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ORIGINAL PAPER

Burkholderia cepacia XXVI siderophore with biocontrol capacity against *Colletotrichum gloeosporioides*

Sergio de los Santos-Villalobos · Guadalupe Coyolxauhqui Barrera-Galicia · Mario Alberto Miranda-Salcedo · Juan José Peña-Cabriales

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Abstract *Colletotrichum gloeosporioides* is the causal agent of anthracnose in mango. *Burkholderia cepacia* XXVI, isolated from mango rhizosphere and identified by 16S rDNA sequencing as a member of *B. cepacia* complex, was more effective than 6 other mango rhizosphere bacteria in inhibiting the model mango pathogen, *C. gloeosporioides* ATCC MYA 456. Biocontrol of this pathogen was demonstrated on Petri-dishes containing PDA by $>$ 90 % reduction of surface colonization. The nature of the biocontrol metabolite(s) was characterized via a variety of tests. The inhibition was almost exclusively due to production of agar-diffusible, not volatile, metabolite(s). The diffusible metabolite(s) underwent thermal degradation at 70 and 121 °C (1 atm). Tests for indole acetic acid production and lytic enzyme activities (cellulase, glucanase and chitinase) by *B. cepacia* XXVI were negative, indicating that these metabolites were not involved in the biocontrol effect. Based on halo formation and growth inhibition of the pathogen on the diagnostic medium, CAS-agar, as well as colorimetric tests we surmised that strain XXVI produced a hydroxamate siderophore involved in the biocontrol effect observed. The minimal inhibitory concentration test showed that 0.64 μg ml^{-1} of siderophore (Deferoxamine mesylate salt-equivalent) was sufficient to achieve 91.1 % inhibition of the pathogen growth on Petri-dishes containing PDA. The biocontrol capacity against *C. gloeosporioides* ATCC MYA 456 correlated directly with the siderophore production by *B. cepacia* XXVI: the highest concentration of siderophore production in PDB on day 7, 1.7 μg ml^{-1} (Deferoxamine mesylate salt-equivalent), promoted a pathogen growth inhibition of 94.9 %. The growth of 5 additional strains of *C. gloeosporioides* (isolated from mango "Ataulfo" orchards located in the municipality of Chahuites, State of Oaxaca in Mexico) was also inhibited when confronted with *B. cepacia* XXVI. Results indicate that *B. cepacia* XXVI or its siderophore have the potential to be used as a biological control agent against *C. gloeosporioides*; thus diminishing environmental problems caused by the current practices to control this disease.

Keywords Rhizobacteria · Hydroxamate · Anthracnose · Lytic enzymes · Indole acetic acid

S. de los Santos-Villalobos · G. C. Barrera-Galicia · J. J. Peña-Cabriales (✉)
Centro de Investigación y de Estudios Avanzados-IPN, Unidad Irapuato, 36500 Irapuato, Guanajuato, Mexico
e-mail: jpena@ira.cinvestav.mx

M. A. Miranda-Salcedo
Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Apatzingán, Michoacan, Mexico

Introduction

Anthracnose, caused by *Colletotrichum gloeosporioides*, is the main disease in mango production, causing up to 60 % of losses in world-wide in pre- and post-harvest when climatic conditions are optimal to its development, particularly, high relative humidity (Ann et al. 1997; Thahir Basha et al. 2010). This disease has been controlled by excessive applications of fungicides, generating disadvantages such as environmental contamination, resistance development of pathogens and residual contamination in fruits (de los Santos-Villalobos et al. 2011).

Recently, many antagonistic microorganisms have been studied to control diseases of agricultural importance, i.e. *Burkholderia cepacia* complex (Bcc), a group of remarkably versatile bacteria that have been found naturally in a

wide diversity of clinical and environmental habitats (Parke and Gurian-Sherman 2001; Bevivino et al. 2002; Mahenthiralingam et al. 2008). This complex includes plant and human pathogens, plant growth promoting bacteria, bioremediation agents and strains capable of biological control of plant diseases (Hebbar et al. 1998; Okoh et al. 2001; Hwang et al. 2002; LiPuma 2003; Stoyanova et al. 2007). Several secondary volatile or non-volatile metabolites from Bcc with the capacity for pathogen biocontrol have been reported, such as antibiotics, alkaloids and siderophores (Roitman et al. 1990; Cartwright et al. 1995; Xi et al. 1996). Siderophores have gained attention as the major metabolites involved in this biocontrol activity (Cartwright and Benson 1995; Páez et al. 2005).

Siderophores are relatively low molecular weight, ferric ion-specific chelating agents, biosynthesized by bacteria and fungi growing under low iron stress (Neilands 1995). Iron is one of the most important nutrients of organisms because it has an essential metabolic role, i.e. transport, storage and activation of molecular oxygen, amino acid syntheses, respiration, DNA biosynthesis, nitrogen fixation, methanogenesis, reduction of ribonucleotides and dinitrogen, activation and decomposition of peroxides and electron transport (Faraldo-Gómez and Sansom 2003; Katiyar and Goel 2004; Miethke and Marahiel 2007; Sandy and Butler 2009).

Under iron limiting conditions, many microorganisms biosynthesize siderophores in order to solubilize, capture and transport inorganic iron to the cell (O'Sullivan and O'Gara 1992; Carrillo-Castañeda et al. 2005; Sandy and Butler 2009); once the iron is depleted in this environment, other microorganisms that need this element for their growth cannot grow, leading to growth inhibition. Therefore, microorganisms that capture iron via siderophores can effect biological control (Wong et al. 1996; Sritharan 2000).

From an agricultural-management perspective, the control of anthracnose in mango would best be achieved at least two ways: (1) pre- harvest control through the use of biocontrol agents native to mango orchards [involving inoculation/re-inoculation of these microorganisms or increasing their populations using specific agricultural practices (weeds and/or fertilization managements)] and (2) post-harvest control, focusing on the application of metabolites responsible for effectiveness of the biocontrol agents—thus diminishing the hazards inherent in the use of intact microbial cells (and the associated potential risk to human health). Key steps toward the above-described biological control of anthracnose include: obtaining a potential biological control agent and exploring mechanistic details. Here we characterize a metabolite conferring biological control of the causal agent of anthracnose (*C. gloeosporioides*). The metabolite was produced by a bacterium (*B. cepacia* XXVI) isolated from mango tree rhizosphere.

Materials and methods

Sampling site

Soils of ten "Ataulfo" mango trees were sampled according to Avilán (2008) from orchards located in the municipality of Apatzingan, State of Michoacan, an important mango-producing state in Mexico (SAGARPA 2007). The collected samples were transferred to moist chambers and transported in a cooler at 4 °C.

Isolation of microorganisms

Baz agar medium (g L^{-1}) had the following composition: solution 1 (0.4 K_2HPO_4, 0.4 KH_2PO_4, 0.2 $MgSO_4$, 0.02 $CaCl_2$, 0.01 $FeCl_3$, 0.002 Na_2MoO_4, 0.5 yeast extract and 15 agar) and solution 2 (5 arabinose), both solutions were adjusted with HCl to pH 5.7 and autoclaved separately at 121 °C (1 atm) for 15 min. Later solutions were combined-mixed and filter-sterilized cycloheximide (80 mg ml^{-1}) was then added (Estrada-de los Santos et al. 2001). This medium was used to isolate bacteria with biocontrol capacity against *C. gloeosporioides* ATCC MYA 456. This isolation was performed using the direct method as well as by serial dilutions. Thus, 1 g of soil was placed onto Petri-dishes containing Baz agar. Also 10 g of soil was homogenized with 90 ml of 10 mM $MgSO_4 \times 7H_2O$ (autoclaved at 121 °C and 1 atm for 15 min) during 1 h in a rotary shaker at 100 rpm. Next, serial dilutions were prepared up to 10^{-3} and 1 ml of these dilutions was spread onto the surface of Petri-dishes containing Baz agar and incubated at 28 °C for 7 days. All experiments were performed in triplicate.

Biocontrol of *C. gloeosporioides* by isolated bacteria

As reported by Silveira Mello et al. (2004) and Cuervo-Parra et al. (2011), confrontation assays were performed on Petri-dishes containing Potato Dextrose Agar (PDA) inoculating, in the center, 1×10^6 spores of *C. gloeosporioides* ATCC MYA 456 and around the pathogen, in two equidistant points, 1×10^4 CFU of each bacterium isolated. These Petri-dishes were incubated at 28 °C for 7 days. The percentage of growth inhibition of the pathogen (as determined by surface colonization) was calculated using the following equation: $\%\,\text{of growth inhibition} = \frac{Ac-Ab}{Ac} * 100$, where Ac: control mycelial area and Ab: mycelial area in treatment (Schmidt et al. 2009). This assay was completed using three independents replicates.

The bacterial strain presenting the highest biocontrol capability against *C. gloeosporioides* ATCC MYA 456 was used, under same conditions mentioned above, against 5 strains of *C. gloeosporioides* (VI, X, XI, XII and XIV). These isolates were obtained from fruits collected at mango orchards located in the municipality of Chahuites, State of Oaxaca in Mexico.

Molecular identification

Bacterial DNA was extracted from the isolate that presented the major biocontrol against the photogenic fungi studied using the Bacterial/Fungal DNA kit (Cat D6005) Zymo Research and used for the 16S ribosomal DNA gene amplification, according to Weisburg et al. (1991). The product generated was purified using the GFX PCR DNA kit and Gel Band Purification (Cat. 28-9034-70) Illustra, sequenced and compared with sequences deposited in the NCBI GenBank.

Biocontrol of *C. gloeosporioides* ATCC MYA 456 by volatile metabolites of *B. cepacia* XXVI

1×10^6 spores of *C. gloeosporioides* ATCC MYA 456 was inoculated in the center of Petri-dishes containing PDA and another was spread with 1×10^4 CFU of *B. cepacia* XXVI, both plates were placed face to face and they were sealed to prevent the loss of potential volatile metabolites. This experiment was carried out at 28 °C for 7 days. The inhibition of the pathogen was determined according to Schmidt et al. 2009. This assay was performed using three independent replicates.

Biocontrol of *C. gloeosporioides* ATCC MYA 456 by diffusible metabolites of *B. cepacia* XXVI

1×10^4 CFU of *B. cepacia* XXVI was inoculated in 250 ml of Potato Dextrose Broth (PDB) at 28 °C for 4 days in a rotary shaker at 100 rpm. 1 ml of culture was centrifuged at 10,000 rpm for 10 min and filtered through hydrophilic Millipore membrane with pore size 0.45 µm. Petri-dishes containing PDA were inoculated, in the center, with 1×10^6 spores of *C. gloeosporioides* ATCC MYA 456 and 300 µl of the filtered supernatant was placed in each of three points around the pathogen. The inoculated Petri-dishes were incubated at 28 °C for 7 days. The inhibition of the pathogen was determined according to Schmidt et al. 2009. This assay was performed using three independent replicates.

Characterization of metabolite(s) causing inhibition of *C. gloeosporioides*

In order to characterize the nature of the inhibitory metabolite(s), 1×10^4 CFU of *B. cepacia* XXVI was inoculated in 100 ml of PDB and incubated at 28 °C for 4 days in rotary shaker at 100 rpm. The culture was centrifuged at 10,000 rpm for 10 min and filtered through hydrophilic Millipore membrane, with pore size 0.45 µm to obtain a stock of supernatant containing the biocontrol metabolite(s) for further experiments.

Thermostability of metabolite(s)

Three supernatant aliquots of 25 ml each were treated for 15 min to three temperature conditions: 28, 70 °C, or autoclaved to 121 °C (1 atm). Then, each supernatant aliquot was evaluated to determine its biocontrol capability, as described above.

Assay for lytic enzyme activity

Supernatant aliquots obtained above were used to quantify cellulase, chitinase and glucanase activities. These were measured according to the method described by Ghose (1987), Suresh and Chandrasekaran (1998) and Kulminskaya et al. (2001), respectively. One unit of activity was defined as the amount of enzyme that releases one micromole equivalent of GlcNAc or glucose per minute under the specified assay conditions. Enzyme yield was expressed as µmol g^{-1} sust min^{-1}. In relation to chitinase and glucasase/cellulose activities, the reducing sugar released was measured at 565 nm by the 3,5-dinitrosalicylic acid (DNS) modified method (Miller 1959) using N-acetyl-D-glucosamine and glucose as standard, respectively.

Assaying for production of indole acetic acid (IAA)

This test was carried out by inoculating 5×10^3 CFU of *B. cepacia* XXVI in 50 ml of PDB supplemented with 100 ppm of tryptophan and incubated at 28 °C for 6 days at 100 rpm. After incubation, 1 ml of culture was centrifuged at 13,000 rpm for 10 min, then 2 ml of Salkowski reagent was added to the supernatant and incubated for 20 min at room temperature (Glickmann and Dessaux 1995). The samples were measured at 540 nm in a TECAN A-5082 spectrophotometer, model Genius. The production of IAA was performed using three independent replicates.

Assaying for siderophore production

As established by Schwyn and Neilands (1987), the Chrome Azurol S (CAS)-agar assay was used to determine siderophore production by *B. cepacia* XXVI. 1×10^4 CFU of this strain were added to Petri-dishes containing CAS-agar, incubated during 7 days at 28 °C. [CAS-agar was

prepared by combining four sterile solutions. The Fe-CAS indicator solution (solution 1) was prepared with 10 ml of 1 mM $FeCl_3$ (dissolved in 1 mM HCl) and 50 ml of CAS (1.21 mg ml^{-1}), to the resulting blue solution were added 40 ml of CTAB (1.82 mg ml^{-1}). The buffer solution (solution 2) was prepared by dissolving 30.24 g of PIPES in 750 ml salt solution containing 0.3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl, the pH was adjusted to 6.8 with KOH at 50 %, the volume was adjusted to 800 ml and 15 g agar was added. Solution 3 was prepared by dissolving 2 g glucose, 2 g mannitol, 493 mg $MgSO_4$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4$, 1.2 mg $ZnSO_4$ and 1 mg Na_2MoO_4 in 70 ml of water. A solution of 30 ml of 10 % casamino acids was sterilized by filtration (solution 4). All the solutions were mixed carefully adding at the end the solution 1 (Alexander and Zuberer 1991).] The experiments to explore the production of siderophores were performed in triplicate.

Characterizing the siderophore produced by *B. cepacia* XXVI

Hydroxamate

$FeCl_3$ test 2 ml of 2 % $FeCl_3$ were added to 1 ml of the filtered supernatant. The presence of a peak between 400 and 450 nm indicates the nature of hydroxamate (Neilands 1981). The samples were analyzed in a spectrophotometer UV visible VARIAN CARY 3E model.

Tetrazolium test A pinch of tetrazolium salt and 1–2 drops of 2 N NaOH were added to 1 ml of the filtered supernatant. The instant appearance of a deep red color is indicative of hydroxamate siderophores (Snow 1954).

Catecholate

$FeCl_3$ test 1 ml of 2 % $FeCl_3$ was added to 1 ml of the filtered supernatant. The presence of a peak at 495 nm indicates the presence of catecholate (Neilands 1981). The samples were analyzed in a spectrophotometer UV visible VARIAN CARY 3E model.

Arnow's test To 1 ml of culture filtrate was added 0.1 ml of 5 N HCl, 0.5 ml of reagent containing 10 g each of $NaNO_2$ and $Na_2MoO_4 \times 2H_2O$ in 50 ml water. After the formation of yellow color at this point, 0.1 ml of 10 N NaOH (a red color resulted) and enough distilled water was added to reach a volume of 5 ml. Absorbance was read at 515 nm (Arnow 1937) using a spectrophotometer UV visible VARIAN CARY 3E model.

Carboxylate 1 ml of 250 µM $CuSO_4$ and 2 ml of acetate buffer pH 4 were added to 1 ml of the filtered supernatant. The copper complex solution was observed in a UV visible spectrophotometer VARIAN model CARY 3E between 190 and 280 nm (Shenker et al. 1992).

Minimum inhibitory concentration

Different volumes of supernatant were added to Petri-dishes containing PDA reaching concentrations of 0.04, 0.08, 0.16, 0.32, 0.64, 0.80, 0.96 µg ml^{-1} of siderophore (Deferoxamine mesylate salt-equivalent). 1×10^6 spores of *C. gloeosporioides* ATCC MYA 456 was inoculated in the center of these Petri-dishes and incubated at 28 °C during 7 days. After this period, the growth of *C. gloeosporioides* ATCC MYA 456 was measured. This experiment was performed using three independent replicates.

Growth kinetics and siderophore production by *B. cepacia* XXVI and growth inhibition against *C. gloeosporioides* ATCC MYA 456

These tests were carried out incubating $1x10^4$ CFU of *B. cepacia* XXVI in 30 ml of PDB at 28 °C and 150 rpm determining growth kinetics and siderophore production every 24 h during 7 days. Growth kinetics was carried out using optical density at 600 nm using a Varian Cary 3 E model spectrophotometer. Siderophore production was quantified using an equivalent standard calibration curve of hydroxamate nature (Deferoxamine mesylatem salt, Sigma-Aldrich), in a range between 0.1 and 10 µg ml^{-1}, subjecting to the $FeCl_3$ test and measuring absorbance in UV visible range in a Varian Cary 3E spectrophotometer at 420 nm. These tests were performed using two independent replicates. Growth inhibition of *C. gloeosporioides* ATCC MYA 456 by siderophore was performed by inoculating 1×10^6 spores of *C. gloeosporioides* ATCC MYA 456 in 30 ml PDB containing the same siderophore concentration produced by *B. cepacia* XXVI every 24 h during its growth kinetics, centrifuged at 10,000 rpm for 10 min and filtered through hydrophilic Millipore membrane, with pore size 0.45 µm. The growth inhibition of the pathogen was calculated using its mycelia dry weight at 70 °C and expressed as a percentage.

Statistical analysis

Data were analyzed by one-way analyses of variance (ANOVA) test and Tukey–Kramer method ($P = 0.05$) using JMP-SAS software v. 8.0.2.

Results and discussion

Growth inhibition of *C. gloeosporioides* by *B. cepacia* XXVI

Anthracnose, the main disease of world-wide mango production, has been controlled by several alternatives: (1) physical methods such as pruning, ultraviolet light, modified atmosphere, etc. (Stevens et al. 1997; Karabulut and Baykal 2004) and (2) chemical methods as fungicides, copper, ergosterol inhibitor, etc. (Ker 2001; Arias and Carrizales 2007). These alternatives present disadvantages mainly in regard to efficiency of disease control (physics methods) and environmental hazards (chemical methods) as well as high economical costs (de los Santos-Villalobos et al. 2011).

Therefore the discovery and optimization of alternatives to control efficiently this disease is necessary. We studied a biological control using bacterial strains isolated from mango orchards or its metabolite(s) produced. Seven bacterial isolates were obtained in this study, one of them, B4, was identified as a member of Bcc and named strain XXVI. According to Coenye et al. (2001), members of Bcc show similarities in the 16S ribosomal DNA higher than 97.7 %. *B. cepacia* XXVI showed a 99 % similarity with sequences of these strains deposited in the NCBI GenBank. Therefore, we can refer strain XXVI as a member of the Bcc.

This isolate showed the highest growth inhibition against *C. gloeosporioides* ATCC MYA 456, 91.5 ± 0.3 %, when they were co-inoculated onto Petri-dishes containing PDA in confrontation assays (Fig. 1a, b). In addition, *B. cepacia* XXVI was evaluated against five strains of *C. gloeosporioides* VI, X, XI, XII, XIV, which were isolated from mango "Ataulfo" orchards located in the municipality of Chahuites, State of Oaxaca in Mexico. *B. cepacia* XXVI was able to inhibit the growth of these strains, observing 73.1 ± 4.2, 64.2 ± 3.4, 58.2 ± 1.7, 50.6 ± 2.3, 48.2 ± 5.6 %, respectively, suggesting the potential use of this strain or its metabolite(s) as a biocontrol agent against at least *C. gloeosporioides*, the causal agent of anthracnose. Similar results have been reported for others strains of *B. cepacia* showing biological control against several phytopathogenic fungi such as *Botrytis cinerea*, *Schizophyllum commune*, *Fusarium oxysporum*, *Phytium*, *Rhizoctonia solani*, causal agents of blue molt of apple, seed rot of oil palm, wilt of tomato, root rot of pea, root rot of Poinsettia, respectively (Kang et al. 1998; Kamaruzaman and Dikin 2005).

With regard to the potentially volatile metabolites that may contribute to the inhibition of *C. gloeosporioides* ATCC MYA 456, the results of our plate-colonization assays did not indicate that volatile metabolites were significantly involved, 0.3 ± 0.1 % of inhibition. That was dominated by non-volatile, diffusible metabolites reaching 93.2 ± 1.6 %. A similar tendency for both classes of metabolites has been reported by Rahman et al. (2007), observing that the contribution of diffusible metabolites was more significant than by volatile metabolites corresponding to 100 and 26.61 % of inhibition of radial growth of *C. gloeosporioides* using a *B. cepacia* strain as antagonist microorganism.

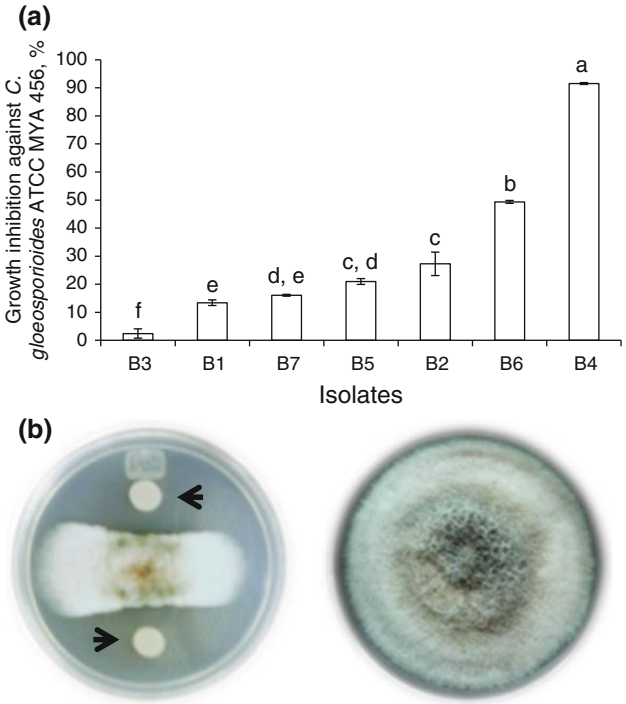

Fig. 1 Confrontation assays **a** of the seven isolates obtained in this study, (*b1*) *B. cepacia* XXVI (*arrowhead*) against *C. gloeosporioides* ATCC MYA 456 and (*b2*) only pathogen inoculated, onto Petri-dishes containing PDA incubated at 24 °C during 7 days. Values with the *same letter* are not significantly different according to ANOVA test ($P = 0.05$)

Once the diffusible metabolites were identified as the main fraction with biological control against *C. gloeosporioides* ATCC MYA 456, the impact of a variety of heat treatments on their biocontrol efficiency was evaluated, observing the loss of activity when they were subjected, during 15 min, at 70 °C and autoclaving at 121 °C (1 atm) compared when they were incubated at 28 °C for the same time period; the latter showed 93.2 ± 1.6 % of inhibition of the pathogen. This thermal degradation of diffusible metabolites produced by *B. cepacia* XXVI suggests that these have a different chemical nature compared with cyclic antibiotic lipopeptides such as iturins and pyrrolnitrin produced by *Bacillus* sp. and *B. cepacia*, which resist these thermal degradations (Bernal et al. 2002; Kadir et al. 2008).

Characterization of metabolites involved in biocontrol

With the aim to characterize the nature of diffusible metabolites involved in the biocontrol against *C. gloeosporioides* ATCC MYA 456 produced for *B. cepacia* XXVI, the activity of lytic enzyme produced by this strain was evaluated, observing no activity of cellulase, glucanase and chitinase contained in the supernatant presenting biocontrol capacity; this eliminated the possible role of lytic enzymes studied in the biocontrol activity of *B. cepacia* XXVI, in contrast to reports by several authors indicating that *B. cepacia* produces beta 1, 3, glucanases, chitinases which are involved in the degradation of cell wall in phytopathogenic fungi (Fridlender et al. 1993; Compant et al. 2005).

We considered the possibility that the inhibitory factor may be IAA. Our rationale was that, in addition to its role as a phytohormone, IAA has been reported as antifungal metabolite against *Phymatotrichum omnivorum*, *Penicillium herquei*, *Fusarium nivale*, *Thielavia terricola*, and *Cunninghamella echinulata* in high concentration up to 400 ppm, evidenced by early studies (Leonian and Lilly 1937; Leelavathy 1969). Results for IAA assays were negative for *B. cepacia* XXVI, indicating non-role of this phytohormone on the inhibition of *C. gloeosporioides* ATCC MYA 456.

Assays for siderophore production by *B. cepacia* XXVI were conducted using CAS-agar. We observed that strain XXVI is able to produce siderophore as indicated by the presence of an orange halo (Fig. 2a) (Schwyn and Neilands (1987). To explore the role of this siderophore in the biological control against *C. gloeosporioides* ATCC MYA 456, a confrontation assay on CAS-agar between the pathogen and *B. cepacia* XXVI was conducted. We observed that this metabolite is involved in the biocontrol capacity against *C. gloeosporioides* ATCC MYA 456, as shown by its growth inhibition in the orange halo zone, with the iron limiting condition of less than 0.56 μg ml^{-1} of iron (Fig. 2b). *C. gloeosporioides* is dependent on iron availability, thus, in an environment with iron limitation imposed by siderophore action, *C. gloeosporioides'* growth is inhibited, and it is restored when this element is added (Santoyo et al. 2010). Several authors have reported the role of siderophores in biocontrol of a wide diversity of bacteria genera such as *Pseudomonas*, *Streptomyces*, *Ochrobacterium*, *Rhizobium* and *Burkholderia* as biocontrol metabolite against *Pyricularia*, *Fusarium*, *Alternaria*, *Macrophomina*, *Sclerotium* and *Phytophotora* (Arora et al. 2001; Díaz de Villegas et al. 2002; Ezziyyani et al. 2004; Chaiharn et al. 2009).

The class of siderophore produced by *B. cepacia* XXVI, involved in its biocontrol capability, was identified using colorimetric test (Tetrazolium test) and spectrophotometric assays ($FeCl_3$), showing that this metabolite belongs to hydroxamate class, due to the appearance of a deep red color and the presence of an only peak at 420 nm, respectively.

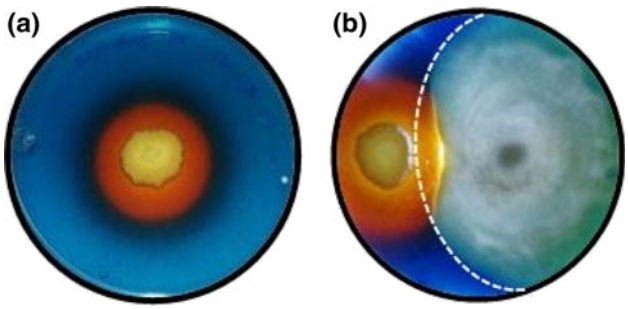

Fig. 2 Identification of siderophore production by CAS-agar indicated by an orange halo at 28 °C, **a** *B. cepacia* XXVI after 3 days of inoculation and **b** confrontation assays of co-inoculated *B. cepacia* XXVI and *C. gloeosporioides* ATCC MYA 456 after 3 days of inoculation (the pathogen was inoculated 3 days prior to *B. cepacia* XXVI), the dotted line represents the expected radial growth of the pathogen in absence of *B. cepacia* XVVI

Minimum inhibitory concentration

Inhibition of *C. gloeosporioides* ATCC MYA 456 was quantitatively correlated with siderophore concentration. We found that a concentration of 0.64 μg ml^{-1} of siderophore (Deferoxamine mesylate salt-equivalent) is sufficient to inhibit 91.1 ± 0.5 % of pathogen growth and this percent did not increase significantly when a siderophore concentration of 0.96 μg ml^{-1} was added to Petri-dishes containing PDA (Fig. 3). These results suggest that 0.64 μg ml^{-1} of siderophore is sufficient to chelate and decrease the major iron concentration in the medium. Similarly, Santoyo et al. (2010) had reported that siderophore concentration is inversely related to the amount of iron available. These data are consistent with the notion that the increased siderophore production by the biocontrol strain triggers inhibition of pathogen fungi due to the starvation of iron. This inhibition can be restored when iron is added to the medium.

Growth kinetics, siderophore production by *B. cepacia* XXVI and its growth inhibition against *C. gloeosporioides* ATCC MYA 456

As shown in Fig. 4, *B. cepacia* XXVI reached its exponential phase at 4th day of incubation, observing a minimal production of siderophore, 0.2 ± 0.0 μg ml^{-1} (Deferoxamine mesylate salt-equivalent), probably due to background iron concentration in the medium, leading to low inhibition of the pathogen, 9.8 ± 0.1 %, suggesting that siderophore is a secondary metabolite as previously

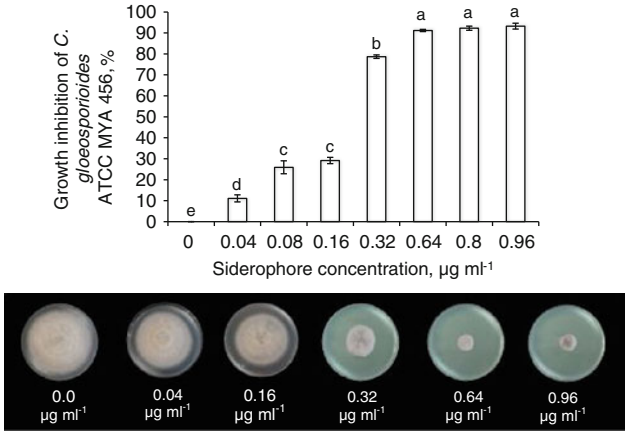

Fig. 3 Minimum inhibitory concentration of siderophore produced for *B. cepacia* XXVI against *C. gloeosporioides* ATCC MYA 456 onto Petri-dishes containing PDA incubated at 28 °C during 7 days. Means with the *same letter* are not significantly different, according to ANOVA test ($P = 0.05$)

reported by Drechsel and Jung (1998) and Fischbach et al. (2006). Siderophore production increased, during the stationary phase (at 4th day of incubation) correlating with the inhibition of *C. gloeosporioides* ATCC MYA 456, observing a high production of this metabolite, 1.7 ± 0.1 µg ml^{-1} (Deferoxamine mesylate salt-equivalent), and inhibition of the pathogen, 94.9 ± 4.3 %, at 7th day of incubation. Data suggest that in the stationary phase, the amount of siderophore was increased due to a state of iron starvation attributed to the consumption of this element in the culture medium as a result of bacterial multiplication (Crosa 1997; Lim et al. 1998; Cowart 2002; González-Carreró et al. 2002), resulting in the pathogen inhibition when this siderophore was added to the culture medium. These complex iron-siderophores present a high dissociation constant ($\sim$pKa = 29) generating a strong starvation of iron in the environment when the concentration of this element is low (Chen et al. 1994), helping to capture and supply this element to siderophore-producing strains under that condition (Loper and Henkels 1999).

This capability has great environmental significance, during the interaction (colonization and establishment) of siderophore-producing strains/plants, conferring competitive advantages for space and nutrients by these strains (Harrison et al. 2008; Eberl and Collinson 2009).

Our results suggest the role of siderophore produced by *B. cepacia* XXVI (isolated from mango orchards) as an alternative to biocontrol the pathogenic fungus causing anthracnose in mango, *C. gloeosporioides*.

It is important to mention that *B. cepacia* strains have been reported as human or plant pathogens, therefore, the potential application of this biocontrol alternative requires further studies focusing mainly on the characterization of these pathogenic traits. Alternatively, the optimization of siderophore production in large scale to apply this metabolite, alone, against anthracnose in pre and/or post-harvest opens an attractive alternative.

Conclusions

Burkholderia cepacia XXVI is a promising biological control agent against the causal agent of anthracnose, *C. gloeosporioides*, through the production of hydroxamate siderophore. The siderophore is produced in high concentration at the stationary phase, when the iron concentration in the medium was decreased by bacterial growth, reaching 1.7 ± 0.1 µg ml^{-1} (Deferoxamine mesylate salt-equivalent), and showing a growth inhibition of the pathogen of 94.9 ± 4.3 % at 7th day of incubation.

These results support the potential use of *B. cepacia* XXVI or its siderophore as a microbial alternative to control pathogens involved in high losses of agricultural production, diminishing the environmental problems caused by current practices.

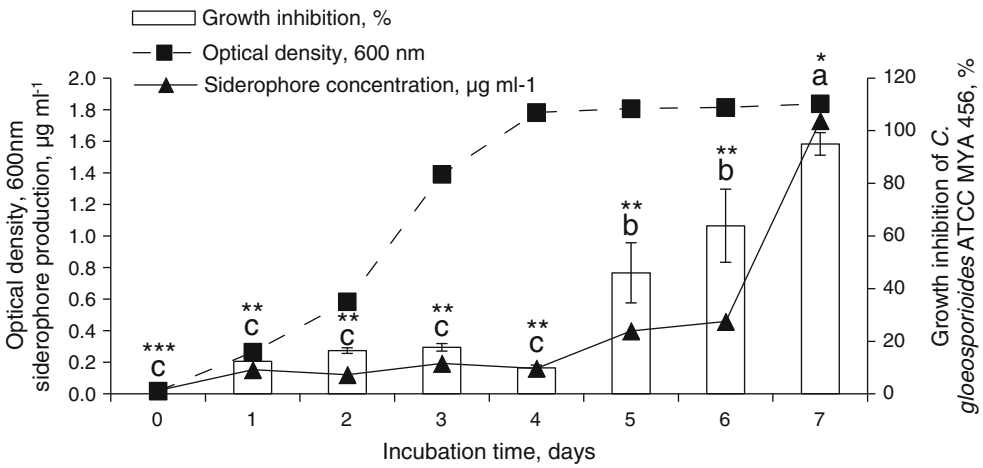

Fig. 4 Siderophore production (Deferoxamine mesylate salt-equivalent) during the growth of *B. cepacia* XXVI measured every 24 h over 7 days, contrasting this siderophore production with growth inhibition of *C. gloeosporioides* ATCC MYA 456 in broth culture by suspension of *C. gloeosporioides* spores in PDB. Means with the *same letter* (growth inhibition) and *asterisk* numbers (siderophore production) are not significantly different according to ANOVA test ($P = 0.05$)

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