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Full Length Research Paper

Identification and partial characterization of antifungal and antibacterial activities of two *Bacillus* sp. strains isolated from salt soil in Tunisia

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Two *Bacillus* sp. strains (B_{29} and B_{27}) isolated from soil in the South of Tunisia were tested for their abilities to produce antimicrobial compounds. Both strains showed antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and fungi. The produced compounds were extracted by using four different solvents. The hexane extract allowed to obtain maximum of activity of the strain B_{29}. The activity of the strain B_{27} was not elucidated by the four solvents used. Bio-autography results of B_{29} hexane extract revealed presence of different antibiotics and antifungal compounds with different R_f values of 0.3 and 0.76 for antifungal compounds and of 0.12, 0.14, 0.19 and 0.3 for antibiotics. Two active fractions were isolated from the culture broth of the strain B_{29} using semi-preparative HPLC. Partial sequencing of the 16S RDNA gene was used to identify both of *Bacillus* strains. They may be assigned to new *Bacillus* species.

Key words: Antimicrobial activity, *Bacillus* sp., characterization, salt soil, Tunisia.

INTRODUCTION

An analysis of the number of pathogenic bacteria and fungi indicated that they become resistant to antibiotics in common use. These antibacterial and antifungal resistances are presently an urgent focus of research and new antibiotics are necessary to combat these pathogens. An investigation of the potential sources of this molecules indicated that over 60% of approved drugs are derived from natural compounds (Cragg et al., 1997; Newman et al., 2003). Many organisms, including micro-organisms, plants and some animals, have the capability to synthesize bioactive natural products. These natural products constitute a library of compounds with a large and privileged structural diversity, showing a variety of biological activities. In fact, many of them have been used for agricultural or pharmaceutical applications (Salas and Mendez, 2007). The search for new, safer, broad-spectrum antifungal antibiotic with greater potency has been progressing slowly (Gupte et al., 2002). In the course of screening for new antibiotics, several research studies are currently oriented towards isolation of new microorganism's species from different soils and ecosystems (Mellouli et al., 2003; Errakhi et al., 2007). Within microorganisms, *Bacillus* represents one of the largest sources of bioactive natural products. Various studies have confirmed that *Bacillus* species have a wide range of antimicrobial activities since they are used as antifungal (Milner et al., 1995), antibacterial (Yilmaz et al., 2006), antiviral (Steller et al., 1999), antiameobocytic (Galvez et al., 1994) and antimycoplasma agents (Peypoux et al., 1999). These are often complex secondary metabolites having great potential for biotechnological and biopharmaceutical applications. A well-known class of such compounds includes the lipopeptides surfactin (Cho et al., 2003), fengycin

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(Vanittanokom et al., 1986), and the iturin compounds (Cho et al., 2009), iturin A, B and C (Peypoux et al., 1978), mycosubtilins (Peypoux et al., 1976), bacillomycins (Peypoux et al., 1984), plipastatin (Tsuge et al., 1996) and di- and tripeptides such as bacilysin (Walker and Abraham, 1970) are peptide antibiotics with potent antimicrobial activities.

In fact, the list of novel microorganisms, especially *Bacillus*, and products found in microbiologically unexplored ecosystems around the world suggest that a careful exploration of other habitats might continue to be useful. South of Tunisia is an explored ecosystem and may be a source of new microorganisms producing new compounds.

In the present work, we describe a preliminary characterization of two bacterial strains isolated from south of Tunisia, the evaluation of their antimicrobial activity by molecular methods as well as the production, isolation and the partial characterization of their antibiotics.

MATERIALS AND METHODS

Microorganism's strains

Bacillus strains, denoted B_{27} and B_{29}, were previously isolated from salt soil in the south of Tunisia and identified as *Bacillus* sp. These bacteria were routinely grown on nutrient agar (NA) medium at 30°C for further treatments. *Escherichia coli* CIP 102 400, *Staphylococcus aureus* CIP658, *Saccharomyces cerevisiae* ATCC 9226, *Mycobacterium phlei* CIP105 389, *Micrococcus loteus* CIPA270, *Pseudomonas aerugenosa* CIP 100720, *Mucor ramannianus* NRLL 1829 (strain collection of Laboratoire de génie chimique, Toulouse) and *Botrytis cinerea* (strain isolated from leaves of vine plants) were used as indicator microorganisms for the biological assays.

Antimicrobial activity

Antimicrobial and antifungal effect of the isolates were determined using the plate diffusion method (Barakate et al., 2002; Errakhi et al., 2007) against Gram-positive bacteria (*M. luteus, S. aureus*, and *M. phlei*), Gram-negative bacteria (*E. coli* and *P. aeruginosa*), yeasts (*S. cerevisiae*), and filamentous fungi (*M. ramannianus* and *B. cinerea*). Isolates were grown on NA medium and two discs (6 mm in diameter) were cut and placed on Bennett medium (beef extract (Merck, Germany) 1 g/l; glucose (Merck) 10 g/l; peptone (Merck) 2 g/l; yeast extract (Merck) 1 g/l and agar (Difco) 15 g/l; for bacteria) and Potato dextrose agar (PDA) medium (for fungi and yeasts) which were seeded with appropriate test organism. Plates were first kept in a refrigerator (4°C) for at least 2 h to allow the diffusion of any antibiotics produced, then incubated at 30°C. Inhibition zones were determined after 24 h for bacteria and yeast and after 48 h for fungi.

Production of antimicrobial activity in various media

Antimicrobial activity of the two *Bacillus* strains was evaluated on 4 media that is, YMA (peptone 0.3%, yeast extract 0.5%, mannitol 2.5% and agar 15 g/l), NA, PDA and Bennett (BT). Bacteria was inoculated on plates of 90 mm diameter on each of this media and incubated at 30°C for 24 h.

The activity against *M. luteus, E. coli*, *P. aeruginosa, S. cerevisiae* and *M. ramannianus* was recorded using a plate diffusion method. Three discs (10 mm in diameter), for each bacteria and each medium, were cut and placed on BT (for bacteria) and PDA (for fungi and yeast) which were seeded with appropriate test organisms. Plates were first kept in a refrigerator (4°C) for at least 2 h to allow the diffusion of antibiotics produced, then incubated at 30°C. Inhibition zones were determined after 24 h for bacteria and yeast and after 48 h for fungi.

Kinetics of antimicrobial products of the *Bacillus* sp. strains in potato dextrose medium

Bacillus strains were selected for their interesting antifungal properties. Kinetics of antimicrobial products was assessed on Potato dextrose broth (PDB) medium. The pH medium was adjusted to 7.2 and 100 ml aliquots were dispensed into 1-L Erlenmeyer flasks. The inoculated flasks were put on a shaker (250 rpm; 30°C). 2 ml of the liquid culture were centrifuged to remove most of the bacterial cells, the supernatant was passed through a sterile filter 0.20 µm membranes, and the resulting broth was used for antifungal activity determination. The activity against *M. luteus*, *S. cerevisiae*, *M. ramannianus*, and *B. cinerea* was regularly recorded each 2 h by the agar diffusion method (well technique; each well of 10 mm in diameter was filled with 50 µl of supernatant). Fungi, bacteria, and yeast spore suspensions were prepared by suspending loopfuls of hyphae and colonies from PDA slant incubated at 22°C *(B. cinerea*) and 30°C for the other organisms. The suspension was filtered with sterile cotton (for *B. cinerea*). A 10^8 CFU/ml spore suspension was obtained through dilutions and counting with a hemaecytometer and adjusted to 10^5 CFU/ml by serial dilution. 4 ml of the spore suspension was suspended under the PDA plates, containing the wells with the supernatant. The Petri dishes were incubated for 4 h at 4°C to allow the diffusion of produced antimicrobial metabolites and then incubated at 30°C for 24-48 h. The relative inhibition data is presented as the diameter of development inhibition around wells.

Production, extraction and detection of antibiotics

Each *Bacillus* strain was cultivated in PDB medium in four 500-ml Erlenmeyer flasks each containing 100 ml of medium. The cultures were incubated at 30°C for 3 days under constant agitation of 250 rpm. The liquid culture was centrifuged for 20 min at 8000 g to remove the bacteria. The supernatant was shared in 4 equal volumes of 100 ml, and then each was extracted with 200 ml of organic solvent. A range of extraction solvents was screened for effectiveness, including n-hexane, dichloromethane, n-butanol and ethyl acetate. The organic extracts were evaporated to dryness using a Rotavapor (Laborota 4000). The resulting dry extracts were recuperated in 2 ml of methanol and subjected to biological assay (25 µl) (disk of 6 mm in diameter, Institut Pasteur) against *M. luteus*, *S. cerevisiae*, *M. ramannianus*, *P. aeruginosa*, and *B. cinerea.* The solvents which gave the highest inhibition diameter were then retained for the detection of antibiotics by bioautography (Figure 1 and 2).

Purification and partial characterization of antifungal products of *Bacillus* sp. strains

Preparative chromatography with silica gel plates (Merck Art. 5735,

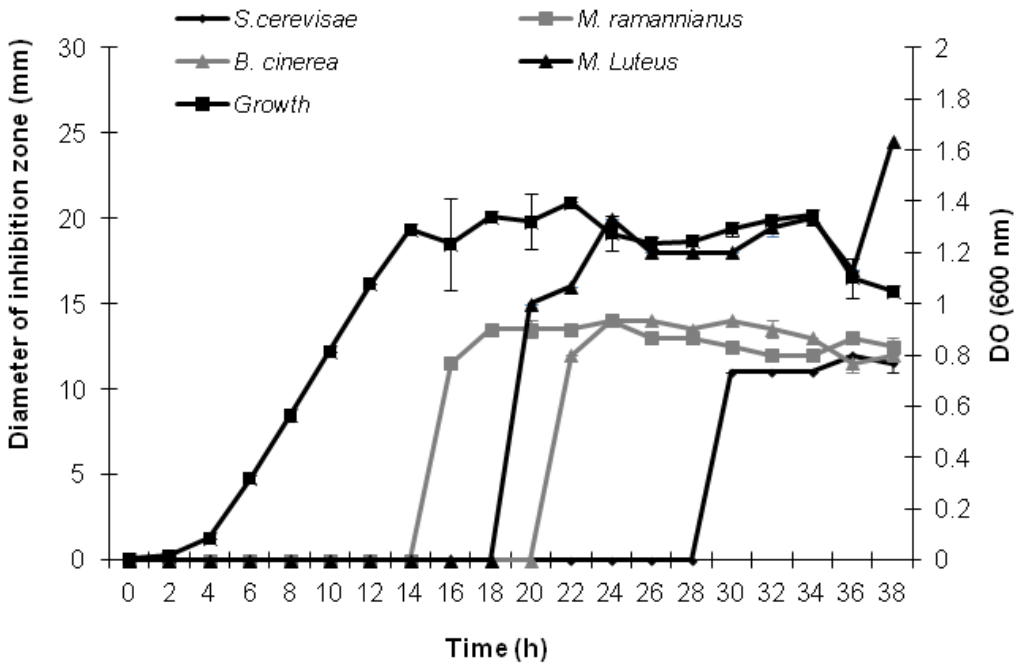

Figure 1. Time course of growth and antibiotics production of *Bacillus* sp. strain B29 on PDB medium at 30°C. The measure of the diameter inhibition against microorganisms was undertaken by the discs method (25 µL extract by disc). Values represent diameter inhibition in mm, including the discs diameter (6 mm).

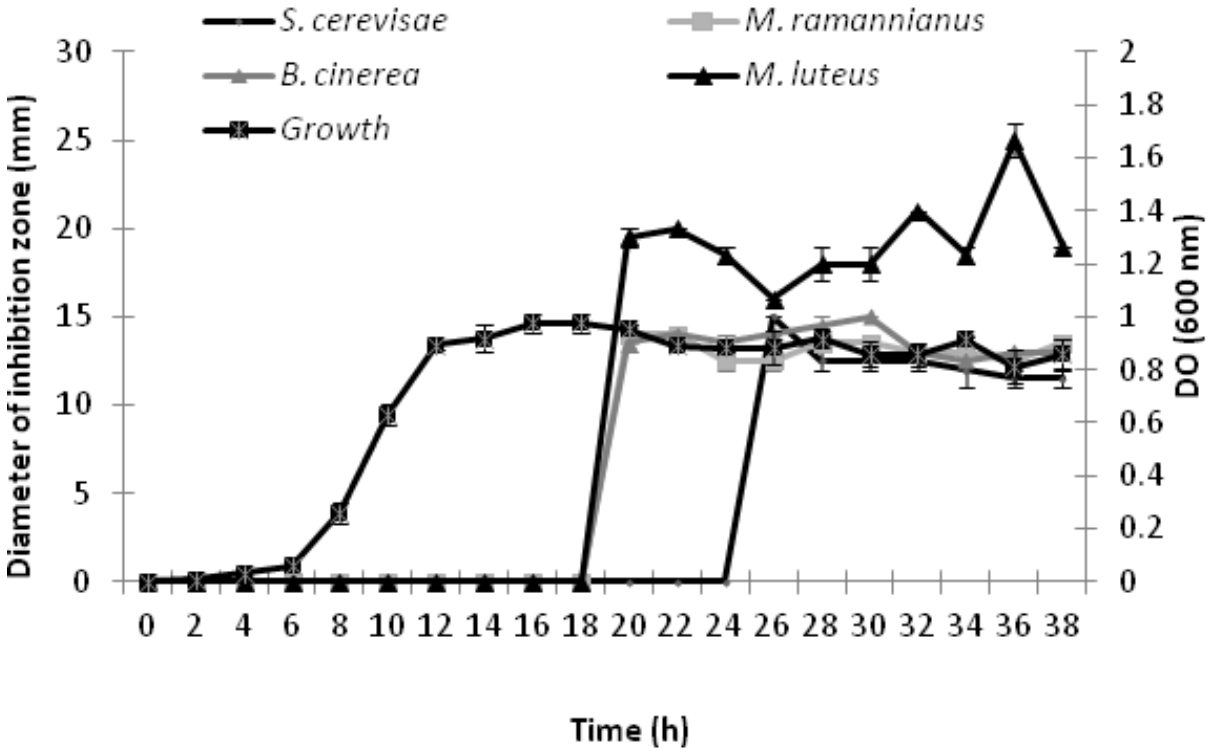

Figure 2. Time course of growth and antibiotics production of *Bacillus* sp. strain B27 on PDB medium at 30°C. The measure of the diameter inhibition against microorganisms was undertaken by the discs method (25 µL extract by disc). Values represent diameter inhibition in mm, including the discs diameter (6 mm).

Kiesel gel 60 F_{254}) was used for the partial purification of antimicrobial products. Thus, 20 µl of n-hexane extract were spotted onto 20 × 20 cm silica gel plates, and then developed with butanol-acetic acid-water (3:1:1). The developed TLC plates were air-dried overnight at 37°C to remove all traces of solvents. They were then placed in a plastic bioassay dish (23 × 23 × 2.2 cm^3, Fisher Scientific Labosi) and overlaid with 100 ml of PDB medium (containing 7 g/l of agar) seeded with *M. luteus* or *M. ramannianus*. After the agar had set, the plate was incubated at 30°C. After 2 days, reddish brown-colored fungal growth or opaque bacterial growth was visible. Clear areas due to inhibition of microorganism growth indicated the location of antibiotic compounds on the TLC plates. The retention factor (RF) of each spot was recorded.

The liquid cultures of B_{27} and B_{29} strains at 30°C for three days were harvested to remove the biomass. The cell-free supernatant fluid was extracted with hexane for B_{29} and with ethyl acetate for B_{27}.

Antibiotics were purified by HPLC (Waters: controller 600, pump 600, dual λ absorption detector 2487, Linear Recorder); Column C_{18} (250 × 7.8 mm UP ODS); mobile phase: Linear gradient of MeOH-H_2O from 0 to 100% for 40 min, Flow rate: 1.5 ml/min, detection: UV at 220 nm. Different peaks were collected separately, concentrated and then tested against *M. ramannianus* and *M. luteus*. After purification, the antibiotics were subjected to spectroscopic studies. Absorption spectra of active extracts in methanol were recorded in the UV region (180-500 nm) using a UV-visible Prekin-Elmer (Lambda 20) spectrophotometer and compared with those of known antifungal antibiotics.

Identification of bacteria

DNA extraction

Single isolated colonies of the two bacterial cultures were taken from agar plates and suspended in 500 µl of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). The tube is kept at room temperature for 10 min. After adding 150 µl of potassium acetate (pH 4.8), the tube is vortexed briefly and spun at >10 000 g for 1 min. After transferring the supernatant to a new Eppendorf tube, an equal volume of isopropyl alcohol is added. The tube is mixed by inversion briefly, spun at >10 000 g for 2 min and the supernatant is discarded. The resultant DNA pellet is washed in 300 µl of 70% ethanol. After the pellet is spun at 10 000 rpm for 1 min, the supernatant is discarded. The DNA pellet is air dried and dissolved in 50 µl of sterile ultrapure water (Liu et al., 2000).

PCR amplification

PCR amplification of almost full length 16S rRNA gene was carried out with specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). A 50 µl reaction volume PCR was performed using about 100 ng of genomic DNA, 10X Reaction buffer, 10 mM (each) deoxynucleoside triphosphates, 25 mM $MgCl_2$ and 0.5 U of DNA polymerase. The PCR was performed in an automated Gene Amp PCR System thermal cycler under the following conditions. The amplification conditions were as follows 98°C for 4 min (denaturation), 55°C for 1 min (annealing), 72°C for 3 min (elongation) and 72°C for 10 min final elongation. Expected PCR product of around 1.5 Kb was checked by electrophoresis of 5 µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethium bromide 0.5 µg / ml.

The PCR products obtained were submitted to GenomExpress for sequence determination. The same primers as above and an automated sequencer were used for this purpose. The analysis of sequences was done at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). All the analyzed sequences have been deposited in genbank.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using softwares included in MEGA version 3.0 (Kumar et al., 2004) package. The 16S RDNA sequence of B_{27} and B_{29} strains was aligned using the CLUSTAL W program (Thompson et al., 1994) against corresponding nucleotide sequences of representatives of the genus *Bacillus* retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was inferred by the Neighbor joining method (Saitou and Nei, 1987). Tree topologies were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resembling of the neighbor joining data set.

RESULTS

Antimicrobial activity

In order to assess whether the two *Bacillus* strains isolated from the salt soil originating from south of Tunisia habitats may be a useful source for natural bioactive compounds, the antimicrobial activity of the isolates was determined (Table 1).

The two isolates exhibited antibiotic activity towards the Gram-negative bacteria *E. coli* and *P. aeruginosa*, the Gram-positive bacteria *M. luteus*, the yeast *S. cerevisiae* and the filamentous fungi *M. ramannianus* and *B. cinerea.* The activity against the Gram-positive bacteria, *S. aureus* and *M. phlei*, was not recorded.

Choice of media for production of antibiotic

The antifungal and antimicrobial productions in various media are illustrated in Table 2. PDA was found to be the better medium in promoting the production of antifungal and antimicrobial compounds active against micro-organisms tested and was therefore retained for later studies.

Kinetics of antimicrobial products of the *Bacillus* sp. strains in potato dextrose medium

Bacillus sp. strains produce metabolites that display anti-fungal activity. The growth inhibition of the two fungi *B. cinerea* and *M. ramannianus*, the yeast *S. cerevisiae* and the bacteria *M. luteus* by the broth of steady-state-cell-free supernatants at different time of growth of the bacteria show the presence of halo of inhibition that began at the stationary phase of the growth of the two bacteria.

The results of antagonism tests revealed an important antimicrobial activity of the two *Bacillus* strains, especially

Table 1. Antimicrobial activity of B27 and B29 *Bacillus* sp. strains against microorganisms tested.

Microorganisms	Diameter of inhibition (mm)	
	B27	B29
Gram-positive bacteria		
M. luteus CIPA270	24.25 (± 0.85)	25 (± 0)
S. aureus CIP658	-	-
M. phlei CIP105 389	-	-
Gram-negative bacteria		
E. coli CIP102 400	20.5 (± 0.5)	21.75 (± 0.75)
P. aerugenosa CIP 100720	20.5 (± 0.5)	21.25 (± 0.47)
Yeasts		
S. cerevisiae ATCC 9226	22.5 (± 0.28)	24 (± 0.40)
Filamentous fungi		
M. ramannianus NRLL 1829	17.25 (± 0.28)	19.25 (± 0.47)
B. cinerea	21.75 (± 0.62)	24 (± 0.40)

Values given are the mean of four samples (± standard error of the arithmetic mean); (-) were not inhibited by the strain.

Table 2. Antimicrobial activity of B27 and B29 *Bacillus* sp. strains in various culture media.

Microorganisms	YMA		NA		PDA		BT	
	B27	B29	B27	B29	B27	B29	B27	B29
M. luteus CIPA270	32(±0.4)	35(±0.3)	30(±0)	35(±0.3)	45(±0.22)	55(±0.13)	35(±0.31)	45(±0.4)
E. coli CIP102 400	12(±1.1)	19(±0.7)	12(±0.65)	18(±0.8)	13(±0.13)	20(±0.4)	12(±0.51)	16(±0.12)
P. aerugenosa CIP 100720	15(±0.75)	21(±0.1)	15(±0.01)	24(±0.21)	30(±0.11)	25(±0)	22(±0)	23(±0)
S. cerevisiae ATCC 9226	12(±0.4)	16(±0)	14(±0.43)	16(±0)	17(±0.21)	17(±0.22)	15(±0)	16(±0.11)
M. ramannianus NRLL 1829	12(±1.1)	17(±0.2)	12(±1.0)	12(±0.12)	17(±0.22)	23(±0.11)	12(±0.13)	22(±0.21)

Values given are the mean of four samples (±standard error of the arithmetic mean)

against filamentous fungi. Indeed, their activity is very strong against the two species *M. ramannianus* and *B. cinerea*. Concerning the antibacterial activity, both *Bacilli* are strongly active against *M. luteus* and *S. cerevisiae*.

The kinetic of production of the antibiotics was realized on PDB medium. Antifungal and antibacterial activities appear after 14 h against *M. ramannianus*, 18 h against *M. luteus*, 20 h against *B. cinerea* and 28 h against *S. cerevisiae* for the B_{29} strain While for B_{27}, the antimicrobial activity has been recorded after 18 h of culture against *M. ramannianus*, *M. luteus* and *B. cinerea* and 24 h against *S. cerevisiae*. These activities increase immediately and remain more or less stable thereafter.

Extraction of antimicrobial activities

The results of the antimicrobial assays of the isolate B_{29} are shown in Figure 3. The bacterium B_{29} exhibited antibiotic activity towards *M. Luteus*, *S. cerevisiae*, *M. ramannianus* and *B. cinerea.* The activity against *P. aeruginosa* was absent. The different fractions obtained from B_{27} isolate culture did not show any antibiotic activity against all microorganisms tested.

The highest activity was exhibited by the strain B_{29}, with an inhibition zone of 25 mm against *S. cerevisiae*, 20 mm against *M. ramannianus* and 15 mm against *B. cinerea* for hexane fraction (Figure 3). This bacterium showed

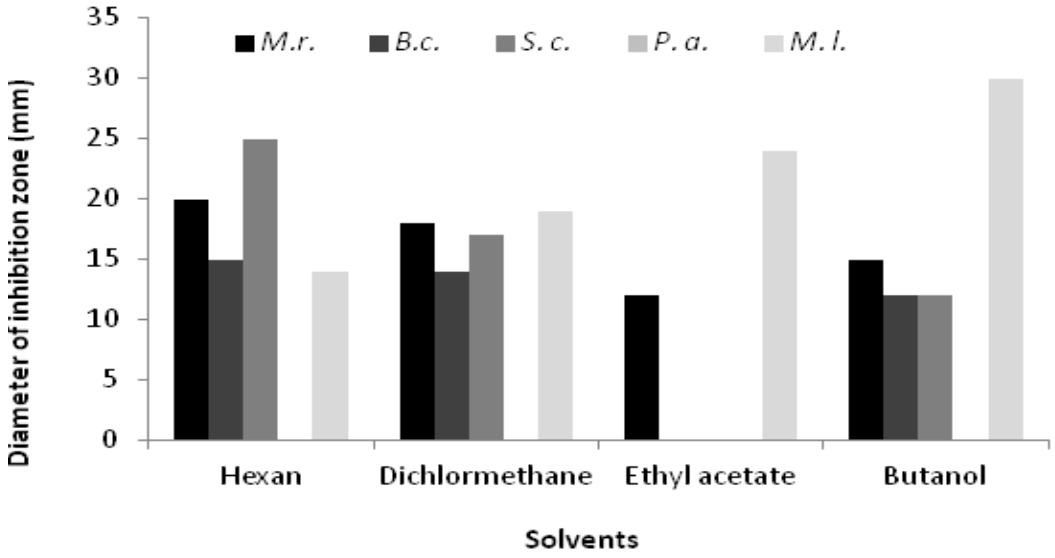

Figure 3. Choice of extraction solvent of antibiotic activities produced on PDB media by the *Bacillus* sp. strain B29. Values represent diameters of inhibition in mm (including the disc diameter that is 6 mm) against M. r. *M. ramannianus;* B. c. *B. cinerea;* S. c. *S. cerevisiae;* P. a. *P. aeruginosa* and M. l. *M. luteus.*

activities against bacteria, in fact the ethyl acetate fraction inhibit 25 mm of the development of *M. luteus* while Butanol fraction inhibit 30 mm of its development.

The hexane has been therefore chosen as extraction solvent for the *Bacillus* strain B_{29}. Therefore, this fraction was retained for later studies.

The B_{27} strain exhibited strong antifungal and anti-microbial activity against the microorganisms tested. This activity was not extracted by n-butanol or by the other organic solvents such as ethyl acetate, dichloromethane and n-hexane.

Partial characterization of antifungal products of *Bacillus* B_{29} strain

The cell-free supernatant of 1.5 L of liquid culture of B_{29} strain was extracted by hexane. The organic phase was concentrated to dryness, recuperated in methanol and chromatographed by TLC. On analytic TLC plates developed in BAW solvent system, many active spots were detected by bioautography: Antifungal spots at 0.30 and 0.76 Rf values and antibacterial spots at 0.12, 0.14, 0.19 and 0.30 Rf values. The antibiotic localized at 0.30 Rf value showed strong antibacterial and antifungal activities.

Antimicrobial activity guided fractionation of the broth of B_{29} culture showed that the hexane extract exhibited inhibitory activity against the filamentous fungi tested, and the butanol extract showed activity against the bacterial strains. Further fractionation of the hexane extract was undertaken by reverse phase HPLC. Figure 4 shows a representative HPLC trace of hexane extract when separated on a C18 semi-preparative HPLC column and detected at 220 nm. Seventeen fractions could be clearly resolved under the conditions employed. Of the 17 fractions that were tested for antimicrobial activity, fractions 15 and 16 were found to be active against *M. ramannianus* and *M. luteus.* The remaining fractions did not exhibit antimicrobial activity.

Antibiotic 15 showed maxima, UV-VIS spectrum absorbance in methanol, at 210, 224 and 272 nm. Antibiotic 16 exhibited the most interesting activity. Its UV-VIS spectrum in methanol showed maxima at 203, 224 and 270 nm.

Molecular identification and phylogenetic analysis of bacteria

Data from the 16S RDNA gene provide a limited number of characters that are informative for the phylogeny of the *Bacillus* strain. Aspect of tree presented in Figures 5 and 6 appears to be informative. Based on the sequence homology and phylogenic analysis, organism was found to be *Bacillus* (1104513.1). It is more closely related to *Bacillus* sp. Z19 (EU236746.1). For the *Bacillus* B_{29} strain (1104513.2), it was found to be more closely related to *Bacillus amyloliquefaciens* strain EXWB3-03 (EU334107.1).

The partial sequence analysis of the 16S RDNA gene and BLAST sequence comparison in the GenBank

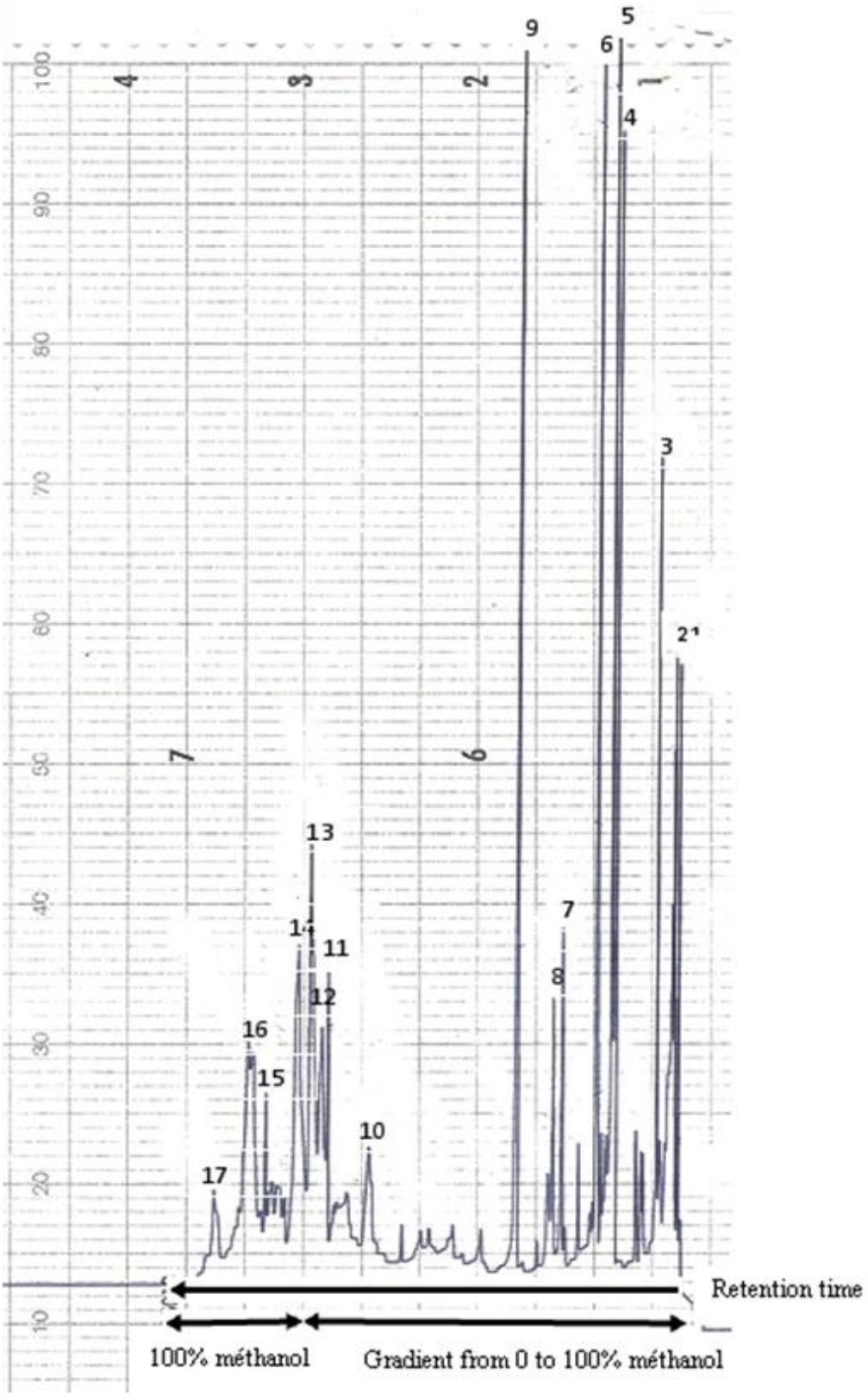

Figure 4. HPLC trace of hexane extract of the culture broth of *Bacillus* sp. strain B29. Seventeen fractions are of relatively high intensity, are easily identified. In addition several peaks of lower intensity are also observed. Majority of the observed antimicrobial activity appears to arise from compounds in peaks 15 and 16.

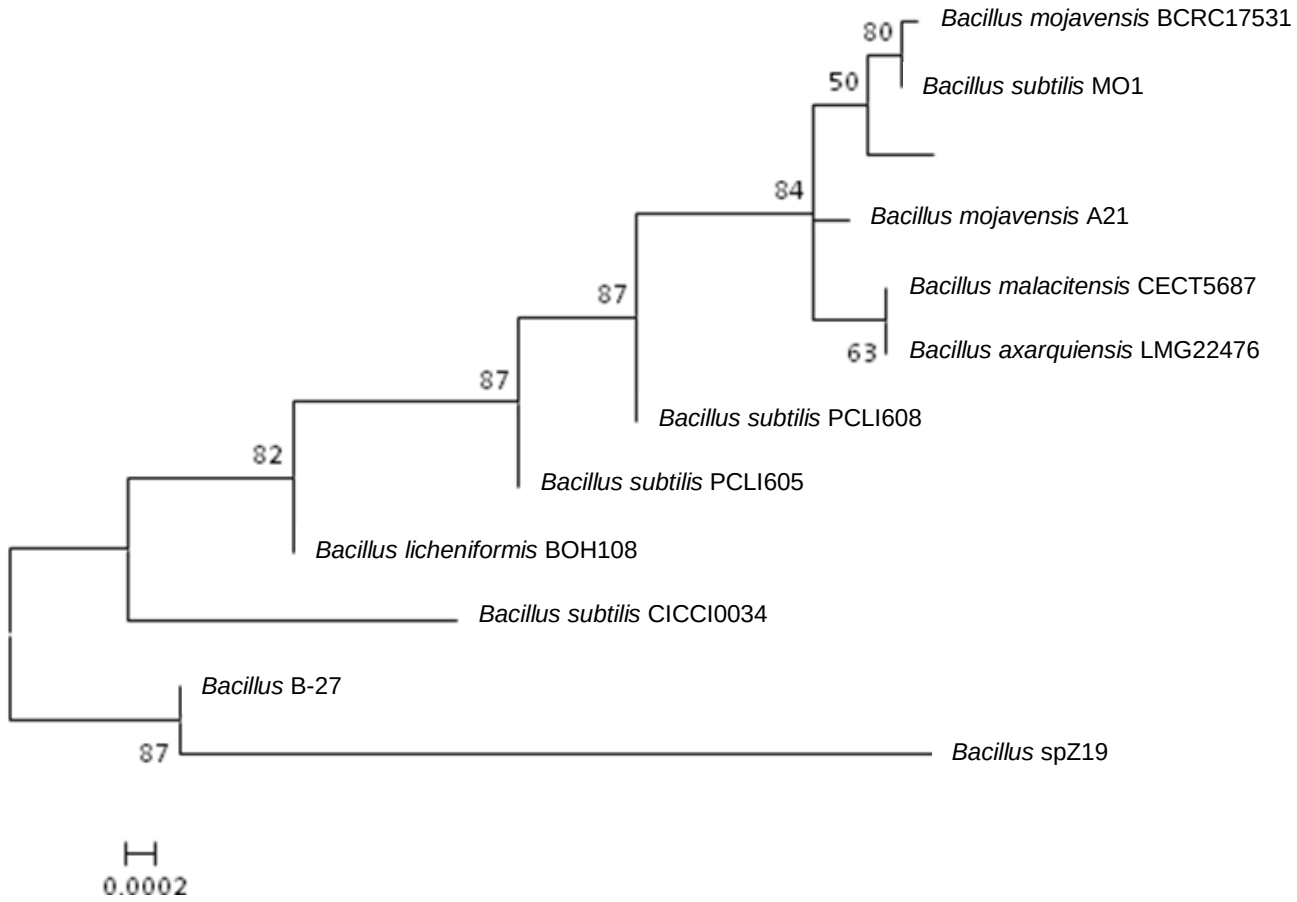

Figure 5. Phylogenetic tree derived from parsimony analysis of 16S rDNA gene from *Bacillus* sp. strain B27 (accession number 1104513.1). The 1415 bases determined for B27 were aligned to the corresponding regions from bacteria. The number at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets; only values over 50% are given. Bar, 0.0002 nt substitution per nt position.

database showed an alignment of 1414/1415 bases (99% similarity) of the strain B_{27} (accession number 1104513.1) with the sequence of *B. subtilis* strain PCL 1605 (accession number DQ779882.1); similar results have also been obtained for *B*. *mojavensis* strain BCRC 17531 (accession number DQ993678.1). For the strain B_{29} (accession number 1104513.2), the 16S RDNA gene partial sequence aligned with the sequence of *B*. *amyloliquefaciens* strain TB2 (accession number EU359773.1), though at the lower similarity level of 99% with an alignment of 1414/1415 bases and with the *B. subtilis* strain Pab02 (accession number EU346662.1).

DISCUSSION AND CONCLUSION

Microorganisms produce some of the most important compounds with pesticidal activity. They are the source of lifesaving treatments for bacterial and fungal infections. In spite of the tremendous success of the past secondary metabolite research, the number of terrestrial antibiotics seems currently to approach a saturation curve with an apparent limit in the near future. The increasing number of duplications and the urgent demand for few leading structures in pharmacology and agriculture, have enforced the search for metabolites in so far untouched habitats.

Our interest focused on microorganisms belonging to the *Bacillus* genus, the members of which have demonstrated interesting antimicrobial activity. In this article, we have described the use of *Bacillus* strains B_{27} and B_{29} isolated from salt soil as well as their secreted secondary metabolites to act as antimicrobial product against a large range of microorganisms tested (bacteria, yeasts and filamentous fungi). Cho et al. (2009) have recently described the use of many strains of *Bacillus* sp. as antifungal and antibiotic agents.

The extent of the antibiosis of the two *Bacillus* isolates against the test organisms, evaluated in terms of reduced radial growth, is almost similar. The two isolates exhibited

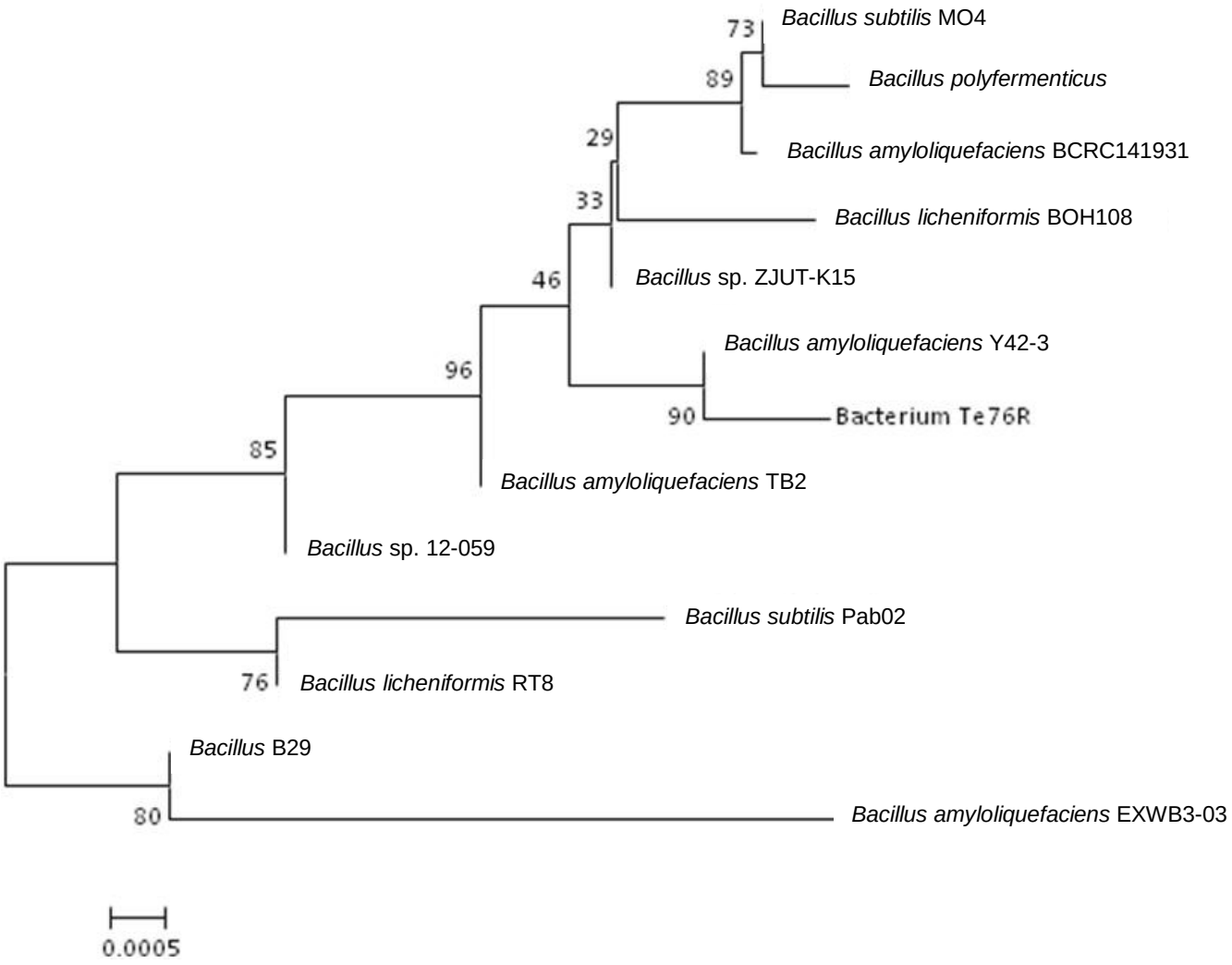

Figure 6. Phylogenetic tree derived from parsimony analysis of 16S rDNA gene from *Bacillus* sp. strain B29 (accession number 1104513.2). The 1415 bases determined for B29 were aligned to the corresponding regions from bacteria. The number at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 resampled data sets; only values over 50% are given. Bar, 0.0005 nt substitution per nt position.

a strong activity (inhibition zone > 20 mm) against Gram-positive and Gram-negative bacteria, yeast and filamentous fungi. The strong activity expressed by a large zone of inhibition on agar plates indicated, as mentioned by Barakate et al. (2002), that those two isolates produce water soluble antimicrobial metabolites which may play an important role in the biocontrol of plant diseases.

Results of antibiotics activity expressed in terms of the diameter of the inhibition zone showed differences in the percentage of antibiosis and specificity of efficacy; this may imply that the two investigated *Bacillus* isolates belong to different species or to the same one but they produced different bioactive compounds exhibiting inhibitory activity against a large number of microorganisms.

These results were confirmed by the time course of growth and antibiotics production of the two strains. In fact, we noted many differences that concerned the specificity of the antibiotics and their time of production.

The chemical nature of the compounds responsible for the observed activity in the organic solvent extracts, respectively for B_{29} and B_{27} strains, are being elucidated. The *Bacillus* species are widely recognized as a rich source of antimicrobial agents (Gebhardt et al., 2002). Many antibiotics including cyclic peptides, cyclic lipopeptides and novel thiopeptides have been reported from *Bacillus* sp. (Nagai et al., 2003).

Bioautography of hexane extract of the B_{29} strain has revealed several products active against bacteria and fungus suggesting the presence of antibiotics having probably different chemical structures.

The B_{29} strain active fractions 15 and 16 have retention times of 47.5 and 48.35 min respectively. The UV-visible spectra in methanol of these two fractions exhibited maxima at 270 nm suggesting the presence of an aromatic ring compound (Badji et al., 2005; Zitouni et al., 2005).

The 16S RDNA gene sequence analysis has been described to be necessary mainly to detect some misidentification of *Bacillus* and related strains (Guinebretiere et al., 2001; Wu et al., 2006). Sequencing the nucleotide positions 70–344 of the 50 end region of the 16S RDNA gene may represent a very efficient index for the identification of *Bacillus* and *Paenibacillus* species being this region's most informative one. In fact, about 275 pb of the 50 end region of the 16S RDNA gene were the hypervariant region in the gene and it was highly specific for each *Bacillus* type strain, but also highly conserved within the species (Goto et al., 2000). The 16S RDNA sequence of B_{27} and B_{29} strains were compared with those of other *Bacillus* species. The similarity level was about 99% with *B. subtilis* and *B. mojavensis* for the B_{27} strain and was about 99% with *B. amyloliquefaciens* and *B. subtilis* for the B_{29} strain. However, it is clear from phylogenetic analysis that these two strains did not cluster with neither of *B. subtilis*, *B. amyloliquefaciens* nor *B. mojavensis* and represented distinct phyletic lines suggesting new genomic species.

All results suggest that both strains may be new species of *Bacillus*. The antifungal activities exhibited especially against *B. cinerea* showed that both strains may play an important role in biological control. Further studies are needed to determine the ability of these strains to control disease caused by *B. cinerea* on *Vitis vinifera*.

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