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Identification and control of endophytic bacteria during *in vitro* cultures of *Staphylea pinnata* L.

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ABSTRACT

This study focused on the identification and elimination of endophytic bacterial contaminations during *in vitro* propagation of European bladdernut (*Staphylea pinnata*). Axillary shoots were propagated on Murashige and Skoog medium with 20 mg · dm^{-3} FeEDDHA, 5 µM BA and 0.5 µM NAA at 20/18°C (day/night) and a 16-h photoperiod. Clouding by endophytic bacterial colonies was observed where shoots contacted the media. Bacteria were isolated and separated by repeated streaking as two strains. Gram staining revealed that both strains were Gram-negative. The colonies were very precisely identified as *Acinetobacter johnsonii*, strain ATCC 17909, and *Methylobacterium rhodesianum*, strain DSM 5687, using VITEK®2—a rapid bacterial identification system—and the 16S rRNA gene sequencing method. The agar disc-diffusion test proved that both bacterial strains were susceptible to 13 antibiotics (out of 25 tested), derived from the groups of fluoroquinolones, aminoglycosides and tetracyclines. Doxycycline or gentamicin (100–300 mg · dm^{-3}) was added to the *S. pinnata* shoot propagation medium to eliminate bacteria. Gentamicin 100 mg · dm^{-3} showed the best effect, inhibiting the growth of endogenous bacteria (63%) when applied in the medium for 4 weeks. After the following transfer to media without antibiotics, shoots developed axillary buds and bacterial colonies were not observed.

Keywords: *Acinetobacter*, antibiotics, decontamination, gene sequencing, *Methylobacterium*, VITEK®2 system

INTRODUCTION

Endogenous bacteria are specific microorganisms that inhabit the interior of plant organs. They live in herbaceous and woody plants, in various plant parts, most often in the root system. They often benefit the plant by producing growth regulators or substances that can play their function, absorption of nitrogen and phosphorus or prevention of infections by competing with pathogenic species (Rosenblueth and Martínez-Romero, 2006; Ryan et al., 2008; Orlikowska et al., 2017).

The presence of bacteria in *in vitro* cultures is considered a negative symptom associated with the neglect of aseptic working conditions. Surface disinfection of plant material is unable to remove the latent endogenous bacteria present in tissues (Moreno-Vázquez et al., 2014). Bacterial infections can inhibit the culture development by producing phytotoxic metabolites and competing between the plant and bacteria for nutrients (Leifert and Cassells, 2001). There is often a limited culture growth without the visible symptoms of a bacterial infection that looks like the exudation of substances from tissues to the medium, and it is transferred with subcultures (Viss et al., 1991). These infections are latent and manifest themselves during drastic changes in the *in vitro* environment (Almeida et al., 2009).

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Staphylea pinnata L. (European bladdernut) is a deciduous woodland shrub micropropagated due to difficulties with a standard generative propagation (Tylkowski, 2007). It is native to Central and Eastern Europe and Asia Minor and is endangered and strictly protected in Poland. *S. pinnata* has a high ecological and ethnobotanical value. It is planted in contemporary gardens due to its high ornamental quality (Łuczaj, 2009, Heiss et al., 2014). Recent studies have demonstrated its high antiproliferative, anticancerogenic and antioxidant activities (Laciková et al., 2007, 2009). The development of an effective and reliable *in vitro* method would ensure species protection as well as plant material to study the potential of bioactive compounds and secondary metabolites. The first, successful multiplication experiments were conducted by our group (Szewczyk-Taranek and Pawłowska, 2016; Szewczyk-Taranek et al., 2017). Despite the propagation success, bacterial contaminations occurring in 2-year-old *S. pinnata* cultures were the bottleneck.

Contaminations are detrimental, especially in three micropropagation systems. Woody plant *in vitro* cultures (e.g. *S. pinnata*) are long-term ones that are more likely to develop latent infections caused by internal bacteria (Reed et al., 1998). In laboratory tissue cultures, microbial contaminations significantly increase the time, effort and financial expenses of the experiments and the risk of losing valuable cultures and affecting the results. In commercial production, severe losses due to contamination are one of the most economically important problems (Cassells and O'Herlihy, 2003). Detection and elimination of infections is one of the most important tasks in laboratory tissue culture management. Rapid bacterial identification and further susceptibility testing can improve the decontamination and suppression of endophytic bacteria and provide the necessary data to effectively circumvent the decline problem in *in vitro* cultures (Thomas, 2004).

Modern molecular techniques that allow to save time while maintaining high efficiency increasingly support bacterial identification. Three types of sequences are usually analysed using the polymerase chain reaction (PCR) method: pathogenesis-related genes, plasmid genes and ribosomal operons. The 16S rDNA gene is most useful for determining bacterial strains at the molecular level (Moreno-Vázquez et al., 2014). 16S rDNA sequencing has played an essential role in the accurate identification of bacterial isolates and the discovery of novel bacteria in microbiology over the last decade. The 16S rRNA gene is currently established as the 'gold standard' not only in bacterial identification but also in microbial ecology studies. The 16S rRNA is present in all known bacteria and encodes a structural RNA that is a ribosome component. It has a size of ~1,500 bp and includes both conserved and variable regions; this method proved more sensitive and accurate compared to VITEK2 (Bosshard et al., 2006). The 16S rRNA method is extremely useful due to the possibility of easily detecting bacteria that are rare, multiplying slowly or not growing under artificial conditions (uncultivable bacteria). The speed of the method allows for quick reactions and combating the infection at the early stages of its development (Woo et al., 2008).

Antibiotics are used to control bacterial infections in *in vitro* cultures; they should have an adequate biocidal effect on the bacteria to be controlled, without being toxic to plants. Before the antibiotic is used, the infection should be identified to select the appropriate agent and minimise the risk of developing a resistant strain (Falkiner, 1997).

The objective of the study was to identify and control the manifestations (growth) of the endophytic bacteria in *in vitro* cultures of bladdernut. Our research hypothesis assumed that the phenotypic VITEK®2 system and the fast molecular analysis of 16S rDNA sequencing would identify endophytic colonisation in *S. pinnata* cultures. The addition of well-selected antibiotics to the medium would allow to repress bacteria, while preserving the potential for shoot propagation in *in vitro* culture.

MATERIALS AND METHODS

Biological material and experiment program

In vitro cultures of *S. pinnata* were initiated from shoot tips and propagated through axillary bud development on Murashige and Skoog (1962) multiplication medium with 20 mg · dm^{-3} FeEDDHA and plant growth regulators 5 μM BA (6-Benzylaminopurine) and 0.5 μM NAA (1-naphtaleneacetic acid), pH 5.7. The cultures were incubated at 20/18°C (day/night), 80% relative humidity, photosynthetic photon flux density 35 μM · m^{-2} · s^{-1} and a 16-h photoperiod (Szewczyk-Taranek and Pawłowska, 2016). The cultures were transferred to fresh medium every 5 weeks. Clouding and pale pink color were detected on media where shoots came in contact with the medium after 16 months of culture. This suggested the development of latent infection. Bacterial colonies were observed in 75% of containers. Elimination of contaminations from the culture was a relevant next step due to the valuable plant material and the decrease in plant quality. Decontamination strategy stages included (Figure 1): (1) isolation and identification of bacteria; (2) determination of antibiotic susceptibility; and (3) antibiotic effectiveness in the control of the bacterial manifestation by antibiotic therapy and the antibiotic toxicity test to *in vitro* plant material.

Isolation of microorganisms from plant in vitro cultures

Microorganisms occurring in plant *in vitro* cultures (Figure 2A) were collected directly from the medium or plants and isolated using a streaking technique on microbiological plates containing enriched agar medium (2.5%, Biocorp, Warsaw, Poland). Bacteria were streaked onto nutrient agar plates and incubated at room temperature. After 24–48 h incubation, the developed microbial colonies were purified by

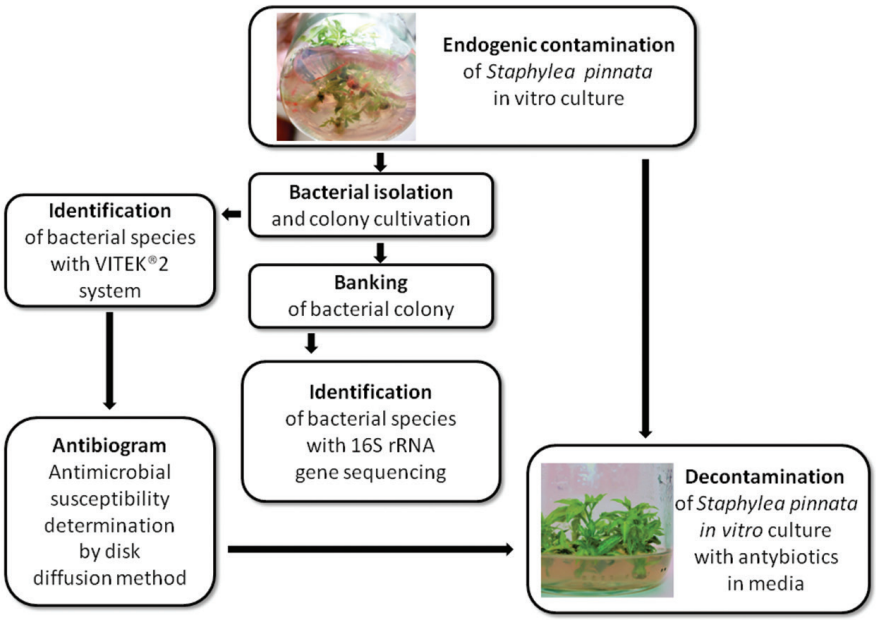

Figure 1. Schematic presentation of the experimental procedures in the study.

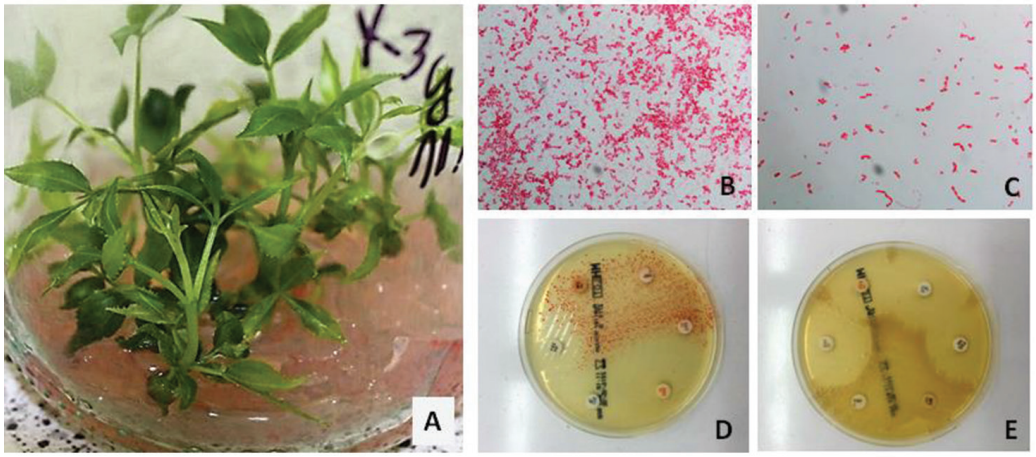

Figure 2. Bacterial contamination in *in vitro* culture of *Staphylea pinnata* (A); Gram-staining test: *Acinetobacter johnsonii* colony (B); *Methylobacterium rhodesianum* colony (C); susceptibility test with agar disc-diffusion method: *A. johnsoni* (D); *M. rhodesioanum* (E).

repeated streaking. Morphology, size, shape and growth pattern were recorded for each colony type. The microbiologically pure strains were then cultivated and preliminarily characterised by the Gram-staining technique (Lia et al., 2018). A microbial bank of isolates was established employing the Microbank™ system (Pro-Lab Diagnostics, www.pro-lab.com) and stored at −80°C for further use.

Bacterial identification by VITEK®2

The isolated strains were identified by the VITEK®2 BioMerieux™ system (Pincus, 2015) using the cards for Gram-negative bacteria, GN 21 341. The identification was further verified with a Brüker MALDI Biotyper® (Sogawa et al., 2011).

Bacterial identification by 16S RNA

Previously collected bacterial isolates were streaked onto TSA plates (Tryptic Soy Agar—Oxoid, Basingstoke, Hants, UK) and cultured at room temperature for 72 h. For genomic DNA extraction, a single colony from each plate was used to inoculate 5 ml of TSB culture medium (Tryptic Soy Broth, Oxoid). After 72 h of incubation, the cells were harvested by centrifugation at 4°C, 3,890 g for 15 min (Sigma 3–16K). Pellets were used to extract genomic DNA with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol for bacteria. Briefly, bacterial cells were lysed with Proteinase K, and cell content was loaded onto spin columns. During brief centrifugation, DNA was selectively bound to a silica-gel membrane, while contaminants passed through.

The remaining impurities were removed in two wash steps, and DNA was then eluted.

For PCR amplification of the 16S gene, 63F (5′-CAGGCC TAA CAC ATG CAA GTC-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) primers were used (Marchesi et al., 1998). The PCR reaction used GoTaq Green Master Mix (Promega), which already contained Taq DNA polymerase, dNTPs, $MgCl_2$ and two dyes for electrophoresis monitoring.

PCR conditions were as follows: 5 min of initial denaturation at 95°C, then 30 cycles consisting of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min followed by a final extension step of 5 min at 72°C. PCR products were visualised by agarose gel electrophoresis with Gel Red (Biotium) for DNA staining. The expected size of the PCR products was 1320 bp (Table 1). PCR products were purified using the QIAquick PCR purification kit (Qiagen); DNA concentration was assessed with a NanoDrop spectrophotometer. PCR products were sent for sequencing using both 63F and 1387R primers to Eurofins MWG Operon. The sequences were analysed with the Basic Local Alignment Search Tool for nucleotides (BLASTn) against the '16S ribosomal RNA sequences (Bacteria and Archaea)', NCBI database (2016). The sequences were subsequently aligned with the top hit genes from the BLASTn search, using Clone Manager 9. Sequencing chromatograms were analysed using BioEdit.

Antibiotics susceptibility

The antibiotic susceptibility test was carried out using the agar disc-diffusion method as recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST). Petri dishes with Mueller-Hinton agar medium were inoculated with the tested bacterial isolates (Balouiri et al., 2016; Deote et al., 2018). Then, filter paper discs (6 mm in diameter), containing 25 antibiotics from 6 classes (manufactured by Bio-Rad, Warsaw, Poland), were aseptically placed on the agar surface (6 on each plate). The test is based on the application of standard class of antibiotics: β-lactam (penicillin 6 μg, ampicillin 10 μg, amoxicillin 10 μg, ampicillin+sulbactam 10–10 μg, piperacillin 100 μg, ticarcillin 75 μg and cefuroxime 30 μg); fluoroquinolones (ciprofloxacin 5 μg, levofloxacin 5 μg, norfloxacin 10 μg and ofloxacin 5 μg); aminoglycosides (amikacin 30 μg, gentamicin 10 μg, netylmicin 30 μg and tobramicin 10 μg); macrolides (azithromycin 15 μg, clarithromycin 15 μg, erythromycin 15 μg and clindamycin 2 μg); tetracyclines (tetracycline 30 μg and doxycycline 30 μg) as well as several others: chloramphenicol 30 μg, nitrofurantoin 100 μg, trimethoprim 5 μg and co-trimoxazole 25 μg. The inoculated plates were incubated at 37°C for 24 h. The strains were characterised as sensitive or resistant to antibiotics based on their ability to grow in the presence of a given antibiotic. Diameters of the developed inhibition zones around the discs were measured and recorded (Figure 2D, 2E).

Table 1. PCR reaction mixture for amplification of gene coding 16S

	Volume (μl)	Final concentration
GoTaq Green Master mix 2x	25	1×
Upstream primer 63F	5	1 μM
Downstream primer 1387R	5	1 μM
Genomic DNA	5	< 250 ng
Nuclease-free water	10	–
Total	50	

Culture decontamination using antibiotics

S. pinnata shoots were grown *in vitro* on the multiplication medium, supplemented with antibiotic solutions sterilised by filtering (Milex® 0.22 μm filter, Millipore Ireland) and added after media autoclaving. Antibiotics were used in the following concentrations: doxycycline (Doxycylinum TZF Polfa, Poland) 100, 200, or 300 mg · dm^{-3}; gentamicin (Gentamicin KRKA, Poland) 100, 200, or 300 mg · dm^{-3}. Media without antibiotics served as control. This experiment was carried out using shoots with visible contaminations. The cultures were cultivated in a growth chamber for 4 weeks in the same conditions as during shoot micropropagation. After this time, observations took into account: viable and weakened shoots, with the visible clouding of bacteria colonies or without bacteria at the base of shoots. Retarded shoots with yellowish and brownish leaves, without multiplication were considered as weakened shoots.

Only viable shoots without manifestations of endophytic bacteria were transferred to the standard medium for the propagation of axillary shoots.

Data analysis

The experiment was conducted in seven combinations, each with six repetitions, five explants in each repetition (30 explants in one combination). After 4 weeks, the percentage of viable shoots without visible bacterial colonies, the percentage of viable shoots with visible bacterial colonies, weakened shoots without visible bacterial colonies and the percentage of weakened shoots with visible bacterial colonies were recorded. The results were calculated as mean values. Data were analysed with ANOVA after Bliss transformation, and differences between the means were assessed according to Duncan's multiple range test, at the confidence level of $\alpha = 0.05$ using the Statistica software (StatSoft).

RESULTS

The presence of bacteria was detected by visual observations near the base of *S. pinnata* shoots during *in vitro* multiplication. In some plant containers, bacteria formed visible clouding in the medium where the cut-surface of the shoot was in contact with the medium (Figure 2A).

After contaminant isolation, it was possible to separate the strains into two distinct types. Gram staining revealed that both bacteria were Gram-negative and had a rod shape (Figure 2B, 2C). Bacterial strains were identified as *Acinetobacter johnsonii* and *Methylobacterium rhodesianum* according to the results obtained using the VITEK®2 system.

The study used a molecular analysis of the 16S rRNA gene sequence because of its proven presence in all known bacterial species, containing both conservative and variable regions, to independently confirm the VITEK®2 test. Two primers were used for sequencing to compare and align the outcomes. The results refined the observations by confirming the strain identity: *A. johnsonii*, strain ATCC 17909 and *M. rhodesianum*, strain DSM 5687 (Table 2). In the case of A. johnsonii, there was a single nucleotide difference between the used primers. This error was related to the quality of sequencing, which was confirmed by the chromatogram.

The agar disc-diffusion susceptibility test identified 13 antibiotics as potentially useful in eliminating both of these contaminants among 25 antibiotics tested (Table 3 and Figure 2D, 2E). The most effective antibiotics were: two from the β-lactam class (piperacillin and tikarcillin); both tested tetracyclines (doxycycline and tetracycline); all fluoroquinolones (ciprofloxacin, evofloxacin, norfloxacin and ofloxacin) and aminoglycosides (amikacin, gentamicin, netylmicin and tobramicin) (Table 3).

Based on the susceptibility results, two antibiotics, doxycycline and gentamicin, were selected for a further analysis of culture decontamination efficiency and toxicity tests to *in vitro S. pinnata* plants. Different concentrations of both antibiotics were applied to the multiplication medium. After 4 weeks, about 70–93% of shoots on medium with gentamicin or doxycycline were free from visible contaminating bacteria, but 10–70% were weakened, and this effect was dependent on antibiotic concentration (Figure 3). Higher antibiotic concentration inhibited bacterial growth, but at the same time led to shoot dieback. The highest toxic effect on plants was observed on the medium with antibiotics at a concentration of 300 mg · dm^{-3}. At a concentration of 300 mg · dm^{-3}, gentamicin killed 82% of shoots without visible bacteria and 63% of doxycycline. The best results were recorded on the medium with gentamicin 100 mg · dm^{-3}, as the highest percentage (63%) of shoots survived without visible symptoms

Table 2. Basic bioinformatics parameters characterised by 16S rRNA sequencing analysis of the endogenous bacteria isolated from the *Staphylea pinnata* tissue culture

Sample code	Primer	The strain with the highest compatibility	Compatibility (%)	NCBI access number
16–31	63F	*Acinetobacter johnsonii*	99	NR_117624.1
	1387R	Strain ATCC 17909	100	
16–35	63F	*Methylobacterium rhodesianum*	100	NR_041028.1
	1387R	Strain DSM 5687	100	

Table 3. Antibiotics susceptibility test with agar disc-diffusion method performed on bacterial strains isolated from *in vitro* cultures of *Staphylea pinnata*

Antibiotic class	Antibiotic	AJ*	MR	Antibiotic class	Antibiotic	AJ	MR
β-lactams	Penicillin	R**	S	Aminoglycosides	Amikacina	S	S
	Amoxicillin	R	S		Gentamicin	S	S
	Ampicillin+ sulbactam	R	S		Netilmicin	S	S
	Ampicillin	R	S		Tobramycin	S	S
	Piperacillin	S	S	Macrolides, Lincosamides, Streptogramins	Azithromycin	R	S
	Tikarcillin	S	S		Clarithromycin	R	S
	Cefuroxime	R	S		Erythromycin	R	S
Tetracyclines	Doksycycline	S	S		Clindamycin	R	R
	Tetracycline	S	S	Others	Chloramphenicol	R	S
Fluoroquinolones	Ciprofloxacin	S	S		Nitrofurantoin	R	R
	Levofloxacin	S	S		Trimethoprim	R	R
	Norfloxacin	S	S		Co-trimoxazole	S	R
	Ofloxacin	S	S				

*AJ, *Acinetobacter johnsonii*; MR, *Methylobacterium rhodesianum*.

***R*, *antibiotic-resistant*; *S*, *antibiotic-sensitive*.

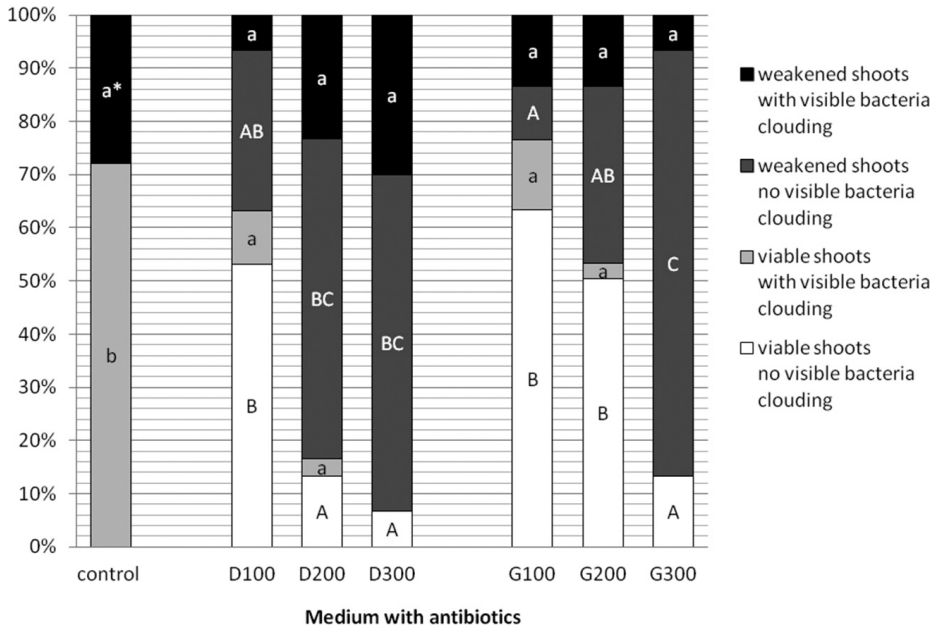

Figure 3. Effect of antibiotics on *Staphylea pinnata in vitro* culture: D100, D200 and D300—Doxycycline at concentration 100 to 300 mg · dm^{-3} in the multiplication medium; G100, D200 and G300—Gentamicin at concentration 100–300 mg · dm^{-3} in the multiplication medium; Control—without antibiotics. *Mean values with the same letter do not differ significantly at α = 0.05, shoots with visible bacteria and shoots without visible bacteria were analysed separately.

of bacterial infection. However, both antibiotics were phytotoxic to *S. pinnata* shoots on all tested media with different antibiotic concentrations, as the shoots were visibly weakened, shorter and some were chlorotic in comparison to control. All shoots in the control medium (without antibiotics) were infected (100%), which led to a slow growth retardation and reduction of multiplication efficiency and dieback of 28% of explants (Figure 3). Application of tested antibiotics in the medium has limited bacterial growth in subcultures. Viable shoots without manifestations of endophytic bacteria were transferred to the standard medium for the propagation of axillary shoots. New shoots developed properly, and bacterial colonies were not noted. The *Staphylea* cultures were a reliable source of explants in our *in vitro* experiments following 2 years.

DISCUSSION

The association of woody plants with endophytic bacteria is common in nature and brings several advantages to both sides. However, in *in vitro* cultures, even the presence of beneficial endophytic bacteria might result in a decline in culture performance or its complete dieback and is considered undesirable (Pirttilla et al., 2008; Orlikowska et al., 2017). Often, bacteria remain latent in culture for several weeks or even several years (Reed et al., 1998). Pathogenic *Agrobacterium vitis* was able to persist in the latent stage within *Vitis vinifera in vitro* shoot cultures for 14 weeks (Poppenberger et al., 2002). In *in vitro* vine cultures, the infection appeared after 8 years of culture and resulted in a decrease in its effectiveness (Thomas and Prakash, 2004). In the case of *S. pinnata*, culture infection became a problem 2 years after its establishment and bacteria limited shoot propagation and led to shoot dieback. Growth of endophytes in the culture medium is a response to imbalance some *in vitro* conditions, and not due to failures in micropropagation techniques (Esposito-Polesi et al., 2015, Thomas and Kumari, 2010).

The identification of these contaminations by the molecular method, based on the sequencing of the bacterial 16S rRNA gene, confirmed the results obtained with the VITEK®2 method and allowed for the precise determination of bacterial strains sampled from *S. pinnata* cultures. The molecular method is accurate and it brings many benefits and improvements in the case of bacteria that are difficult to grow. Previous studies have shown that the VITEK®2 system could give reliable identification and susceptibility results with pure bacterial cultures in medicine and clinical practice (Ling et al., 2003; Wallet et al., 2005; Woo et al., 2008; Moreno-Vázquez et al., 2014) and detect antimicrobial activity in plant extracts or essential oils (Bubulica et al., 2012; Sakkas et al., 2016). Although this test is modern, fast, simple and precise, it is not commonly used for the identification of bacteria in plant tissue cultures. Liu et al. (2005) applied the VITEK®2 test to precisely identify endophytic contamination in *Limonium sinuatum* shoot tip cultures. Bacteria isolated from *S. pinnata in vitro* cultures belonged to *Acinetobacter* and *Methylobacterium*. The bacteria identified in the study were beneficial endophytic microorganisms promoting plant growth by production of phytohormones, reported in the literature (Forchetti et al., 2007).

The presence of endophytic bacteria of the genus *Acinetobacter* has also been confirmed in *in vitro* cultures of other species, for example, pineapple (Abreu-Tarazi et al., 2010), hosta and raspberry (Zawadzka et al., 2014); however, these contaminations were not harmful. The presence of *Acinetobacter* and *Methylobacterium* has been investigated in pineapple and orchids cultures (Esposito-Polesi et al., 2017). In *S. pinnata* cultures the strains of the following endophytic bacteria have been determined: *Acinetobacter johnsonii*, strain ATCC 17909 and *Methylobacterium rhodesianum*, strain DSM 5687. *Acinetobacter johnsonii* belongs to the bacterial genus commonly found in the soil and water environment, whose relationship with plants has not been fully understood (Álvarez-Pérez et al., 2013). *Acinetobacter* has the ability to take up (solubilise) and accumulate phosphorus, which is important for plants, and is quickly immobilised in soil. *Methylobacterium* is a genus of bacteria characterised by the production of a pink pigment that is capable of using one-carbon compounds, including, for example, methanol as a carbon source. They are bacteria commonly found in nature in various environments, and their relationship with plants has been described (Holland, 1997; Green, 2006). These bacteria, thanks to enzymes that facilitate the uptake of nitrogen from urea, influence the metabolism of this element in plants, and the cytokines produced by them affect plant growth (Holland, 1997).

Antibiotic can be used prophylactically or therapeutically in plant tissue cultures. Addition of the antibiotics to the media allows the effective control of *in vitro* contamination and antibiotics are used in bacterial infections that occur during plant micropropagation that aim at eliminating microorganisms from the culture environment. These compounds, however, are not neutral to plant material by being phototoxic or by changing the direction of organogenesis (Grzebelus and Skop, 2014).

Testing different antibiotics during *in vitro* cultures is necessary because that may not be as effective *in vitro* as against isolated microorganism. This might be due to factors like fitotoxicity and poor intake of some antibiotics by the plants (Reed et al., 1995). Aminogycosides antibiotics have been specifically found effective against *in vitro* phytopatogens. Lower concentration of gentamicin have been successfully used against endophytic bacteria in *Aglanoema in vitro* cultures (Fang and Hsu, 2012). Gentamicin in concentrations of 100 and 200 mg · dm^{-3} was effective in decontaminating *S. pinnata* culture, however, it caused chlorosis on the leaves and plant dwarfism relative to control. The application of doxycycline in a lower concentration of 100 mg · dm^{-3} did not result in the expected effect of infection elimination, while the greater concentration was highly phytotoxic. Protein synthesis inhibitor, like tetracycline, has proven toxic for plants micropropagation (Luna et al., 2008).

The 4-week application of antibiotics in the medium has limited bacterial growth in subcultures of *S. pinnata*. Similarly, gentamicin at a concentration of 12.5 μm · ml^{-1} together with streptomycin (1,000 μm · ml^{-1}) as well as the group of amyglicoside antibiotics were effective in clearing hazel cultures from bacteria; however, they exerted significant toxicity to this species (Reed et al., 1998).

CONCLUSIONS

1. Endophytic bacteria manifestations found in 2-year-old *in vitro* cultures of *S. pinnata* were very precisely identified as *Acinetonobacter johnsonii*, strain ATCC 17909, and *Methylobacterium rhodesianum*, strain DSM 5687, using VITEK®2, a rapid bacterial identification system, and the 16S rRNA gene sequencing method.
2. The agar disc-diffusion test showed that both bacterial strains were susceptible to 13 antibiotics, derived from the groups of fluoroquinolones, aminoglycosides and tetracyclines.
3. Gentamicin 100 mg · dm^{-3} applied in the medium for 4 weeks showed the best effect, inhibiting the growth of endophytic bacteria (in visual assessment 63% shoot without clouding by endophytic bacterial colonies, which were not visible in next 18 months of shoot *S. pinnata in vitro* cultures.
4. The described procedure has an overall reliable performance and provides better woody plants *in vitro* culture management.

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AUTHOR CONTRIBUTIONS

B.S.T. and B.P. designed and investigated the research, supervised the finding of this work, analysed all data and wrote the final manuscript. A.J. carried out the experiment concerning gene sequencing and performed analytic calculations and results. P.K. and P.S. carried out the experiment concerning the isolation of bacteria culture, Vitek test and antibiogram. P.P. collected data and contributed in writing the paper. J.M. collected data and designed the figures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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