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Pseudomonas aeruginosa produces secondary metabolites that have biological activity against plant pathogenic *Xanthomonas* species

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ABSTRACT

The bioactivity of compounds produced by *Pseudomonas aeruginosa* (LN strain), against three *Xanthomonas* species was investigated under greenhouse conditions and using electron microscopy. Chromatographic fractions EAP, VLC3, VLC4, VLC3d and VLC4f were tested against *Xanthomonas axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *citri* by disc diffusion. Fractions with antibiotic activity were tested *in vivo* under greenhouse conditions and their bioactivity was evaluated. Scanning electron microscopy showed that VLC4f affects biofilm formation while VLC4f and VLC3d both affect cell morphology. The semi-purified fractions controlled bacterial diseases caused by *Xanthomonas* spp. when sprayed on plants under greenhouse conditions. The VLC4f fraction showed superior results in disease management, reducing the number of lesions on cotton and orange leaves by 94%, and reducing disease severity in bean leaves by 73%. The data suggest that the fractions were effective and have potential as an alternative to conventional bactericides in the control of plant diseases caused by *Xanthomonas* sp under greenhouse conditions.

1. Introduction

Xanthomonas is a large genus of gram-negative, yellow-pigmented, plant-associated bacteria. Pathogenic species and pathovars within species show a high degree of host plant specificity and are known to cause foliar disease on approximately 400 plant hosts, including dicots and monocots (Lu et al., 2008). The phyllosphere is a stressful environment, but it provides a selective habitat and substrates for Xanthomonal bacteria to colonize the host phylloplane before entering leaf tissues and becoming pathogenic (Mhedbi-Hajri et al., 2011). Some important diseases caused by xanthomonads include common bacterial blight of beans (CBB), bacterial leaf blight of cotton (BLB) and citrus canker, all of which lead to serious quality and yield losses in the host crops.

CBB, caused by *Xanthomonas axonopodis* pv. *phaseoli*, is one of the most economically important diseases of bean worldwide. Yield losses may range from 10 to >50% depending on disease severity, environmental conditions and cultivar susceptibility (Izquierdo et al., 2004; Asensio-S.-Manzanera et al., 2005). The disease reduces seed and pod quality, leading to yield loss (Jacques et al., 2005). BLB, caused by *X. axonopodis* pv. *malvacearum* is a serious disease of cotton which occurs in all cotton-growing countries of the world and affects yield and fiber quality. The pathogen is internally seed-borne and yield losses have been estimated at up to 50% (Salah Eddin et al., 2007). Citrus canker, caused by *X. axonopodis* pv. *Citri*, is a serious disease of citrus (*Citrus* spp.), leading to economic losses in citrus production worldwide (Lins et al., 2009). The symptoms consist of erumpent lesions on fruit, leaves and young shoots, reducing fruit quality and fruit yield (Oliveira et al., 2011).

The control measures for these diseases include quarantine procedures, planting disease-free seedlings, use of resistant cultivars, windbreaks to hinder inoculum dispersal, and use of bactericides such as copper-based compounds. These control measures have limited efficacy, with only a few bactericides available on the market and the added issue that copper compounds are toxic, which impact the environment (Lo Cantore et al., 2009).

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The potential of *Pseudomonas* spp. to suppress plant pathogen is been demonstrated in all the world (Haas and Defágo, 2005). Secondary metabolites isolated from *Pseudomonas* have been detected and investigated because of their antibiotic activity. These bacteria can produce phenazines, pyrrolnitrin-type antibiotics, pyo compounds, indol derivates, peptides, glycolipids, lipids and aliphatic compounds (Leisinger and Margraff, 1979), that could be an alternative to the use of chemical compounds in the control of plant disease.

The challenge is to find new methods that guarantee the sustainability of crop production while minimizing the impact on the environment, preferably using a low-cost approach to manage these diseases. The aim of this work was to isolate, purify and test secondary metabolites from *Pseudomonas aeruginosa* (LN strain) against *X. axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *citri* and *X. axonopodis* pv. *phaseoli* under greenhouse conditions and to determine the bioactivity of these metabolites with regard to biofilm formation and cell morphology using electron microscopy.

2. Material and methods

2.1. Bacterial strains and growth condition

Bacteria were routinely grown for 48 h on culture medium at 28 °C and stored in 30% glycerol (v:v) at −20 °C for up one month. For longer-term storage, 200 μL of each culture were mixed with 800 μL of glycerol (30%), and frozen in liquid nitrogen. All *in vitro* and *in vivo* experiments were carried out with cell concentration adjusted to 10^{12} CFU mL^{-1} (OD 590 nm). The culture media used were Luria broth agar, nutrient agar, peptone sucrose agar (Lopes et al., 2008) and tryptic soy agar plus 100 mg L^{-1} $CuCl_2.2H_2O$ (Cain et al., 2000) for the growth of *X. axonopodis* pv. *malvacearum*, *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *phaseoli* and *P. aeruginosa*, respectively.

The antagonist bacterium, *P. aeruginosa* (LN strain), was isolated from a recently formed citrus canker lesion on a leaf of an orange tree (C. *sinensis* cv. Valencia), collected in Astorga, Brazil [23°13′29.11″ S; 51°39′47.20″ W] (Rampazo, 2004).

2.2. Production and separation of antibiotic substances

The initial inoculum of the LN strain was obtained from a culture stored in glycerol and grown as described above. During the log phase of bacterial growth (10^8 CFU mL^{-1}, O.D $\lambda = 590$ nm $= 0.09$), a 150-μL aliquot of cell suspension was inoculated in 1.5 L of tryptic soy broth (TSB) plus 100 mg L^{-1} $CuCl_2.2H_2O$ and cultured for 15 days at 28 °C and 100 rpm. Subsequently, the culture was centrifuged (20 min, 4 °C; 18,000 *g*), and 500 mL aliquots of cell-free supernatant were extracted 10 times by liquid–liquid partition with ethyl acetate 1:1 (v:v). The ethyl acetate phase (EAP) was concentrated at 60 °C in a rotary evaporator (Rotavapor R 215, Büchi) under reduced pressure. The sample was frozen in liquid nitrogen and lyophilized for 24 h. The final quantity obtained was approximately 1 g EAP per 17 L of supernatant.

2.2.1. Fractionation of EAP by vacuum liquid chromatography (VLC)

VLC was conducted in a glass column (20 mm diameter × 350 mm height) filled with 30 g of silica gel 60 (0.063–0.200 mm, Merck) coupled to a vacuum pump at 51 kPa. EAP (1 g mixed with 3 g of silica gel 60) was fractionated using the following mobile phase (v/v): hexane (100; VLC1), dichloromethane (100; VLC2), ethyl acetate (100; VLC3), methanol (100; VLC4), methanol:water (1:1; VLC5) and water (100; VLC6). A volume of 50 mL of each organic solvent was passed eight times through the column, and the eluant was concentrated at 60 °C in a rotary evaporator under reduced pressure, frozen in liquid nitrogen and lyophilized for 24 h.

2.2.2. Purification of the VLC3 and VLC4 by VLC

VLC was carried out as described above. Each phase was fractionated using the following mobile phase (v/v): hexane (100; VLC3a; VLC4a), hexane:dichloromethane (1/1, VLC3b; VLC4b), dichloromethane (100; VLC3c; VLC4c), dichloromethane:ethyl acetate (1/1, VLC3d; VLC4d), ethyl acetate (100, VLC3e; VLC4e), ethyl acetate:methanol (1/1, VLC3f; VLC4f), methanol (100; VLC3g; VLC4g), methanol:water (1:1; VLC3h; VLC4h) and water (100; VLC3i; VLC4i).

2.3. Evaluation of antibiotic activity by disc diffusion

EAP and the 24 purified phases were tested against the three specific phytopathogenic bacteria. Each phase (250 μg) was dissolved in the specific organic solvent used in the VLC, and 5 μL were added to a diffusion disc. After evaporation of solvents, the discs were placed on 20 mL of culture media streaked with a swab with the respective bacterial species (10^{12} CFU mL^{-1}). As a negative control, only the solvent was added to the disc. The plates were incubated at 28 °C for 48 h before the inhibition halos were measured (mm). The experiment was repeated three times.

2.4. Determination of minimum inhibitory concentration (MIC) of EAP, VLC3, VLC4, VLC3d and VLC4f fractions against Xanthomonas strains

The MIC of each fraction was tested by adding 100 μL of the test fraction dissolved in nutrient broth (NB) plus 100 μL of cell suspension (10^{12} CFU mL^{-1}) in NB; the following concentrations of fractions were added to 96-well plates with three replicates: 3000, 1500, 750, 375, 187.5, 93.75, 46.88, 23.44, 11.72 and 5.86 μg mL^{-1}. A positive control of 100 μL of NB and 100 μL of cell suspension (10^{12} CFU mL^{-1}) in NB was included. As a negative control, 200 μL of NB were used for each concentration of the fraction. A control to test metabolite sterility was set up by incubating the fractions in NB alone. The plates were incubated for 48 h at 28 °C and the optical density subsequently determined at 590 nm (ASYS UVM 340). The experiment was repeated three times.

2.5. Electron microscopy study

Xanthomonas strains were grown in tubes containing their specific culture broth (24 h, 28 °C, 100 rpm). When the culture was in log phase, the cell concentration was adjusted to 10^{12} CFU mL^{-1} and 10-mL aliquots of cell suspension were placed in 12 tubes with 200 μg mL^{-1} of the VLC4f or VLC3d fraction. Five other tubes with culture broth in the absence of the fraction served as controls. The tubes were incubated on a shaking incubator for 3 h at 28 °C and 100 rpm. After 30, 60 and 180 min, four 1-mL aliquots were collected to prepare the material for scanning electron microscopy. Each aliquot was fixed by immersion in 1 mL of 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0) for 1 h. Afterward, 20 μL of the cell suspensions (10^{12} CFU mL^{-1}) were spotted onto glass slides previously coated with a thin layer of polylysine, and 1 mL of fixer was added to each glass slide. The slides were left for 3 h. The samples were post-fixed in 1% OsO_4 for 1 h, and the fixed material was dehydrated in an ethanol series (70, 80, 90 and 100%). Scanning electron microscopy (SEM) samples were critical point dried in CO_2 (BALTEC CPD 030 Critical Point Dryer), and the slides were taped onto stubs, coated with gold (BALTEC SDC 050 Sputter Coater) and viewed under a FEI Quanta 200 scanning electron microscope.