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Endophytic Bacteria in Banana In Vitro Cultures: Molecular Identification, Antibiotic Susceptibility, and Plant Survival

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Abstract: Microbial contamination is a common problem that causes significant losses in plant micropropagation systems. The present study reports on the identification and control of bacterial contaminants in banana in vitro cultures. Twelve isolates belonging to *Bacillus pumilus* (S2), *Bacillus subtilis* (R2 and M4), *Geobacillus stearothermophilus* (S1, S3, S4, P2, M3 and R3) and *Paenibacillus* spp. (P1, R1 and M2) were identified by sequencing of 16S rRNA, *gyrA* or *gyrB* genes. Antibiotic susceptibility testing was performed with the disk diffusion method on bacterial isolates using 36 antimicrobial agents. Some antibiotics, notably Ticarcillin, Penicillin, Ampicillin, Cefazolin and Imipenem, had a broader range of bactericidal activity than others did. When contaminated axillary shoot cultures of banana were treated with 100 or 200 mg·L^{-1} of ticarcillin, ampicillin or penicillin the bacteria were eliminated, but a reduction in shoot multiplication and growth was observed. These findings contribute to minimizing the losses in the commercial micropropagation of banana.

Keywords: 16S rDNA; chlorosis; micropropagation; microbial contamination; tissue browning

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1. Introduction

Tissue culture is one of the key tools of plant biotechnology and has been extensively exploited to meet the growing demands for elite planting material to produce healthy and pathogen-free plants at any time of the year in reduced time and physical space. However, microbial contamination is one of the major restrictions in commercial micropropagation systems. It causes difficulties in culture initiation, reduction of multiplication and rooting efficiency, tissue necrosis, and culture mortality [1,2]. Plant tissue cultures can be contaminated by endophytes or rhizosphere microorganisms that colonize plants in their growth habitat. Internal bacterial contamination (intra- or inter-cellular) can survive surface disinfection. Although these bacteria are not necessarily pathogenic or harmful to plants in the natural system, they can induce serious problems during in vitro culture. Endophytic bacteria are beneficial to host plants as they enhance the plants' defense against diseases [3], but they cause serious problems in tissue culture systems. The elimination of endophytic bacteria is usually difficult because systemic sterilizers, such as mercuric chloride and systemic fungicides, can kill the explants [4]. Contaminants are usually not evident at culture establishment, but appear after several subcultures. Although some explants survive and continue to grow with the bacteria present, bacterial infection could result in high levels of damage to the plant material because of bacteria overgrowth. Several microbial

genera, including *Bacillus*, *Burkholderia*, *Erwinia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *and Ochrobactrum*, have been reported as banana colonizers [5–10].

The bacterial 16S ribosomal DNA (rDNA) target gene sequencing is the most commonly used method for bacterial identification. It includes both conserved and variable regions of about 1500 bp encoding the 30S ribosomal protein subunit, which can be used to identify bacteria at the genus level [11,12]. The analysis of the 16S rDNA, GyrA and GyrB gene sequences has been widely used for bacterial taxonomy and identification in different contaminated samples [13–17]. However, the expanded use of 16S rDNA gene-based analysis for the detection and identification of bacteria in many environments, and the application of this approach to identify bacterial contaminants in in vitro plant cultures, is limited. Microbial contamination is a major challenge to the initiation and maintenance of viable in vitro cultures as the endogenous microbes cannot be observed by microscopic examination prior to culture establishment. Following excision of these cultures and transfer to culture medium, massive bacterial and/or fungal contamination usually appears within one week. Identifying the microbes in plant tissue cultures facilitates not only the selection of effective and specific antibiotics to eliminate the contaminants, but also the identification of the possible contamination sources. Therefore, the objectives of this study were to identify microorganisms contaminating banana in vitro cultures using 16S rDNA sequencing and to investigate their antibiotic susceptibility and toxicity to the in vitro cultures.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

In vitro cultures of banana (Musa × paradisiaca L. 'Grand Naine') were established according to the protocol described by Kacar and Faber [18]. Shoot tips were sub-cultured twice (4 weeks per culture cycle) for multiplication on MS medium [19] containing 3% sucrose and supplemented with 3 mg·L^{-1} 6-benzylaminopurine (BAP) and 1 mg·L^{-1} Kinetin. The medium was gelled with 0.2% gellan (Dephyte, Hannover, Germany) and the pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C and 118 kPa for 15 min. The cultures were incubated for 4 weeks at 25 ± 1 °C under a 16-h photoperiod at 25 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool-white fluorescent tubes.

2.2. Isolation of Contaminants, Microbial Growth Media and Microscopic Observations

The visible microbial contaminants that appeared around the base of the in vitro grown axillary shoots within 1–3 weeks on the initiation medium were streaked onto different microbial growth media. Four different media were prepared for bacterial and/or fungal growth as follows: (1) trypticase soy agar (TSA), composed of 30.0 g trypticase soy broth and 15 g agar; (2) potato dextrose agar (PDA), composed of 200 g potato starch, 20 g dextrose, and 15 g agar; (3) Middelbrook medium, composed of 19 g bacto Middelbrook H10 agar and 5 mL glycerol; (4) R2A medium, composed of 0.5 g yeast extract, 0.5 g protease peptone, 0.3 g K_2HPO_4, 0.3 g $MgSO_4$, 5 g Na-pyruvate, 0.5 g soluble starch, 0.5 g glucose, and 0.5 g casamino acids. All media were adjusted to 1 L volume and solidified with 15 g L^{-1} Bacto-agar. The plates were incubated for 48 h at 28 °C. The media were used for bacterial isolation and further culturing. Microbial isolates were purified to single colonies by repeated culturing on the same media. The contaminants were preliminarily categorized as bacteria. Morphological analysis of the bacterial isolates were performed using a simple phase contrast microscope (Zeiss Axioskop 2, Oberkochen, Germany).

2.3. Fatty acid Methyl Ester (FAME) Analysis

The whole cell fatty acid methyl ester (FAME) was performed at DSMZ institute, Germany using standard procedures for further identification and grouping of the bacterial isolates. Cellular fatty acids are analyzed after conversion into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction using minor modifications of the method of Miller [20] and Kuykendall et al. [21]. The fatty acid methyl esters mixtures

were separated by gas chromatography and detected by a flame ionization detector using Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 USA). Peaks are automatically integrated and fatty acid names and percentages were calculated using the MIS Standard Software (Microbial ID).

2.4. Total DNA Extraction, PCR Conditions and Sequencing

Before DNA extraction, all obtained bacterial isolates were grown individually in 30 mL liquid tryptic soy broth (TSB) for 24 h at 37 °C under constant mixing at 180 rpm. Bacterial genomic DNA was extracted with the Master Pure™ DNA purification Kit (Epi-104 centre, Madison, Wisconsin) according to the manufacturer's recommendations. The isolates were identified by partial sequencing of the 16S region for bacteria. The 16S rDNA regions of the bacteria were amplified using the universal [22] forward primer 27F 5′-AGAGTTTGATC (AC) TGGCTCAG-3′) and the reverse primer 1492R (5′-ACGG (CT) TACCTTGTTACGACTT-3′). The PCR reactions consisted of 4 µL of dNTPs (1.0 mM each, Roche, Penzberg, Germany), 2 µL of 10X buffer (Roche), 0.2 µL of each primer (0.5 µg), 0.2 µL of *Taq* polymerase (5 U/µL), 1 µL of 50 ng of template DNA, and 12.2 µL of sterile Milli-Q water to a final volume of 19.8 µL. The amplification conditions consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR-fragments were purified using the Qiagen PCR-purification kit (Qiagen, Hilden, Germany). The bacterial isolates that identified as *Bacillus subtilis* and *Bacillus pumilus* using 16srDNA sequencing were also identified by partial amplification and sequencing of the gyrA and gyrB genes using primer pairs of gyrAF/gyrAR, (5′-AATGATTTAGGCATGACGAGTGAC-3′/5′-TTCATCACGCAAATCAGTTATTCC-3′) and gyrBF/gyrBR (5′-TTGTTAATGCGTTATCTACGACCTTAG-3′/5′-TCCCCGGTAAGCTGGAGAC-3′), respectively [23]. The gyrA and gyrB genes were amplified using touchdown PCR procedure. The reaction mixtures prepared as above described for the 16S rDNA, were first incubated for 3 min at 94 °C, followed by 35 cycles with a denaturation of 30 s at 94 °C; 30 s at a reduced annealing temperature from 62 to 55 °C by one degree Celsius per a cycle, then 54 °C for the last 25 cycles, then 2 min at 72 °C, followed by a final extension for 7 min at 72 °C. The 16S rRNA, GyrA and GyrB genes of bacterial isolates were sequenced using the forward 27F, GyrAF and GyrBF primers, using the Big Dye Terminator Cycle Sequencing kit v1.1. Sequencing reactions were run using the Applied Biosystems a 3500xL Genetic Analyzer, Foster city, California. The nucleotide sequences were compared with NCBI GenBank entries using the nucleotide BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST; accessed on 12 October 2021) for identification. Furthermore, pairwise sequence similarities were calculated using the method recommended by Meier Kolthoff et al. [24] for the 16S rRNA gene found on the GGDC web server [25,26] available at http://ggdc.dsmz.de/ (accessed on 12 October 2021).

2.5. Antibiotic Susceptibility of the Bacteria

Antibiotic susceptibility testing was performed with the disk diffusion method on bacterial isolates using 36 antimicrobial agents according to the European Committee on Antimicrobial Susceptibility Testing [27]. Mueller-Hinton agar was used as the culture medium (Merck 1.05435, Darmstadt, Germany). Depending on the size of the zone of inhibition, results were interpreted as susceptible, intermediary susceptible, and resistant (Table 1).

Table 1. Antibiotics susceptibility of bacterial isolates from banana 'Grand Naine' in vitro culture to different antibiotics based on diameter of inhibition zone (Resistance (R) ≤ 10 mm, intermediate (I), 11–20 mm; susceptible (S) > 20 mm).

Plate Code	Antibiotic Name	µg/ Disk	Bacterial Isolates on Different Media			
			Trypticase Soy Agar	Potato Dextrose Agar	Middelbrook	R2A
			Bacillus pumilus	*Bacillus subtilis*	*Paenibacillus* spp.	*Geobacillus stearothermophilus*
			Diameter of Inhibition Zone (mm)			
1	Oxacillin (OX)	5	30 (S)	32 (S)	32 (S)	34 (S)
	Penicillin G (P)	6	46 (S)	48 (S)	50 (S)	50 (S)
	Ampicillin (AMP)	10	44 (S)	46 (S)	48 (S)	48 (S)
2	Mezlocillin (MEZ)	30	40 (S)	46 (S)	42 (S)	42 (S)
	Ticarcillin (TIC)	75	50 (S)	50 (S)	55 (S)	55 (S)
	Cefalotin (KF)	30	48 (S)	50 (S)	50 (S)	55 (S)
3	Aztreonam(ATM)	30	6 (R)	0 (R)	0 (R)	12 (I)
	Cefotaxim (CTX)	30	32 (S)	38 (S)	30 (S)	34 (S)
	Cefazolin (KZ)	30	40 (S)	42 (S)	40 (S)	42 (S)
4	Chloramphenicol (C)	30	34 (S)	36 (S)	36 (S)	42 (S)
	Imipenem (IPM)	10	48 (S)	52 (S)	48 (S)	50 (S)
	Tetracyclin (TE)	30	38 (S)	40 (S)	40 (S)	42 (S)
5	Vancomycin (VA)	30	24 (S)	26 (S)	24 (S)	28 (S)
	Gentamycin (CN)	30	28 (S)	30 (S)	30 (S)	32 (S)
	Amikacin (AK)	30	30 (S)	30 (S)	32 (S)	34 (S)
6	Erythromycin (OFX)	5	30 (S)	34 (S)	34 (S)	34 (S)
	Lincomycin (MY)	15	24 (S)	24 (S)	22 (S)	24 (S)
	Ofloxacin (E)	15	34 (S)	38 (S)	36 (S)	40 (S)
7	Colistin (CT)	10	12 (I)	12 (I)	12 (I)	12 (I)
	Pipemidsäure (PIP)	20	20 (I)	20 (I)	22 (S)	22 (S)
	Norfloxaci (NOR)	10	28 (S)	32 (S)	32 (S)	32 (S)
8	Polymyxin B (PB)	300	16 (I)	16 (I)	16 (I)	18 (I)
	Bacitracin (B)	10	8 (R)	8 (R)	8 (R)	8 (R)
	Nitrofurantoin (F)	100	22 (S)	25 (S)	24 (S)	24 (S)
9	Neomycin (N)	30	24 (S)	24 (S)	26 (S)	28 (S)
	Kanamycin (K)	30	30 (S)	32 (S)	32 (S)	34 (S)
	Doxycyclin (DO)	30	42 (S)	40 (S)	40 (S)	42 (S)
10	Fosfomycin (FOS)	50	6 (R)	0 (R)	6 (R)	0 (R)
	Clindamycin (DA)	10	34 (S)	32 (S)	32 (S)	34 (S)
	Ceftriaxone (CRO)	30	32 (S)	32 (S)	30 (S)	36 (S)
11	Moxifloxacin (MXF)	5	36 (S)	36 (S)	36 (S)	38 (S)
	Nystatin (NS)	100	0 (R)	0 (R)	0 (R)	0 (R)
	Linezolid (LZD)	10	42 (S)	42 (S)	42 (S)	44 (S)
12	Piperacillin/tazobactam (TZP)	40	36 (S)	38 (S)	36 (S)	42 (S)
	Teicoplanin (TEC)	30	22 (S)	22 (S)	22 (S)	26 (S)
	Quinupristin/Dalfopristin (QD)	15	22 (S)	22 (S)	24 (S)	24 (S)

2.6. Antibiotic Treatment to Prevent Contamination in Banana Shoot Cultures

Axillary banana shoots were cultured in glass jars containing 50 mL of medium used for their axillary shoot multiplication. The medium was autoclaved and then supplemented with filter-sterilized antibiotics (ampicillin, penicillin, and ticarcillin) at concentrations of 25, 50, 100 and 200 mg·L^{-1}. There were ten replicates for each treatment, with each replicate consisting of a glass jar containing three explants. Antibiotic toxicity on plant growth was determined by observing symptoms of tissue necrosis, browning, chlorosis, and morphological changes. Contamination percentage, number of axillary shoots, number of leaves, and shoot length were also recorded.

2.7. Experimental Design and Statistical Analysis

All experiments were set up in a completely randomized design. All data were subjected to ANOVA and to Tukey's multiple range test using the SAS statistical software (Version 8.1; SAS Institute, Cary, NC, USA).

3. Results and Discussion

3.1. Isolation, Morphological and Biochemical Analyses of Bacterial Isolates

The microbial contaminants that appeared around the base of the in vitro grown banana shoots (Figure 1A) were streaked onto four different media, i.e., TSA, PDA, Middelbrook and R2A to permit the growth of different bacterial species. Twelve bacterial isolates were obtained from contaminated banana in vitro cultures. Repeated streaking of bacteria on four different microbiological media was carried out in order to obtain single and pure colonies. Morphological and biochemical characterization was carried out for the obtained pure colonies to identify all isolated bacteria up to the defined species. The results indicated that the P2 isolate showed slow growth on PDA and colonies required about 10 days to become visible. Microscopic examination of these bacterial isolates from each media showed that colonies of different morphology were obtained depending on the media used. All the bacterial isolates have typical bacterial cell appearance of bacilli in reference to long or short rods endospores (Figure 1B).

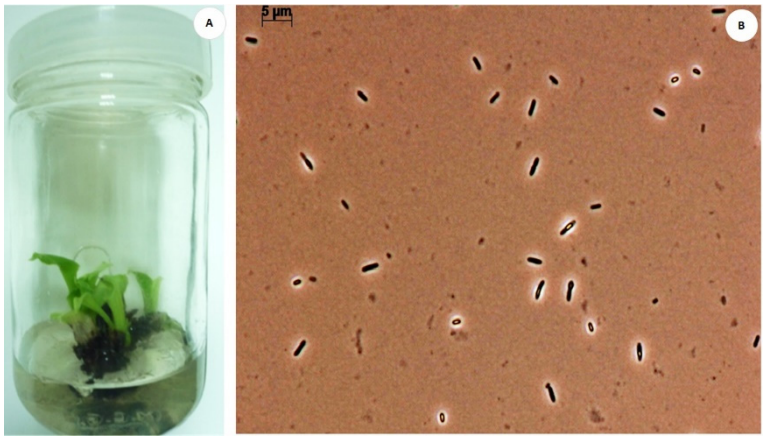

Figure 1. Representative morphology of contaminated in vitro banana culture (**A**) and spore-forming bacillus (Bacillus pumilus) growing on trypticase soy agar medium (**B**).

The identity of these isolates was further confirmed by fatty acid methyl ester analysis (FAME). The bacterial isolates were grouped into four groups (S2; R2, M4; S1, S3, S4, P2, M3, R3 and P1, R1, M2) with a similarity to *Bacillus pumilus* GC subgroup B 99.76%, *Bacillus subtilis* 99.68%, *Geobacillus stearothermophilus*-GC subgroup and *Paenibacillus* spp., respectively. FAME analysis was developed to identify bacterial species more quickly and easily than differential biochemical testing. FAME analysis is currently able to accurately identify 1700 species of bacteria and yeast, many to the subspecies or strain level [28]. In agreement with our findings, Dias et al. [29] reported that twenty endophytic bacteria isolates colonizing in vitro strawberry plants were identified, by FAME profile, into the genera *Bacillus* and *Sphingopyxis*. Bacterial contaminants have been reported to cause culture tissue necrosis, reduced shoot proliferation, and reduced rooting [30]. The recognition of the contamination source is usually difficult [31]. Bacteria are associated with plants as epiphytes or endophytes [32,33]. Therefore, it is impossible to disinfect the explants obtained for plant parts that are attached to the soil due to endophytic microbes [34]. Early findings indicated that bacteria could obviously be detected endogenously in the in vitro plants [35]. However, bacterial growth can be also observed and activated on the surface of the culture media by addition of cytokinins, as performed for rapid multiplication purposes.

3.2. Sequencing Analyses of 16S rDNA, GyrA and GyrB Genes

Genomic DNA was extracted from the twelve bacterial isolates obtained from banana tissue cultures. Bacterial rDNA was amplified successfully 27F and 1492R primers and PCR products were subjected to nucleotide sequencing. The basic local alignment search tool (blast) was used to search nucleotide sequence databases for sequence similarity to DNA sequences. According the sequences alignment, the twelve bacterial isolates were identified as *Bacillus pumilus*, *Bacillus subtilis*, *Geobacillus,* and *Paenibacillus* (Figure 2) using 16S rDNA sequencing, Most of the isolates (S1, S3, S4, P2, M3 and R3) could be clearly identified by more than 99.8% match to *Geobacillus* in the database. Two isolates (R2 and M4) matched to *Bacillus subtilis* by 99.9% and its 16S rDNA sequence was deposited in the NCBI database (AccessionNumber MT157396). The most closely related species, *Bacillus pumilus* represented by the isolate S2 was also found with 99.7% identity in the sequenced 16S rDNA region. The last three isolates (P1, R1 and M2) were identified as *Paenibacillus* spp. where the match percentage was 99.4. Sequencing of 16S rDNA is used extensively for bacterial identification due to its universal distribution among bacterial species and the presence of variable species-specific regions [36,37]. Identification of bacterial contaminants using 16S rDNA sequence analysis has been successfully carried out for in vitro cultures of many plant species, including *Ilex dumosa* [38], *Aglaonema* [39], *Guadua angustifolia* [2] and *Staphylea pinnata* [14].

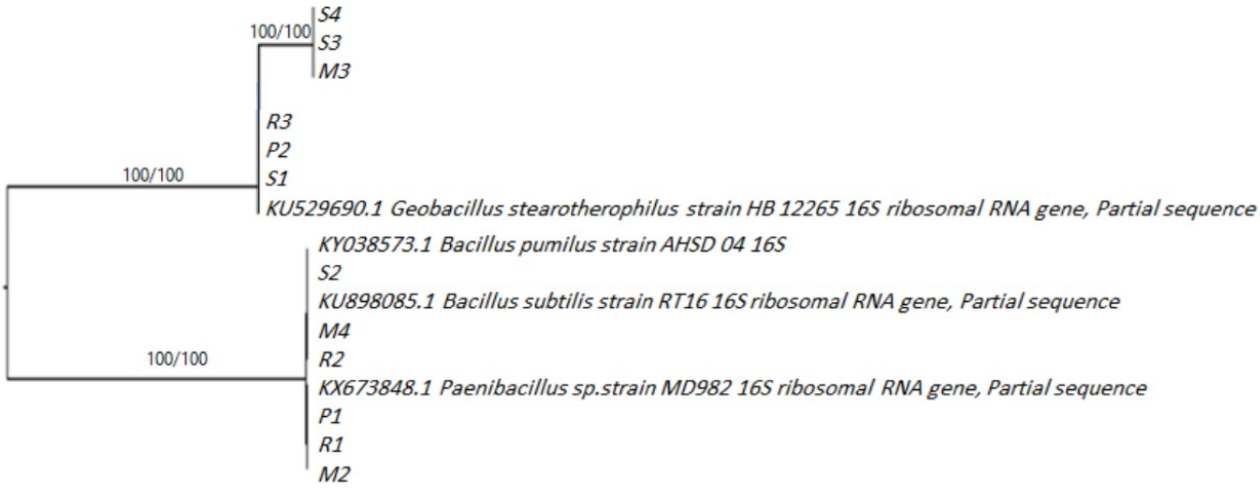

Figure 2. Phylogenetic relationships of 12 isolates belongs to the four references bacteria species based on 16S rDNA gene sequences. S2 represents *Bacillus pumilus; M4 and R2 represent Bacillus subtilis; P1, R2 and M2 represent Paenibacillus* spp. and S1, P2, R3, M3, S3, S4 represent *Geobacillus stearothermophilus*. The branching pattern was generated by the neighbor-joining method. Bootstrap values are indicated at the node.

Previous study on identification of culturable endophytic bacteria isolated from shoot tip cultures of banana 'Grand Naine' revealed the presence of *Enterobacter*, *Klebsiella*, *Ochrobactrum*, *Pantoea*, *Staphylococcus* and *Bacillus* spp. based on partial 16S rRNA gene sequence homology analysis [40]. Moreover, Kneifel and Leonhardt [41] reported that different Gram-positive and Gram-negative bacteria *(Staphylococcus xylosus, S. aureus, S. cohnii, Bacillus* sp., *Corynebacterium* sp., and *Pseudomonas vesicularis)* were isolated from homogenized shoot tips of *Drosera rotundifolia, Spatiphyllum* sp., *Syngonium* 'White butterfly', and *Nephrolepis exaltata* 'Teddy Junior'. In addition, Birmeta et al. [42] identified ten bacterial species using 16s rDNA sequencing in 16 isolates from *Ensete ventricosum* in vitro cultures. The bacterium, *Pseudomonas reactans* was the most frequent in vitro contaminant.

Due to the high level of similarity between the 16S rDNA sequences of *Bacillus subtilis and Bacillus pumilus*, Bacterial isolates designed as S2, R2 and M4 that were identified as *Bacillus pumilus and Bacillus subtilis* on the basis of their 16S rDNA sequences were also identified by amplification and sequencing of the GyrA and GyrB genes. Sequencing and

NCBI BLAST algorithm showed that S2 and R2, M4 were identified at 100% similarity with the sequences available in database of *Bacillus pumilus and Bacillus subtilis* respectively.

The phylogenetic tree of the bacterial isolates was generated by Genome-to-Genome Distance Calculator (GGDC 3.0). The identified bacteria showed a low diversity representing 4 *Bacillus* species in the 12 isolates investigated. The isolates were divided into two main clusters, the first cluster contained 6 isolates subgrouped into S3, S4, M4 and S1, P2, R3 belongs to *Geobacillus* while the second cluster contains two groups; P1, R1 and M2 belongs to *Paenibacillus* wherase the second group comprise the isolates S2 and R2, M4 belongs to *Bacillus pumilus and Bacillus subtilis*, respectively (Figure 2).

Figure 3 shows the phylogenetic tree for the isolates belongs to *Bacillus pumilus* and *Bacillus subtilis* species based on the *GyrA and gyrB* gene sequences. As indicated in Figure 3, the isolate S2 is located in the same cluster with *Bacillus pumilus*; similarly, the isolates R2 and M4 form a cluster with *Bacillus subtilis*. A large number of endophytic microbes have been identified in plant tissue cultures, including species of *Corynebacterium*, *Agrobacterium*, *Pseudomonas*, and *Bacillus* [43,44]. The most common in vitro culture contaminants have been reported to be *Bacillus* spp. [43,45,46]. Rhizospheric soil is one of the possible contamination sources with some *Bacillus* spp. such as *B. pumilus*, which is often found in soil. Explants become contaminated with *Bacillus* spp. because of physical damage to source plants during growth in the field, and surface disinfection of explants during tissue culture initiation is not effective at eliminating endophytic microbes. Microbial contamination with bacilli has recently become more noticeable and easily visible.

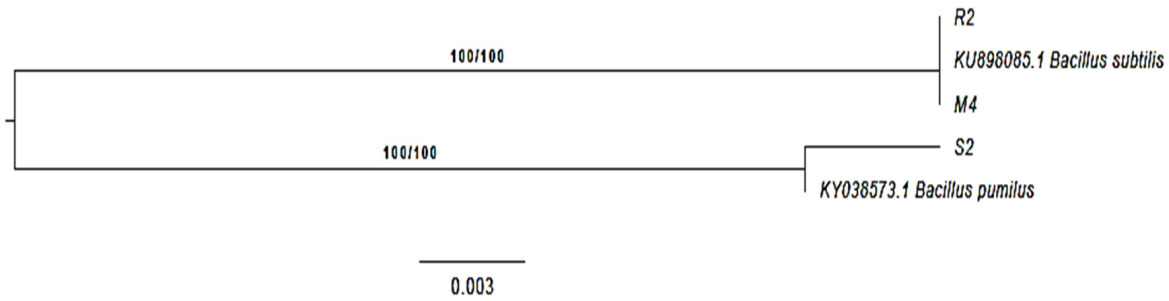

Figure 3. Phylogenetic tree based on the nucleotide sequences of *gyrA and GyrB* genes for three bacterial isolates (S2 and R2, M4) represents *Bacillus pumilus* and *Bacillus subtilis*, respectively.

3.3. Antibiotic Susceptibility and Side Effects on Banana In Vitro Cultures

Thirty-six antibiotics belonging to different antibiotic groups with different mechanisms of action were used to determine the antibiotic susceptibility of the strains isolated from the contaminated in vitro shoot cultures of banana, as shown in Figure 4 and Table 1. Treatment effectiveness varied greatly with the antibiotic tested rather than the bacterial isolates. Antibiotic susceptibility tests showed that 16 antibiotics (Imipenem, Ticarcillin, Cefalotin, Penicillin G, Ampicillin, Mezlocillin, Cefazolin, Chloramphenicol, Tetracycline, Erythromycin, Ofloxacin, Doxycycline, Moxifloxacin, Linezolid, Piperacillin, and Tazobactam) as well as the mixture of antibiotics Piperacillin/tazobactam (TZP) and Quinupristin/Dalfopristin (QD) strongly inhibited the growth of all the bacteria isolated from in vitro cultures. The most effective antibiotics were Ampicillin, Cefalotin, Ticarcillin, Imipenem, and Penicillin G. Conversly, Aztreonam, Bacitracin, Fosfomycin and Nystatin while Colistin, Polymyxin B, and Bacitracin showed low inhibition against all bacterial isolates; their inhibition zones ranged between 6 and 16 mm in diameter. The remaining antibiotics were effective against all bacterial isolates, resulting in inhibition zones ranging between 20 and 36 mm in diameter.

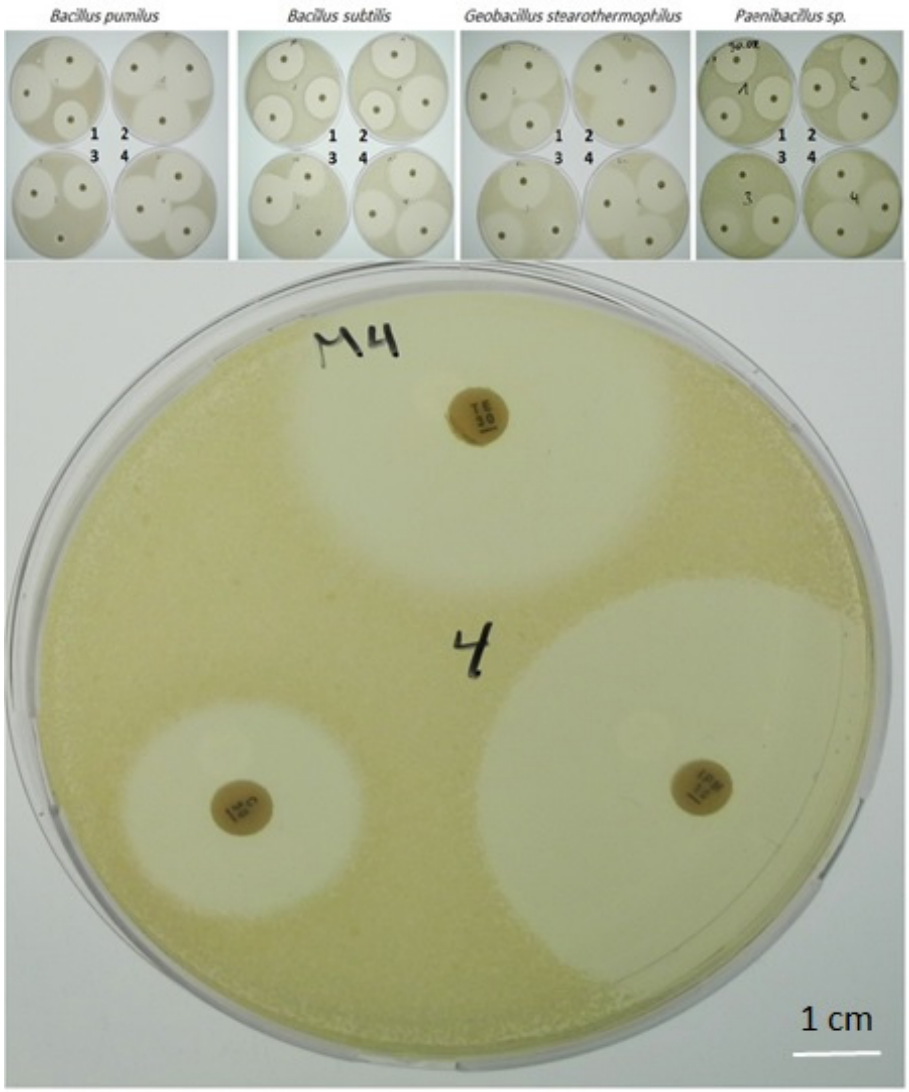

Figure 4. Representative results for an antibiotic susceptibility profile showing the zones of inhibition obtained on Mueller–Hinton (MH) agar for bacterial isolates from banana in vitro plants. Plates coded 1, 2, 3, and 4 represent four different antibiotic groups: (1) Oxacillin, Penicillin G, and Ampicillin; (2) Mezlocillin, Ticarcillin, and Cefalotin; (3) Aztreonam, Cefotaxime, and Cefazolin; (4) Chloramphenicol, Imipenem, and Tetracycline.

In the routine culture procedure, bacterial contaminations appeared after one week and 100% mortality was reached after three weeks. Antibiotic type and concentration significantly influenced the extent of contamination. Bacterial contamination was undetectable and completely eliminated after 6 weeks of antibiotic treatment at concentrations of 100 and 200 mg·L^{-1}, as shown in Table 2. However, the number of shoots and leaves per explant and the shoot length decreased as the concentration of the antibiotic increased. The highest numbers of shoots were recorded at a low antibiotic concentration (25 mg·L^{-1}) of penicillin, ampicillin, and ticarcillin, resulting in 7.3, 7.7, and 8.3 shoots per explant, respectively. However, these growing shoots showed symptoms of browning and bleaching of leaves (chlorosis). Overall, ticarcillin and penicillin treatments at 100 mg·L^{-1} were optimal for elimination of contaminants, despite the reduced shoot multiplication and growth of banana cultures.

Table 2. Effect of ampicillin, penicillin and ticarcillin, on contamination percentage, multiplication and growth of banana 'Grand Naine' shoots after 6 weeks in culture.

Antibiotic	Concentration ($mg \cdot L^{-1}$)	Contamination (%)	Shoots (No./Explant)	Leaves (No./Explant)	Length of the Main Shoot (cm)
Control	0	100.0 a [z]	0.0 h	0.0 f	0.0 g
Ampicillin	25	46.7 c	7.7 ab	4.0 ab	4.7 bc
	50	23.3 de	5.7 cd	3.3 bc	3.7 cde
	100	0.0 f	4.3 ef	3.0 cd	3.0 ef
	200	0.0 f	2.7 g	2.3 de	2.3 f
Penicillin	25	28.3 d	7.3 ab	3.7 abc	5.7 ab
	50	21.7 e	6.7 bc	3.3 bc	4.3 cd
	100	0.0 f	5.0 de	2.3 de	3.3 def
	200	0.0 f	2.7 g	2.0 e	2.3 f
Ticarcillin	25	53.3 b	8.3 a	4.3 a	6.0 a
	50	23.3 de	7.7 ab	3.7 abc	5.7 ab
	100	0.0 f	5.0 de	2.3 de	3.3 def
	200	0.0 f	3.3 fg	2.0 e	2.3 f
Significance [y]					
Antibiotic type (A)		*	*	NS	*
Antibiotic concentration (B)		*	*	*	*
A × B		*	NS	NS	NS

[z] Values followed by the same letter in the same column are not significantly different at $p \leq 0.05$ level, according to Tukey's multiple range test. [y] NS, * not significant or significant at $p \leq 0.01$.

To eliminate the contamination problem, many studies have been conducted to identify an antibacterial agent effective in controlling bacterial contamination without affecting growth and morphology of the in vitro plants. Various antimicrobial agents have been extensively tested to inhibit the growth of microbial contaminants within media during in vitro plant cultures, with varying success in banana [47], *Lilium candidum* [48], *Saraca asoca* [49], *Saccharum officinarum* [50], *Pelargonium hederaefolium* [51], *Ipomoea batatas* [52], *Jatropha curcas* [53], and *Solanum tuberosum* [54,55]. The addition of antibiotics to the medium resulted more effective than when added as surface sterilants in the *Citrus* sp. [56,57]. Moreover, pretreatment of tissue cultures with antibiotics proved effective in in vitro cultures of several plant species such as *Bambusa balcooa* [58,59] and *B. nutans* [60]. However, long duration of antibiotic treatment has a toxic side effect on explants and may reduce the plant growth [61]. In this case, an effective alternative approach could be the addition of antibiotics to the culture media, even though high antibiotic doses can still result in phytotoxic effects. The reduced in vivo growth of treated plants with antibiotics could be due to the elimination of endophytes while the vitality of a plant essentially depends on the core microbiome. However, in vitro plants are grown under aseptic conditions and such endophytes are considered contaminants and must be eliminated to maintain healthy cultures. In the present study, application of the recommended doses of antibiotics proved effective to save the losses and increase the survival rate of regenerated banana plantlets during acclimatization stage (Figure 5).

Figure 5. Impact of antibiotics supplementation (100 mg L^{-1} ticarcillin) to the in vitro contaminated banana culture on its growth and survival during acclimatization in greenhouse. (**a**) Non-treated plantlets (control); (**b**) treated plantlets.

4. Conclusions

We concluded that *Bacillus pumilus*, *Bacillus subtilis*, *Geobacillus*, and *Paenibacillus* endophytic bacteria were identified in banana in vitro cultures by sequencing of 16S rRNA, *gyrA* or *gyrB* genes. The identification of these bacteria facilitated the selection of appropriate antibiotics to prevent their growth. Using a single antibiotic, such as ticarcillin, ampicillin, or penicillin (100 mg·L^{-1} for 6 weeks), proved effective for eliminating the bacterial contamination. Based on these results, it is recommended that the antibiotic should be used for one culture to reduce or avoid the losses in the commercial micropropagation of banana.

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