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ORIGINAL ARTICLE

In vitro growth inhibition of *Curvularia gudauskasii* by *Bacillus subtilis*

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Abstract Five rhizosphere *Bacillus subtilis* isolates were tested for in vitro antagonism against a sugarcane seedling pathogen strain of *Curvularia gudauskasii*. The isolate *B. subtilis* SR/B-16 was selected because it permanently inhibits 71% of fungal growth. Microscopic analysis of the antagonistic bacteria effects on *C. gudauskasii* revealed swelling and bulbous hyphae, vacuolated cytoplasm and no spore formation. The analysis of culture filtrates of SR/B-16 collected from stationary phase showed that they are responsible for the antifungal effects and for the abnormal shapes observed. However, bacterial culture efficiency is higher. The organic concentrated fraction of the extracts caused the same morphological changes as those caused by antifungal lipopeptides secreted by *Bacillus* species. The strain SR/B-16 can be used for the formulation of bioactive compounds for the treatment of *C. gudauskasii* diseases on sugarcane seedlings.

Keywords *Bacillus subtilis* · Culture filtrate · Antifungal · *Curvularia gudauskasii* · Hyphal abnormality · Biocontrol

Introduction

Numerous plant species are affected by *Curvularia* plant pathogen fungi, mainly under climatic stress periods with high temperatures and environmental humidity. *Curvularia* phytopathogen species produce spots in grains and seeds, as well as damage to plant leaves (Barrios and Pérez 2005).

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In Cuba, *Curvularia* plant pathogen species are associated with sugarcane seedlings, producing root rottenness and malformations affecting fertility and seedbed efficiency (Alfonso et al. 1990). The most common species, *Curvularia lunata*, *Curvularia senegalensis* and *Curvularia gudauskasii* affect seed bank yields by up to 30% (Alfonso et al. 1990; Chinea 2005). Treatment of this plant disease is usually by chemical fungicides. Some attempts at selecting biological control agents (BCA) against *Curvularia* phytopathogen species have revealed their in vitro susceptibility to *Trichoderma viridae*, *Trichoderma harzianum* and *Trichoderma lignorum* (Alfonso et al. 1990). Also, *Pseudomonas* and *Bacillus* strains have been reported to have inhibitory activity against *C. lunata* in dual culture assays (Alfonso and Villa 2002; Basha and Ulaganathan 2002; Tendulkar et al. 2007).

Bacillus and its relatives are attractive candidates for bio-controlling plant pathogens because they produce extracellular metabolites such as antibiotics lipopeptides and hydrolytic enzymes, which play an important role in the direct antagonism of plant disease fungi (Kildea et al. 2008; Yang et al. 2008; Saidi et al. 2009). Their interaction with filamentous fungi induces hyphae malformations related to their biological control mechanisms. The predominant morphological alterations are swelling and bulbous formation, hyphae distortion, cell wall darkness, cytoplasm aggregation, inhibition of spore germination and hyphae tip rupture (Rahman et al. 2007; Prapagdee et al. 2008).

In a previous study, five *Bacillus subtilis* rhizosphere strains exhibited a marked inhibitory effect on the plant pathogen fungi *Curvularia lunata*, *Fusarium oxysporum* and *Colletotrichum* sp. (Orberá et al. 2009). This research was aimed at evaluating if the bioactive compounds secreted by those *B. subtilis* antagonistic isolates are able

to generate morphological alterations related to the in vitro growth inhibition of a *Curvularia gudauskasii* strain causing damage to sugarcane seedlings.

Materials and methods

Microorganisms

Bacteria

Five aerobic endospore-forming bacterial isolates with antagonistic properties against plant pathogenic fungi were used (Orberá et al. 2009). The strains were previously identified as *Bacillus subtilis* and the 16S rRNA gene partial sequences accession numbers, deposited at the National Centre for Biotechnology Information Database are: SR/A-1 [HQ025915], SR/B-8 [HQ025916], SR/B-16 [HQ025917], SR/B-17 [HQ025918], and SR/A-17 [HQ025919] (NCBI, http://www.ncbi.nlm.nih.gov).

Fungi

A phytopathogen strain of *Curvularia gudauskasii* was provided by the Phytopathology Laboratory strain collection at the National Sugarcane Research Institute (INICA), Havana, Cuba.

The microorganisms were maintained at −20°C on glycerol, at the Industrial Biotechnology Study Centre Culture Collection (CCEBI) [http://www.aam.org.ar/cultivos_microbianos.shtml]. Bacteria were subcultivated on nutrient agar (NA, Biolife Italiana, Milan, Italy) and nutrient broth (NB, Biolife Italiana). Fungi were grown on potato dextrose agar (PDA, UNI–CHEM Chemical Reagents; http://www.unichem-chemicals.com/).

Bacillus subtilis strains with antifungal activity against *Curvularia gudauskasii*

The dual culture assay was performed in Petri dishes containing PDA medium. A 5-mm diameter mycelial plug taken from a 5-day-old culture of *C. gudauskasii* was transferred to the center of the Petri dish. One milliliter of each *Bacillus subtilis* strain suspension (10^6 CFU mL^{-1}) was inoculated at an equidistant point (20 mm) from the Petri dish center, which was inoculated with the mycelial plug. The plates were incubated in the dark at 30°C. Trials were done in triplicate and a control was prepared with the fungal culture without bacterial inoculation. The appearance of a growth inhibition zone of the fungal colony was taken as a positive result (Muhammad and Amusa 2003). The inhibitory effect was assessed following Ezziyani et al. (2004), by calculating the radial growth inhibition percentage (RGIP) based on the following formula:

$$\mathrm{RGIP} = \frac{\mathrm{R1} - \mathrm{R2}}{\mathrm{R1}} \times 100\% \quad (1)$$

where,

R1 Radial growth of *C. gudauskasii* in the control plate
R2 Radial growth of *C. gudauskasii* interacting with the antagonistic *B. subtilis* strain

Hyphal morphology

Following positive antibiosis test results, hyphal strands from the border of the fungal colony nearest to the growth inhibition zone were removed smoothly using a sterile needle and placed on a microscope slides with lactophenol 28.5% (BDH Chemicals, Poole, UK) and examined under a light microscope (40×), looking for hyphal abnormalities. Microphotographs were taken using a digital camera (Canon Power Shot A640; Rahman et al. 2007).

Bacterial growth kinetics

fresh bacterial culture of *B. subtilis* SR/B-16 from a NA slant previously grown for 18 h at 30°C, was inoculated into a 250 mL Erlenmeyer flask containing NB medium. The culture was incubated at 30°C for 12 h and shaken vigorously at 150 rpm. For growth curve estimation, 1 mL from the pre-inoculum was inoculated into a 500-mL Erlenmeyer flask containing 100 mL NB. The culture was then incubated for 72 h at 30°C. Cellular growth was determined spectrophotometrically by measuring changes in optical density (OD) with time at 620 nm.

Characterization of bacterial culture filtrates

Bacterial culture was transferred to NB and incubated for 6 and 21 h, while shaking vigorously at 150 rpm at 34°C. Cells were removed by centrifugation at 5,000 rpm at 10°C for 10 min. The supernatant collected was filtered through a 0.2 μm pore size Millipore membrane. The protein and carbohydrate content, as well as the qualitative presence of lipids were determined on the culture filtrates. Protein content was determined following the Lowry technique, using a solution of bovine serum albumin (BSA; BDH, Poole, UK) as standard (Lowry et al. 1951). The absorbance was read at 650 nm. Soluble carbohydrates were determined using the phenol–sulfuric method (Dubois et al. 1956). Spectrophotometer readings were taken at 490 nm. A glucose solution (UNICHEM, 1.00 g L^{-1}) was used as

standard. The qualitative presence of lipids was determined using the Sudan III coloring technique (Díaz et al. 1989). A test tube with sterile medium was used as a negative control.

Concentration of free-cell extracts

Sterile extracts were separated with chloroform: methanol mixture (2:1) according to the Folch method (Folch et al. 1957). The organic extract was concentrated in vacuo, yielding a brownish gummy residue, which was suspended in 5 mL phosphate buffer at pH 6.5. The soluble phase was kept at 4°C to be used later.

Antagonism test with free-cell extracts

Antagonism tests with free-cell extracts were carried out using the plate diffusion technique described by Vignolo et al. (1993). Sterile filtrates collected at 6 and 21 h of incubation were evaluated, as well as the organic and inorganic fractions obtained from the 21-h extracts. Aliquots of 100 μL of the crude extract were deposited in sumps opened in the surface of the solid medium surface on Petri dishes, previously inoculated with a mycelium plug. The plates were incubated at 30°C for 7 days until the appearance of mycelium growth inhibition zones. Hyphal abnormalities were studied as described (Rahman et al. 2007).

Statistical analysis

The means and standard deviations of the inhibition of radial growth were calculated. Data were analyzed by one-way analysis of variance (ANOVA). Significant differences ($P \leq 0.05$) among the means were determined by Duncan's multiple range tests, using the software Statgraphics Plus 5.1 for Windows (http://www.statgraphics.com/statgraphics_plus.htm).

Results

In vitro growth inhibition of *Curvularia gudauskasii* by *Bacillus subtilis*

Growth inhibition of *C. gudauskasii* by *B. subtilis* isolates is represented in Fig. 1. Bacteria showed values of RGIP of between 41 and 71%, corresponding to the isolates SR/A-17 and SR/B-16, respectively. Strains SR/B-8 and SR/B-16 had similar significantly higher inhibitory effects; however, the latter was the most efficient. All the strains were classified as moderately toxic according to the scale used by Ros et al. (2008), which considered as toxic only those isolates inhibiting fungal growth by 100%.

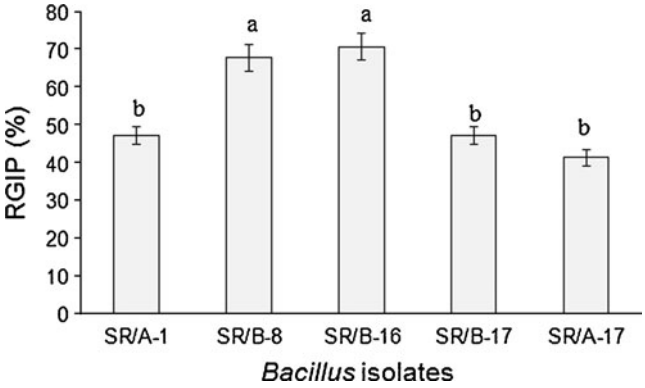

Fig. 1 In vitro radial growth percentage inhibition (RGPI) of *Curvularia gudauskasii* by *Bacillus subtilis* isolates. Different lower case letters indicate statistical differences at $P \leq 0.005$

Curvularia gudauskasii mycelium alterations upon interaction with *Bacillus subtilis* antagonistic strains

Dual culture on Petri dishes of *B. subtilis* strains SR/A-1, SR/B-8 and SR/B-16 with *C. gudauskasii* is shown in Fig. 2. A growth inhibition zone produced by bacteria extracellular metabolites was observed around the fungal mycelium. This zone was visible next to isolates SR/A-1, SR/B-17 and SR/A-1 from the 3rd to the 5th incubation day, when the fungal growth restarted; however, the rate was lower and a loss of the typical dark color of mature *Curvularia* colonies was observed (Fig. 2d). As *Curvularia* pigmentation is attributed to the presence of asexual conidia, this loss of color indicated that antagonistic *B. subtilis* strains inhibited spore formation in this plant pathogen fungus. No mycelial growth was seen around strains SR/B-8 and SR/B-16 after 15 incubation days. Necrosis and darkening colour were also observed in the border of the colony nearest these isolates (Fig. 2d). *B. subtilis* SR/B-16 showed the strongest antifungal effects, and the study was continued using only this strain and its extracellular metabolites. Microphotographs showed hyphal swelling, bulb formation, a granular cytoplasm with an intense vacuolization, and absence of conidia (Fig. 2e–f).

Cell-free extract effects of SR/B-16 on *Curvularia gudauskasii* morphology

The encounter of *C. gudauskasii* growing mycelium and SR/B-16 sterile filtrates collected during its active growth, did not show any alterations on hyphae and colony morphology. However, fungal cultivation with the extracts obtained at stationary phase (21 hours) corroborated that they contain the metabolites implicated in bacterial anti-fungal activity (Fig. 3). The in vitro interaction of *C. gudauskasii* with the collected sterile extract of stationary phase *B. subtilis* SR/B-16 generated a mycelium growth

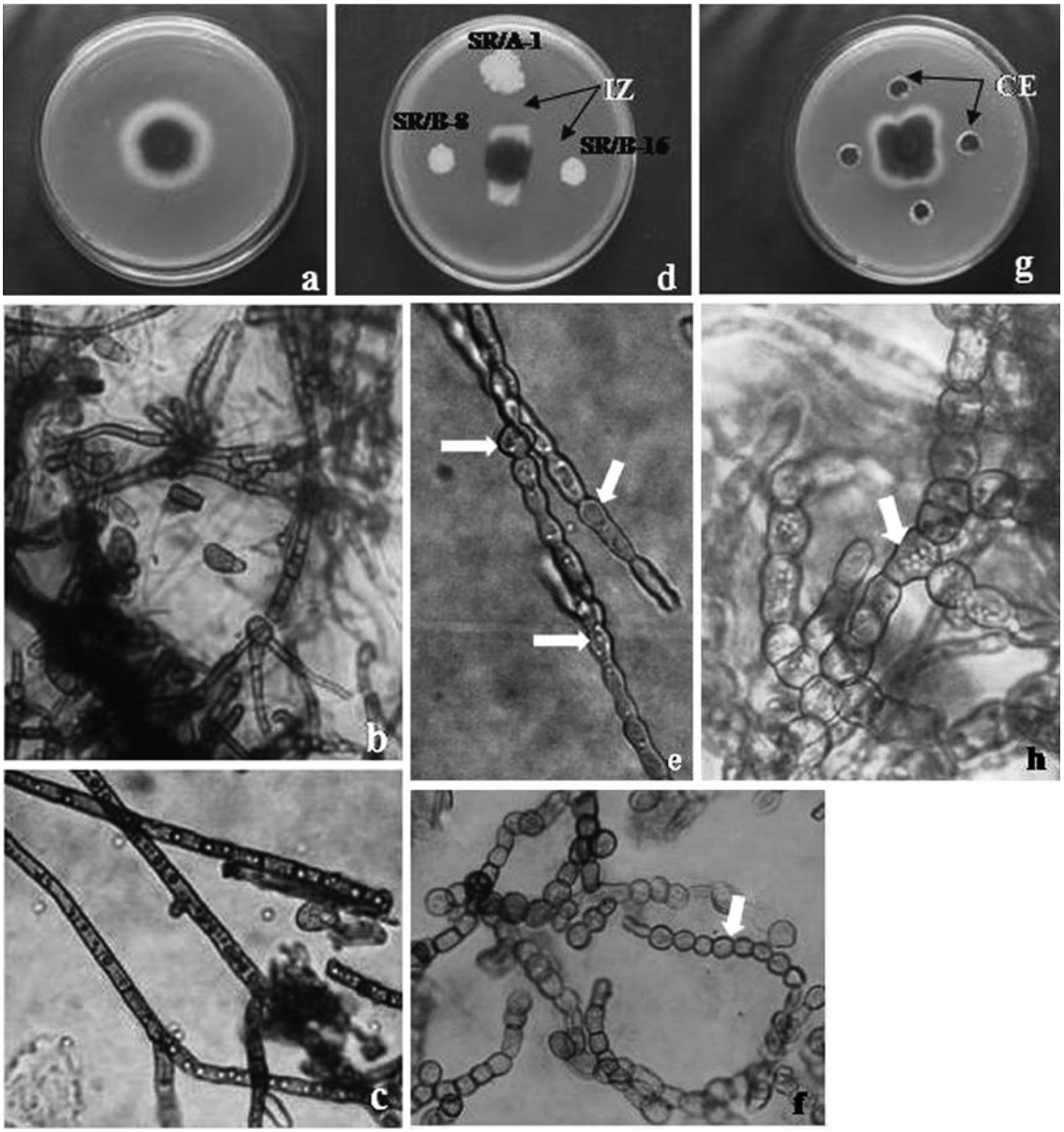

Fig. 2 In vitro interaction effects of *B. subtilis* antagonistic strains and *C. gudauskasii* (40× magnification). **a** Control plate; **b**,**c** control mycelium; **d** dual culture of *C. gudauskasii* and *B. subtilis* isolates; **e**,**f** *C. gudauskasii* hyphae encounter with *B. subtilis* SR/B-16 (*arrows* vacuolization, bulb formation, swelling, no conidia); **g** fungal colony in interaction with SR/B-16 sterile extracts; **h** *C. gudauskasii* hyphae interaction with SR/B-16 sterile extracts (*arrows* swelling, vacuolization, no conidia). *IZ* Growth inhibition zone, *CE* cell free extracts

inhibition zone (Fig. 2g). The corresponding hyphae microphotographs showed swelling, intense vacuolization and no conidia formation (Fig. 2h).

Characteristics of SR/B-16 free-cell extracts

Protein and carbohydrate contents in the sterile extracts collected at 6 and 21 incubation hours were determined (Fig. 3). Total protein content was higher in exponential phase, and decreased when active growth ended. Carbohydrates decreased along with bacterial growth kinetics, showing that they were consumed as nutrients. The qualitative presence of lipids was determined only in extracts collected at stationary phase.

Antifungal effects of concentrated extracts of SR/B-16

The cell-free extract of SR/B-16 strain was partitioned into aqueous and organic phases in order to evaluate their effects on fungal morphology. The in vitro interaction of the organic fraction of *B. subtilis* SR/B-16 sterile extracts with *C. gudauskasii* caused abnormal appearance of the fungal

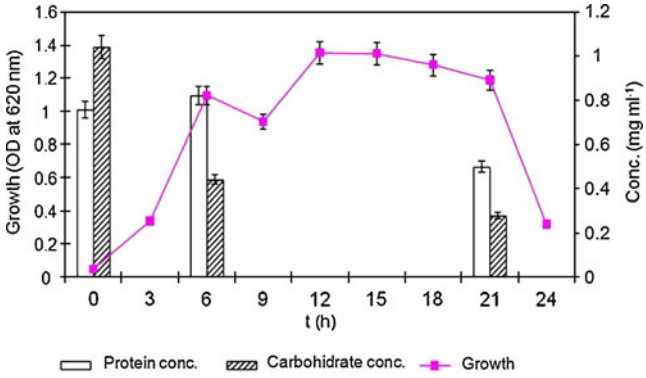

Fig. 3 *Bacillus subtilis* SR/B-16 growth on nutrient broth (NB). Protein and carbohydrate concentrations in the crude extracts during growth stages are indicated

cells. Microphotographs (Fig. 4) show cell wall thickening, a reduction in hyphae diameter and the absence of asexual conidia. The interaction of *C. gudauskasii* with the inorganic fraction did not generate mycelium alterations.

Discussion

The present study explored, for the first time, the potential use of *Bacillus subtilis* rhizosphere isolates for the biological control of the sugarcane seedling pathogenic fungus *Curvularia gudauskasii*. In vitro fungal growth inhibition values coincided with previous results shown by other researchers. In Cuba, inhibition of *Curvularia* phytopathogenic species by *B. subtilis* ATCC 6633 with radial growth inhibition has been reported to be between 66 and 100% (Castellanos et al. 2002). Rahman et al. (2007) isolated *Pseudomonas aeruginosa* and *Burkholderia cepacia* strains that strongly inhibited the growth of *Colletotrichum gloeosporoides* by an average of 74 and 68%, respectively. Numerous *Bacillus* species inhibited the in vitro growth of a *Fusarium oxysporum* f.sp. *radicis-lycopersici* isolated from tomatoes cultivars by more than 50% (Saidi et al. 2009).

In order to determine how rhizobacterial isolates affect fungal growth, the presence of hyphal malformations in *C. gudauskasii* colonies was investigated using a dual culture assay with *B. subtilis* SR/B-16. The bacterial strain and its sterile crude extracts showed the highest antagonistic activity. The swelling and the absence of conidia observed in the plant pathogen fungus under the light microscope are abnormalities caused by antagonistic bacteria and conventional antifungal compounds interacting with filamentous fungi. Swelling has been detected in the presence of quitinases enzymes and antibiotics such as iturins, subtilisins and surfactins (Tendulkar et al. 2007; Rahman et al. 2007; Prapagdee et al. 2008). Swelling is also related to the disorganization of cellular actin filaments produced by secondary metabolites from antagonistic rhizobacteriae (Deora et al. 2010). The absence of conidia has been reported as one of the malformations generated by antifungal isolates of *Paenibacillus lentimorbus*, *Burkholderia cepacia* and *Streptomyces hygroscopicus* and their sterile filtrates (Chen et al. 2003; Rahman et al. 2007; Prapagdee et al. 2008). Both swelling and the absence of conidia were observed on *Neurospora crassa* grown in the presence of conventional chemical fungicides (Pereira and Said 2009).

The necrosis observed in the mycelium border at the interaction zone with the strains SR/B-8 and SR/B-16 is probably related to fungal growth inhibition. It is well known that alterations in the apex of hyphae of filamentous organisms affect major biological processes such as growth, reproduction, morphogenesis, nutrient absorption and protein secretion taking place in the tips (Pereira and Said 2009).

The morphological alterations in *C. gudauskasii* seen upon application of crude extracts of SR/B-16 are less severe than those seen in dual culture assay with bacterial colonies. This suggests that bacteria act through a combination of at least

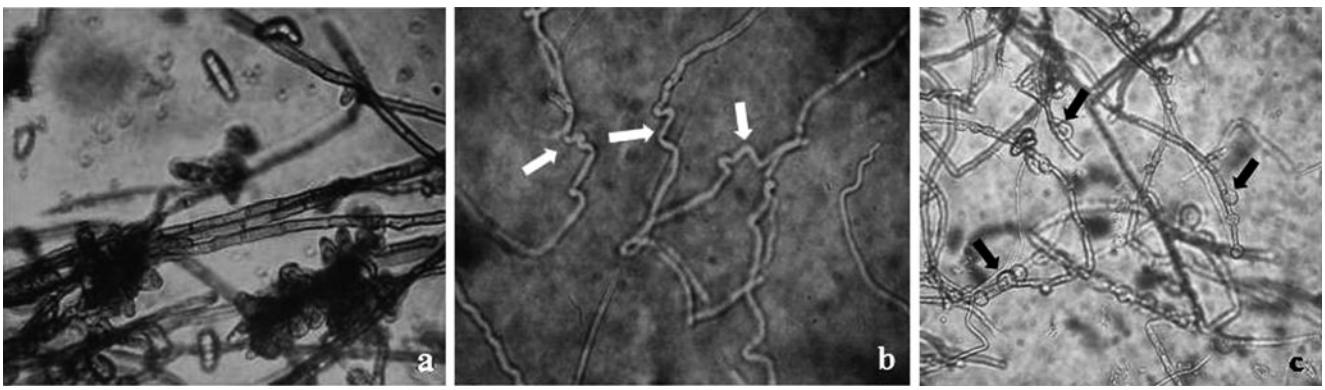

Fig. 4 Morphological abnormalities of *C. gudauskasii* mycelium in interaction with the organic concentrate of *B. subtilis* SR/B-16 crude extract (40× magnification). **a** Control mycelium; **b**,**c** mycelium encounter with organic extract (*arrows* distortion, hialinization, bulbs formation, absence of conidia)

two biological control mechanisms: nutrient competition and antifungal metabolite secretion. A similar phenomenon was produced by a *B. subtilis* strain with inhibitory effects on *Trichoderma* species contaminating commercial mushroom production (Chittihunsa et al. 2007). In vitro dual culture between an antagonistic *Bacillus lentimorbus* isolate and *Colletotrichum gloeosporoides* showed that nutrient competition at early stages and antibiosis at advanced stages are responses to their stress interaction (Lee et al. 2005). This result is invaluable during the selection of an efficient biological control agent (BCA). Antifungal metabolite secretion is known to offer more advantages than competition, but the synergistic effects in a BCA strain increase considerably its effectiveness as a plant disease treatment.

Bacillus subtilis SR/B-16 developed diauxic growth in NB culture medium, which may be attributed to the initial hydrolysis of peptone, a simple carbon source present in the culture medium. After that, SR/B-16 entered a second lag phase, probably developing physiological mechanisms to hydrolyze beef extract, a more complex carbon source present in NB, showing the wider potential of this strain to grow in a variety of carbon sources. Both peptone and beef extract supplied *B. subtilis* SR/B-16 with the nutrients required for growth. In the technical sheet of NB culture medium from Biolife Italiana (http://www.biolifeit.com/biolife/upload/file/Schede/TS-401810.pdf), the culture medium is referred to as beef peptone media. Peptone hydrolysis by *Bacillus* strains has been reported by Vasileva-Tonkova et al. (2007).

The presence of proteins in the sterile extracts of SR/B-16 is attributed to the ability of *Bacillus* and its relatives to excrete numerous enzymes, signals and antifungal peptides (Arguelles-Arias et al. 2009). The decrease in the final protein content may be provoked by the production of bacterial extracellular proteases. Razo et al. (2008) reported maximum levels of proteases in a *B. subtilis* strain at the end of its active growth.

The detection of proteins and lipids in SR/B-16 crude extract collected at stationary phase, led us to infer something about the lipoprotein nature of the antifungal metabolites secreted by this bacteria. Also, hyphae malformations in the presence of the filtrate are similar to those induced by *Bacillus* antifungal lipopeptides. Low spore numbers, bulbous hyphae showing patchy and vacuolated cytoplasm are the morphological changes induced by the antibiotic iturin secreted by a *Bacillus licheniformis* strain (Tendulkar et al. 2007). Distortion, the most evident hyphal abnormality observed in *C. gudauskasii* in the presence of the organic fraction of SR/B-16 crude extract, is the major abnormality generated by antibiotic cyclic lipopeptides; their interaction with fungal cell walls creates membrane channels producing ion extrusion and apical growth pattern alterations (Stein 2005).

Numerous antimicrobials produced by *B. subtilis* and *Bacillus amyloliquefaciens* are cyclic peptides, such as iturins, surfactins, and fengicins, excreted as secondary metabolites. All play different roles in the growth and survival of *Bacillus* species in their natural habitat, rhizosphere colonization and their biological control efficacy (Arguelles-Arias et al. 2009). However, to confirm our suggestions regarding the nature of the antifungal compounds from *B. subtilis* SR/B-16, more studies, including characterization and purification tests, mass spectra and NMR, will be needed.

Conclusions

B. subtilis rhizosphere isolates were evaluated for their potential for biological control of the phytopathogen *C. gudauskasii*. The wild type strain SR/B-16 inhibits the in vitro growth of the plant pathogen fungus by more than 70%, probably through a combination of two biological control mechanisms: nutrient competition and the excretion of antifungal lipopeptides as secondary metabolites. Sterile filtrate collected at stationary phase contains the bioactive compounds responsible for the alterations in morphology and growth pattern, as well as the absence of conidia, which are related to fungal growth inhibition. Therefore, we suggest that *B. subtilis* SR/B-16 may be used as an inoculant for the biological control of diseases caused by *C. gudauskasii* in sugarcane seedbeds.

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