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Acyl-Homoserine Lactones Produced by *Pantoea* sp. Isolated from the "Maize White Spot" Foliar Disease

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The "maize white spot" foliar disease is a problem of increasing importance to Brazilian maize crops. A bacterium isolated from water-soaked lesions from infected maize leaves was pathogenic in biological assays in vivo. It was identified as a Gram-negative, nonsporulating, facultative anaerobic bacterium, belonging to the genus *Pantoea*. Chemical study of the extracts from bacterial cultivation media allowed the identification of (*S*)-(−)-*N*-butanoyl-homoserine lactone and trace amounts of *N*-hexanoyl-homoserine lactone, widely recognized quorum-sensing signaling substances employed in cell-to-cell communication systems. The absolute configuration of natural (*S*)-(−)-*N*-butanoyl-homoserine lactone was determined by gas chromatography−flame ionization detection with a chiral stationary phase and by comparison of circular dichroism spectroscopic data with enantiopure synthetic substances. Biological evaluations with reporter *Agrobacterium tumefaciens* NTL4(pZLR4) were carried out with synthetic and natural products and also with extracts from maize leaves contaminated with the isolated bacterium, as well as from healthy leaves.

KEYWORDS: ***Pantoea*** **sp.; acyl-homoserine lactones; maize foliar disease**

INTRODUCTION

Maize (*Zea mays* Linnaeus) is one of the most important crops in Brazilian agriculture. However, this crop is affected by several insect plagues and fungal and bacterial diseases. Among them, the "maize white spot" foliar disease has emerged as an outstanding drawback for its cultivation in recent years in Brazil. The disease appeared in the country in the 1980s and has now spread over practically all of the country (*1*). The initial symptoms of the disease are water-soaked lesions on the leaves, which become necrotic and straw-colored and can spread to the entire leaf surface in advanced cases (*2*−*4*). A production loss of 63% was reported in a nonresistant maize variety, and the severity increases in rainy and cold seasons (*3*).

Initially, the disease was attributed to *Phaeosphaeria maydis* Henn fungus, whose filamentous structures were detected in advanced lesions (*4*). However, an accurate phytopathological investigation revealed that the initial leaf colonizer is a Gram-negative bacterium, without the presence of fungal structures in young lesions (*2*). The bacterium was identified as *Pantoea ananatis* Serrano using conventional biochemical assays and morphological studies for strain identification. Most probably, fungal colonization followed the initial bacterial attack (*2*).

The *Pantoea* genus encompasses phytopathogenic bacteria and was previously part of the *Erwinia* genus (*5*). Nowadays, it is well-established that many species of the *Erwinia* alliance and other Gram-negative bacteria exploit chemical communication mechanisms known as quorum-sensing, employing mainly acyl-homoserine lactones as signaling substances (*6*). In several bacterial species, the quorum-sensing phenomenon was described and proved to be important for bacterial survival and host infection, since the expression of many virulence phenotypes can be under quorum-sensing control (*6*−*8*).

The production of acyl-homoserine lactones was reported in a strain of *P. ananatis* isolated from pineapple, which produces (*S*)-(−)-*N*-hexanoyl-homoserine lactone, *N*-heptanoyl-homoserine lactone, and *N*-octanoyl-homoserine lactone (*9*). A signaling substance is also produced by *P. stewartii* Smith, responsible for "Stewart's disease" of maize, characterized by necrotic lesions in leaves (*10*). Biological investigations with mutants revealed that *P. stewartii* employs signaling substances to control the expression of many important phenotypic factors related to virulence and host attack, including the production of an exopolysaccharide, which protects bacterial cells from host defense mechanisms and also obstructs fluid circulation in leaves, causing necrosis. Mutants of *P. stewartii* unable to produce acyl-homoserine lactones displayed a dramatically reduced pathogenic activity against maize leaves, which was restored by addition of synthetic acyl-homoserine lactones (*10*). This exemplifies the great importance of quorum-sensing in phytopathogenic bacteria.

Continuing the efforts to characterize the pathogen of the maize white spot foliar disease, we report herein the isolation

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of a bacterium from young lesions in leaves and also infer the production of acyl-homoserine lactones by this strain, including the absolute configuration determination of the major identified acyl-homoserine lactone. Bioassays with specific reporter *Agrobacterium tumefaciens* Smith & Townsend NTL4(pZLR4) using synthetic and natural products and also extracts from maize leaves artificially inoculated with the bacterium were also performed.

MATERIALS AND METHODS

General Experimental Procedures. Fourier-transform infrared (FT-IR) spectra were obtained with a Bomem MB Michelson spectrometer, using KBr (Merck, Darmstadt, Germany) as sample support. NMR spectroscopic data were acquired with a Varian Inova spectrometer, operating at 499.88 MHz for ^{1}H NMR and 125.71 MHz for ^{13}C NMR, or alternatively with a Varian Gemini spectrometer, operating at 300.06 MHz for ^{1}H NMR and 75.45 MHz for ^{13}C NMR. $CDCl_3$ was used as solvent and TMS was used as internal reference (δ_H and δ_C 0.0). Chemical shifts δ were recorded in ppm, and coupling constants J were recorded in Hertz (Hz). Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Absorbance measurements were carried out on a Pharmacia-Biotech Ultraspec 2000 spectrophotometer. Silica gel for column chromatography (0.035−0.070 mm) was from Merck. Thin-layer chromatography analyses were made on silica gel 60 F_{254} plates (Merck) and visualized under exposure to UV light (254 nm) or by spraying with a solution of acidic *p*-anisaldehyde, followed by air heating.

Gas Chromatography−Mass Spectrometry (GC-MS) Analyses. GC-MS analyses were carried out with an Hewlett-Packard (HP) 6890/5973 instrument, equipped with a 30 m × 0.25 mm i.d., 0.25 μm HP5 fused silica capillary column. Mass spectra were recorded over the *m*/*z* 40−450 range at 3.54 scans/s, with an ionization energy of 70 eV. Helium was the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250 °C. The initial oven temperature was 100 °C, increased at 10 °C/min to 290 °C, and then held for 10 min. Samples of 1 μL (1 mg/mL in ethyl acetate) were injected in the splitless mode.

Chiral GC−Flame Ionization Detection (FID) Analyses. Chiral GC analyses were carried out with an HP 6890 instrument with FID, equipped with a 25 m × 0.25 mm i.d., 0.25 μm, chiral capillary column heptakis(2,3-dimethyl-6-pentyl)-β-cyclodextrin (Universidade Federal de Santa Maria, Santa Maria, Brazil). The column consisted of a mixture of 20% of chiral phase and 80% of OV1701. The initial oven temperature was 50 °C, increased at 2 °C/min to 180 °C, and then held for 5 min. Hydrogen was the carrier gas at a flow rate of 1 mL/min. The injector and detector (FID) temperatures were kept at 220 and 240 °C, respectively. Samples of 1 μL (1 mg/mL in ethyl acetate) were injected, with a 1:200 split ratio.

Bacterial Strains and Cultivation Media. Indicator strain *A. tumefaciens* NTL4(pZLR4) was built by Cha and co-workers (*11*). It was provided for us by Dr. Welington L. Araújo (Universidade de São Paulo, Brazil) and maintained in Luria−Bertani (LB) medium supplemented with gentamicin (50 μg/mL). Nutrient broth (NB) medium (20 g/L) was from Oxoid (Hampshire, England). LB medium was composed of 1% peptone (Oxoid), 0.5% NaCl, and 0.5% yeast extract (Oxoid). Solid medium was prepared with 2% agar (Oxoid). Trypticase soy broth (TSB) medium was prepared as previously described (*2*). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Sigma (Aldrich Chemical Co., Milwaukee, WI).

Isolation and Identification of the Bacterium from Young Lesions. Infected maize leaves were collected at the Experimental Farm of the State University of Londrina (Londrina, Paraná State, Brazil) in December 2003. The bacterial strain was isolated as previously described (*2*). Strain identification was carried out by phylogenetic analyses of sequences from 16S rDNA fragments, according to well-established procedures (*12*). The strain was deposited at the Brazilian Collection of Microorganisms from Environment and Industry (CPQBA, UNICAMP, Brazil) under the code *Pantoea* sp. CBMAI 732.

Infection Assays Using Maize Leaves in a Greenhouse. Pathogenicity assays were carried out with maize variety BR HS200, recognized as susceptible to the "maize white spot" disease. Seeds were sown in pots, totaling three plants per pot, and kept in a greenhouse for 35 days. For bacterial inoculum, the bacterium *Pantoea* sp. CBMAI 732 was cultivated in TSB medium at 30 °C for 12 h. Then, 1 mL of this preinoculum was transferred to 100 mL of TSB medium and incubated under shaking for 4 h. Immediately before inoculation, the inoculum was adjusted to 10^6 cfu/mL by absorbance at 700 nm (*2*, *13*). The maize leaves were softly damaged with a sponge, and then, the bacterial suspension was sprayed. For the control test, sterile water was sprayed on damaged leaves. Inoculated plants were placed in a moist chamber for 72 h at room temperature. Then, the vases were maintained in the greenhouse, remaining there for 144 h. After this time, the leaves were examined for the presence of white spots, characteristic of the disease. No spots were observed with the control plants.

Infection Assays in Microhumidity Chamber. Thirty-five day old maize leaves variety BR HS200 were softly injured with a sponge and attached to an acrylic microhumidity chamber (*14*). A bacterial suspension in sterile saline solution (10^8 cfu/mL) was pipetted (1 drop) into the orifices. A blank was performed with saline solution. After 72 h of incubation at room temperature, the acrylic chamber was removed and the symptoms were evaluated. Positive activities, characterized as white necrotic regions, were observed in bacterial inoculations. No activity was observed in control assays.

Detection of Acyl-Homoserine Lactones. The strain *Pantoea* sp. CBMAI 732 was grown in a test tube containing liquid NB medium (10 mL), incubated at 28 °C for 24 h, and then transferred to NB medium (1 L) to be incubated at 28 °C under shaking at 150 rpm. After 24 h, the culture medium was centrifuged at 4472*g* for 20 min, under refrigeration (5 °C). The aqueous medium was extracted with ethyl acetate (3 × 500 mL). The combined organic phases (1.5 L) were washed with distilled water (1 × 500 mL), dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure at 50 °C. This procedure yielded a crude extract (0.228 g), which was first separated by flash column chromatography (2 g silica gel; column diameter, 1 cm), eluted with hexane (50 mL), ethyl acetate (50 mL), and methanol (50 mL). The ethyl acetate fraction (26.4 mg) was assayed with reporter *A. tumefaciens* NTL(pZLR4) in a β-galactosidase assay, providing positive biological activity (*9*). This active fraction was then purified by silica gel (6.5 g; column diameter, 2 cm) column chromatography eluted with solvents hexane, dichloromethane, ethyl acetate, and methanol in increasing polarity. Fractions were combined on the basis of similarity of thin-layer chromatography profiles of main spots and analyzed by GC-MS. Two acyl-homoserine lactones were identified:

(*S*)-(−)-*N*-*Butanoyl-homoserine Lactone*. Yield, 1.1 mg. GC-MS (EI, 70 eV) *m*/*z*: 171 (M^+, 5), 143 (91), 125 (9), 102 (6), 101 (9), 100 (9), 85 (13), 71 (60), 57 (90), 43 (100). ^{1}H NMR (499.88 MHz, $CDCl_3$, TMS): δ_H 0.97 (t, 3, J = 7.3 Hz, H-4′), 1.61 (m, impurity, H-3′), 2.12 (m, 1, H-4), 2.24 (t, 2, J = 7.3 Hz, H-2′), 2.88 (m, 1, H-4), 4.30 (ddd, 1, J = 5.9; 11.4 and 9.1 Hz, H-5), 4.48 (t, 1, J = 8.9 Hz, H-5), 4.54 (ddd, 1, J = 5.9; 8.8 and 11.7 Hz, H-3), 5.94 (broad s, NH). For circular dichroism (CD) spectrum, see **Figure 2**.

N-Hexanoyl-homoserine Lactone. Trace amounts in complex mixture. GC-MS (EI, 70 eV, SIM) *m*/*z*: 199 (M^+, 3), 156 (12), 143 (100), 102 (12), 101 (15), 100 (9), 99 (22), 71 (19), 57 (32), 43 (31).

Acyl-homoserine Lactones Syntheses. The substances were synthesized as previously described (*15*).

(±)-N-Hexanoyl-homoserine Lactone. Yield, 54%. GC-MS (EI, 70 eV) data were similar to the natural product. IR, ^{1}H, and ^{13}C NMR data were consistent with those previously reported (*7*, *16*).

(±)-N-Butanoyl-homoserine Lactone. Yield, 19%. GC-MS (EI, 70 eV) data were similar to the natural product. IR, ^{1}H, and ^{13}C NMR data were consistent with those previously reported (*7*, *16*).

(S)-(−)-Butanoyl-homoserine Lactone. Yield, 19%. GC-MS, ^{13}C and ^{1}H NMR, DEPT-135, and DEPT-90 data were identical to those obtained for the racemic compound. $[\alpha]_D^{20}$ −23.68° (*c* 0.38 MeOH). For CD spectrum, see **Figure 2**.

(R)-(+)-Butanoyl-homoserine Lactone. Yield, 19%. GC-MS, ^{13}C and ^{1}H NMR, DEPT-135, and DEPT-90 data were identical to those obtained for the racemic compound. $[\alpha]_D^{20}$ + 20.48° (*c* 0.38 MeOH). For CD spectrum, see **Figure 2**.

Preparation of Maize Leaf Extracts for Biological Assays. One maize leaf artificially infected with the strain *Pantoea* sp. CBMAI 732 exhibiting necrotic lesions (cultivated in the greenhouse, BR HS200 variety, described above) was pricked and extracted with ethyl acetate. The suspension was filtered, and the solvent was evaporated under reduced pressure. The crude extract obtained was filtered through a silica gel column (2.0 g, 50 mL of ethyl acetate; column diameter, 1 cm). The same procedure was applied to obtain a healthy leaf extract as the control. Maize leaves (100 days old) were also collected from the cultivation field at EMBRAPA (Londrina, Paraná, Brazil) in March 2005. Leaves were collected from infected plants exhibiting necrosis, from infected plants but without necrosis, and from healthy leaves from BRS1010 maize variety resistant to the disease and without necrosis. Ethyl acetate extracts were obtained and treated as described for the leaves collected from greenhouse plants. Stock solutions of the extracts were prepared in ethanol and employed in qualitative biological assays with reporter *A. tumefaciens* NTL4(pZLR4). The tests were performed in duplicate.

Qualitative Bioassays of Extracts, Fractions, and Synthetic Compounds with Reporter *A. tumefaciens* NTL4(pZLR4). β-Galactosidase expression/induction activities of the synthetic products, ethyl acetate extract, and fractions from *Pantoea* sp. CBMAI 732 strain cultivation medium and extracts from maize leaves were evaluated with reporter *A. tumefaciens* NTL4(pZLR4) as previously described (*9*, *15*). The tests were performed using solutions (20 μL) of each synthetic product [(±)-*N*-hexanoyl-homoserine lactone, (*S*)-(−)-*N*-butanoyl-homoserine lactone, and (±)-*N*-butanoyl-homoserine lactone], the silica gel-filtered ethyl acetate extract from bacterial cultivation medium and the fraction containing natural (*S*)-(−)-*N*-butanoyl-homoserine lactone in ethanol (2 mg/mL), and with solutions (20 μL) of extracts from maize leaves in ethanol (10 mg/mL). The blanks were performed with ethanol (20 μL) and with silica gel-filtered ethyl acetate extract from nutrient broth (Oxoid) medium. The solutions were visually evaluated for positive or negative results after 24 h of incubation at 28 °C. The test was performed in duplicate.

***N*-Butanoyl-homoserine Lactone Absolute Configuration Determination by GC-FID with Chiral Column [Heptakis(2,3-dimethyl-6-pentyl)-β-cyclodextrin].** The analytical conditions for the enantiomeric discrimination were established using synthetic (±)-*N*-butanoyl-homoserine lactone (two peaks in an approximately 1:1 ratio), according to the conditions described above. The identification of the (*R*)- and (*S*)-*N*-butanoyl-homoserine lactone retention times (50.79 and 50.95 min, respectively) was performed by analysis of synthetic (*S*)-(−)-*N*-butanoyl-homoserine lactone (51.01 min). The absolute configuration of the natural product was determined by comparison of the retention times and relative abundances of the (*R*)- and (*S*)-(−)-*N*-butanoyl-homoserine lactone in the synthetic racemic standard, in the natural product and in both samples coinjected.

CD. CD curves were obtained with a Jasco 720 spectropolarimeter, using a 0.1 cm length cell. Samples were dissolved in methanol HPLC grade (Merck) at a concentration of 1 mg/mL. The spectroscopic range was 205−250 nm. Methanol was used as blank. The curves represent an average of 16 acquisitions, at a scanning speed of 50 nm/min with 0.2 nm of resolution.

RESULTS AND DISCUSSION

The importance of the maize white spot foliar disease in Brazilian maize crops has stimulated studies toward the correct identification of the etiological agent and to understand the infection mechanisms. Among several bacterial strains isolated from the maize white spot disease, one was particularly aggressive and displayed fast growth in vitro, in comparison with other isolated strains, and was selected for the present study. The phylogenetic analysis based on 16S rDNA sequences showed a similarity of 96−97% with the species *P. ananatis* and 97−98% with *Pantoea agglomerans* Ewing & Fife. These species are closely related, and their differentiation is difficult (*17*). In vivo leaf inoculation experiments with a susceptible maize variety demonstrated that this strain was able to colonize

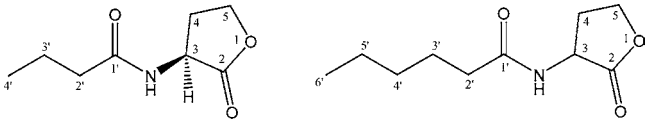

Figure 1. Acyl-homoserine lactones produced by *Pantoea* sp. CBMAI 732.

the plant leaves, causing white lesions characteristic of the disease. Inoculation experiments were performed using greenhouse conditions or employing a microhumidity chamber. The phytopathogenic activity of the strain stimulated a chemical study toward the identification of possible quorum-sensing signaling substances produced by this bacterium.

It is now well-established that many bacterial species exploit intercellular communication mechanisms to regulate the expression of a range of phenotypic factors, such as enzymes, exopolysaccharides, secondary metabolites, and other virulence-important characters. The communication mechanism is based on the production of signaling substances, whose extracellular concentrations are related to the population density. Once a threshold concentration of the signal is reached (meaning a dense bacterial population), the signaling substances bind to specific receptor proteins, and this complex regulates the expression of many genes (*6*). Such orchestrated, multicellular behavior allows the whole population to act coordinately, being an important evolutionary artifice for the host invasion processes (*18*). In Gram-negative bacteria, the acyl-homoserine lactones are the main signaling substances.

Initially, extracts from bacterial cultivation media were assayed with reporter *A. tumefaciens* NTL4(pZLR4) (*11*). This mutant is unable to produce its own acyl-homoserine lactone; however, it can detect exogenous signaling substances present in extracts, fractions, or synthetic products. Exogenous acyl-homoserine lactones bind to the TraR receptor protein, and this complex regulates the expression of an operon, which contains the *lacZ* gene. Expression of *lacZ* generates a β-galactosidase enzyme, which metabolizes the added X-Gal reagent, generating an indigo derivative (*11*). Thus, a blue-green coloration is obtained in the presence of an active substance, while blank controls present the coloration characteristic of the cultivation media (pale yellow). Positive biological activities were observed with extracts from *Pantoea* sp. CBMAI 732 cultivation media, therefore stimulating a more detailed chemical study.

The purification of ethyl acetate extract from bacterial cultivation media allowed the isolation of *N*-butanoyl-homoserine lactone and the detection of trace amounts of *N*-hexanoyl-homoserine lactone in a complex mixture (**Figure 1**). The metabolites were identified by mass spectra comparison and coinjection in GC-MS with synthetic standards and, in the case of *N*-butanoyl-homoserine lactone, also by ^{1}H NMR spectral data comparison with synthetic standard and literature data (*19*). All synthetic products and natural *N*-butanoyl-homoserine lactone provided positive biological activities with reporter *A. tumefaciens* NTL4(pZLR4), corroborating the previous results obtained with *Pantoea* sp. extracts.

The absolute configuration of natural *N*-butanoyl-homoserine lactone was initially determined by GC-FID, using a chiral column (*9*, *15*). Analysis of the synthetic racemic mixture allowed the identification of the (*R*) and (*S*) peaks, eluting at 50.79 and 50.95 min, respectively. The retention time of the (*S*) enantiomer was determined by analysis of synthetic (*S*)-(−)-*N*-butanoyl-homoserine lactone (51.01 min). Analysis of the natural product with the same conditions displayed a signal

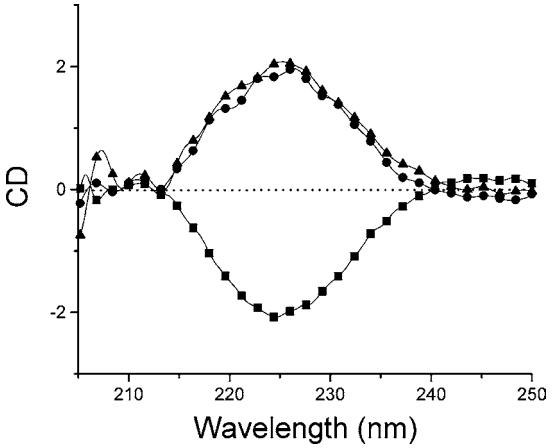

Figure 2. Circular dichroism curves for *N*-butanoyl-homoserine lactone [(*S*) synthetic enantiomer, ●; (*S*) natural product, ▲; and (*R*) synthetic enantiomer, ■].

with retention time (50.90 min) very close to that of the synthetic (*S*) enantiomer. The (*S*) absolute configuration was confirmed by the relative abundance increment of the signal corresponding to the (*S*) enantiomer after coinjecting the natural product with the synthetic racemic mixture. In addition, CD analyses of the natural product and the synthetic enantiomers confirmed without doubt the absolute configuration of natural (*S*)-(−)-*N*-butanoyl-homoserine lactone (**Figure 2**). Both the natural product and the synthetic (*S*) enantiomer displayed a positive Cotton effect between 215 and 240 nm, while the (*R*) enantiomer provided a negative Cotton effect in this range.

The reporter *A. tumefaciens* NTL4(pZLR4) can detect several different acyl-homoserine lactones and mimic compounds, especially those with acyl side chains ranging from hexanoyl to decanoyl, with or without 3-oxo and 3-hydroxy substituents. The short chain *N*-butanoyl-homoserine lactone is not easily detected by this reporter in the usual thin-layer chromatography protocol (*11*, *20*). However, the test tube methodology applied here allowed the biodetection of this substance at the microgram level.

Nowadays, it is recognized that bacterial signaling substances or mimic compounds can occur in hosts in vivo (*21*, *22*). In the present work, the biological activities of maize leaves extracts obtained from healthy and diseased plants cultivated in the greenhouse and in the field were evaluated with reporter *A. tumefaciens* NTL4(pZLR4), and all extracts provided positive biological activity. The presence of acyl-homoserine lactones producing epiphytic/endophytic microorganisms could explain the reporter activation by extracts from healthy leaves. Recently, we have observed the epiphytic behavior of *Pantoea* sp. on maize leaves and also the low disease incidence when the superficial bacterial density is low (*23*). In fact, the disease only appeared after an increase in the bacterial density, and we are presently investigating if this behavior is under quorum-sensing control by the acyl-homoserine lactones described.

Several plants, such as pea, rice, lettuce, soy, and tomato can produce mimic compounds (acyl-homoserine lactones agonists or antagonists), which activate or repress the expression of quorum-sensing controlled phenotypes in different bacterial reporters (*21*). Therefore, plants could interfere in the chemical communication mechanisms exploited by potentially pathogenic bacteria. It is an interesting defense strategy, since the production of mimic signaling substances by the plant could prematurely induce the expression of virulence factors by bacteria before a high population density is reached, improving the chances of successful host defense (*24*). This phenomenon could also be responsible for the positive response observed in the biological assays with healthy maize plant extracts. In spite of the efforts in this field by many groups, the chemical characterization of these agonists has not been achieved up to now, probably due to the very small amounts present in the plants (*21*, *24*).

In conclusion, it was demonstrated here that the strain *Pantoea* sp. CBMAI 732 produces short chain acyl-homoserine lactones, similar to other species of the *Erwinia* alliance (*7*, *9*, *10*, *15*). This is the first report on the occurrence of (*S*)-(−)-*N*-butanoyl-homoserine lactone in the *Pantoea* genus. Efforts are now being focused on investigating whether these substances play any role during the transition of *Pantoea* sp. from the epiphytic to the pathogenic stage.

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