.31 ± 0.26 | 0.80 | 0.9854 |
| B-FE | 1023.8 | 97.48 ± 0.25 | 1.71 | 0.9972 |
| C-FE | 898.8 | 97.80 ± 0.13 | 0.78 | 0.9956 |
| D-FE | 1067.6 | 97.38 ± 0.18 | 0.60 | 0.9850 |
| G-FE | 2172.8 | 96.68 ± 0.88 | 1.99 | 0.9992 |
| Negative control | 40805.2 | - | - | - |

*Mean values obtained at the extracts concentration of 2.1 mg/mL (maximum concentration). MIC90: Extract concentration needed for inhibition of 90% of the cells, in mg/mL. $R^2$: shows how well regression curve fits the original curve obtained from the extracts, with a maximum value of 1.
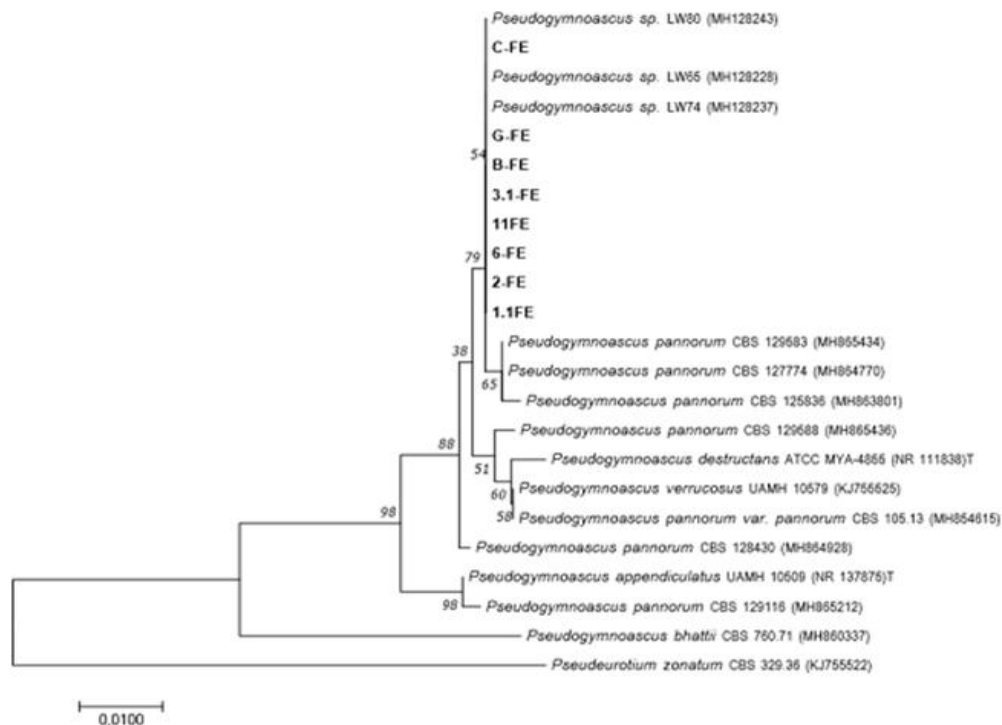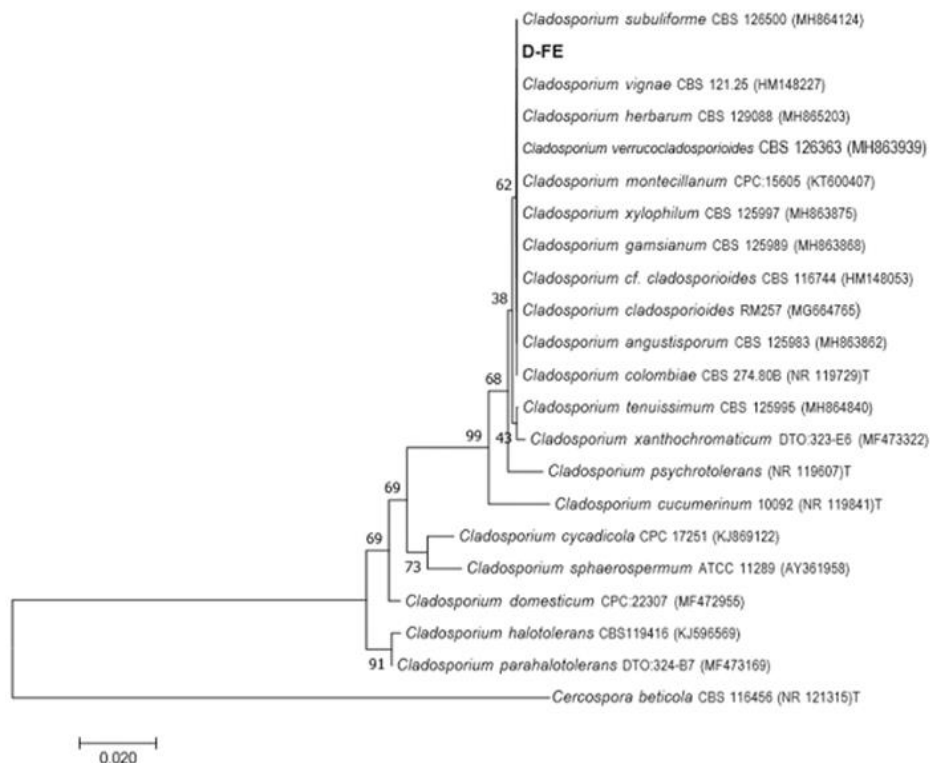


**Figure 1.** Phylogenetic analysis of *Pseudogymnoacus* species evaluated in this study with sequences of the closest species deposited in Genbank. The tree was built base on the ITS sequences using the Neighbor-joining method with Kimura 2-parameters nucleotide substituttion model.

to be applied in order to get speciation for *Pseudogymnoascus/Geomyces.* Representatives of this genus are globally distributed and can develop in different conditions and extreme environment (Hayes, 2012), including Antarctic ecossystems (Arenz et al., 2006; Bridge and Spooner, 2012; Godinho et al., 2015). In the study reported by Wentzel et al. (2018) *Pseudogymnoascus* was the most abundant filamentous

fungi found in antarctic samples of soil and marine sediments.

The isolates from *Pseudogymnoascus* have been reported as having activity against microorganisms (Furbino et al., 2014; Gonçalves et al., 2015). Figueroa et al. (2015) described four new nitroasterric acid derivatives with antibacterial and antifungal activity obtained from the cultures of a *Pseudogymnoascu*s sp.

**Figure 2.** Phylogenetic analysis of Cladosporium sp. (D-FE) evaluated in this study with sequences of the closest species deposited in GenBank. The tree was built base on the ITS region sequences using the the Neighbor-joining method with Kimura 2-parameters nucleotide substituttion model.

fungus isolated from an Antarctic marine sponge. Other studies reported the bioactivity of this genus against *Xanthomonas* (Henríquez et al., 2014), including some from our research group, against *X. citri*, *X. euvesicatoria* and *X. axonopodis* pv. *passiflorae* (Vieira et al., 2018, Puric et al., 2018), but so far no molecules have been described.

Representatives of *Cladosporium* have also been reported in the Antarctic environments (Arenz et al., 2006; Bridge and Spooner, 2012; Wentzel et al., 2018). According to Tosi et al. (2002) *Cladosporium* is the most frequently observed genus in association with antarctic mosses. Antimicrobial molecules were reported as produced by representatives of this genus (Zhang et al., 2001; Xiong et al., 2009; Liang et al., 2018), but studies related to the bioactivity of *Cladosporium* against *Xanthomonas* are still scarce. In a previous study published by our research group, a representative of *Cladosporium* showed potencial to inhibit the cell growth of *X. citri, X. euvesicatoria* and *X. axonopodis* pv. *passiflorae* (Puric et al., 2018), corroborating the results achieved in the present study, where *Cladosporium* sp. (D-Fe) was able to produce bioactive molecules against *X. citri.*

The present study highlights the potential of bioprospecting of extrememophile microorganisms, in the search for new and more sustainable bioactive molecules, potentially able to substitute the use of copper in agriculture and in the combat of phytopathogens.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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