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DETECTION, ISOLATION, AND PRELIMINARY CHARACTERIZATION OF BACTERIA CONTAMINATING PLANT TISSUE CULTURES

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Abstract

In order to limit the contamination problem in plant tissue cultures experiments on selection of media suitable for detection and isolation of bacteria contaminating plant tissue explants, and preliminary characterization of isolates were made. In the first experiment aiming at detection of bacteria in plant explants four strains representing genera most often occurring at our survey of plant tissue cultures, and earlier isolated and identified (*Bacillus, Methylobacterium*, *Pseudomonas* and *Xanthomonas*) were streaked on five bacteriological media (NA, King B, K, R2A and 523) and on the medium used for plant culture initiation – ½ MS with milk albumin (IM). All strains grew on all media but on K and IM at the slowest rate and on 523 medium at the fastest. The IM medium proved to be useful for immediate bacteria detection at the initial stage of culture. In the second experiment, aiming at characterization of isolates on the basis of colony growth and morphology 14 strains (*Agrobacterium*, *Bacillus*, *Curtobacterium, Flavobacterium*, *Lactobacillus*, *Methylobacterium* – 2 strains *Mycobacterium*, *Paenibacillus*, *Plantibacterium*, *Pseudomonas, Stenotrophomonas*, *Xanthomonas,* and species *Serratia marcescens*) were streaked on five microbiological media: KB, NBY, YDC, YNA and YPGA. All strains grew on all those media but at different rates. The only exception was the strain of *Lactobacillus* spp., which did not grow on King B medium. This medium allowed the detection of such characteristic traits as fluorescence (*Pseudomonas*) and secretion of inclusions (*Stenotrophomonas*). The third experiment was focussed on assessment of the sensitivity of detection of specific bacteria in pure cultures and in plant tissue cultures using standard PCR and BIO-PCR techniques with genus specific primers and 2 methods of DNA isolation. Results showed that the use of Genomic Mini kit enabled an increase of the sensitivity by 100 times as compared to extraction of DNA by boiling. Moreover, the application of BIO-PCR increased sensitivity of detection from 10^2 to 10^5 times over the standard PCR. If looking for unknown cultivable bacteria more effective detection seems to be use of microbiological method enabling detection on bacteriological media single cells in the fragments of explants or in wash liquids, in which fragmented explants were shaken.

Key words: bacterial contamination, plant tissue culture, bacteria detection, PCR, BIO-PCR

INTRODUCTION

Bacterial contaminations are a serious problem in plant *in vitro* cultures, both in commercial plant micropropagation, by making difficult culture initiation, reducing efficiency of multiplication and rooting of shoots, as well as in research laboratories, where contamination can be the causal agent of false results in physiological experiments [1, 2, 3]. The diversity and abundance of genera and species of exo- and endobiotic bacteria accompanying donor plants [4, 5] is a major challenge in the sterilization of initial explants, a quick detection of bacteria in the first *in vitro* passages and a minimization of their adverse effect on shoot multiplication and rooting efficiency. In most cases, bacteria are introduced to the cultures together with initial explants.

In practice, initial explants are only surface sterilized, and thus internally living microorganisms are introduced to *in vitro* cultures. If symptoms of bacteria colonizing plant tissues appeared within a short time, the contaminated explants should be immediately removed. In case when bacterial growth is very slow or temporarily retarded in plant culture conditions, they remain in a cryptic state and may appear only when the

culture conditions will drastically change, for example after delayed subculture, increase of temperature, change of medium composition or due to other factors [6]. It is therefore important to use appropriate methods for quick bacteria detection and removal of contaminated cultures before microorganisms' spreading. Our large survey of plant tissue cultures originating from eight laboratories in Poland resulted in obtaining 104 isolates of bacteria, which were assigned by phenotypic tests and 16S rDNA fragments sequencing to 29 genera (data not published).

The aim of present studies was to determine which media are suitable in detecting bacteria inhabiting plant explants. The next task was to assess usefulness of selected media for preliminary characterization of isolated bacteria. The third task was to find a way to identify taxa with genus specific primers in possible minimal population of bacteria, using two PCR techniques and two methods of DNA isolation.

MATERIALS AND METHODS

In the present study, isolates obtained from different plant tissue cultures, identified to the genera , were used**.**

Evaluation of media enabling detection, isolation and preliminary characterization of bacteria. In this experiment, which aimed at selecting medium suitable for detection and isolation of bacteria from initial explants, 4 strains of bacteria: 87 (*Bacillus* spp. isolated from *Phalenopsis*), E (*Pseudomonas putida* from *Rubus ideaus*), 81b (*Methylobacteriumlusitanum* from *Sambucus nigra*), and 78 (*Xanthomonas* from *Sambucus nigra*) were streaked on six media: King B (KB), Nutrient Agar (NA) + 1% sucrose, K [7], R2A [8], 523 [9] and on plant tissue initiation medium (IM), containing ½ Murashige and Skoog inorganic salts [10], vitamins of WPM medium [11], 100 mg l^{-1} inositol, 0.5 mgl^{-1} benzyladenine, 30 g l^{-1} sucrose, 250 mg l^{-1} milk albumin and 6.6 g l^{-1} Plant Agar (Duchefa). Growth and morphology of bacterial colonies were evaluated every 24 h for 7 days and photographed.

Evaluation of media enabling bacteria characterization. In the experiment aimed at selection of medium enabling preliminary characterization of isolated bacteria, 14 strains, representing the genera most often found in surveyed plant tissue cultures: *Agrobacterium*, *Plantibacterium,* and *Xanthomonas* (each from *Sambucus nigra*), *Bacillus* (from *Phalenopsis*), *Flavobacterium* (from *Daucus carota*), *Lactobacillus* (from *Hosta*), *Methylobacterium* (2 strains), *Curtobacterium, Mycobacterium*, *Paenibacillus*, *Pseudomonas, Stenotrophomonas* (each from *Rubus ideaus*) and *Serratia marcescens* (from *Alocasia*), were streaked on the following media: King B (KB), Nutrient Broth-Yeast Extract (NBY), Yeast Dextrose Chalk Agar (YDC), Nutrient Agar supplemented with 0.5% yeast extract (YNA), and Yeast Peptone Glucose Agar (YPGA) [12, 13]. Observations of colony morphology were made every 24 h for 7 days. Time of colonies appearance, their size, and morphology were recorded. The KB, NA, NBY, YDC, YNA and YPGA are known as universal bacteriological media but R2A and 523 (in both low amount of organic nitrogen, 523 contains sucrose) are used for isolation of bacteria from *in vitro* plant cultures. K medium has been recommended for isolation of Methylobacteriaceae (without organic nitrogen and with methanol as the only source of carbon) and IM is usually used as the initial medium for introduction of plant explants to *in vitro* cultures.

Sensitivity of bacteria detection in pure cultures using PCR with genus specific primers. This model experiment aimed at possibility of detection the minimal number of bacteria in a sample. Bacterial suspensions of three strains were prepared by washing off bacterial colonies with sterile water. Strain 87 (*Bacillus*) was cultivated on NA + 1% sucrose, isolate E (*Pseudomonas*) and isolate 81b (*Methylobacterium*) on KB medium. The initial concentration of suspension for strain 87 was $2x10^7$, for 81b – $2x10^6$, and for strain E – $5x10^7$ cfu ml^{-1}. Each of them was then serially tenfold diluted to final concentration 10^0 cfu ml^{-1}. From each dilution, DNA was isolated by two methods. 1/ One ml of each bacterial suspension was boiled for 10 min, cooled on ice and centrifuged at 14 000 rpm; one µl of supernatant was used in standard PCR reactions. 2/ One ml of each bacterial suspension was centrifuged at 14 000 rpm, re-suspended in 100 µl TE buffer and DNA was isolated using Genomic Mini kit for DNA extraction according to manufacturer's instruction (A&A Biotechnology). One µl of DNA (after isolation from each bacterial dilution) was used in standard PCR reactions.

Additionally, to compare detection sensitivity of standard PCR with BIO-PCR (pre-incubation followed by amplification) 100 µl of each dilution of bacterial suspension of each isolate was streaked on appropriate medium, as described above. The plates were incubated at 26°C for up to 5 days. At respective time, single colonies were counted and then washed off with 3 ml of sterile water; 2 ml of the suspension were used for DNA isolation with the same two methods as described above.

To assess the sensitivity of bacteria detection using standard PCR and BIO-PCR and two different DNA isolation methods the following primer pairs were used: for *Bacillus* – Bac-200 and Bac-470 [14], for *Methylobacterium* – Met2F and Met2R [15] and for *Pseudomonas* – primers Ps-for and Ps-rev [16]. Amplifications were performed in 15 µl of a reaction solution containing: 2 µl of DNA, 0.4 U polymerase GoTaq DNA (Promega Corp. USA), 1x buffer GoTaq,

0.2 mM of each DNTP and 1 µM of primers. All amplification reactions were conducted according to the recommendation of the authors with only modification for *Bacillus* (lowering of annealing temperature by 8°C). PCRs were carried out in T3000 Biometra thermal cycler. PCR products were separated on a 1.5% agarose gel with 0.5 TBE buffer and visualized by staining with ethidium bromide (0.5 mg l^{-1}).

Sensitivity of bacteria detection in *in vitro* plant explants artificially contaminated with bacteria. The shoots for the experiment were harvested from the stock cultures „free of cultivable bacteria". The stocks were initiated from microshoots indexed two times, in two subsequent subcultures, for the presence of contamination by placing the basal parts of shoots on two bacteriological media: Nutrient Agar and 523 medium. The shoots, from which bacteria did not grow in both subcultures, were considered as free of cultivable bacteria. The single microshoots were transferred to jars containing perlite saturated with liquid rooting medium appropriate for each plant species and 100 µl of 24 h bacterial suspension was injected in the centre of a jar immediately after shoot transfer. Microshoots of *Gerbera* x *hybrida* 'Kormoran' were contaminated with strain 87 of *Bacillus* spp., *Anthurium* x *andreanum* 'Bolero' – contaminated with strain E of *Pseudomonas putida* and *Sambucus nigra* contaminated with strain of 81b of *Methylobacterium lusitanum.* After 4 weeks, the roots were cut off, and the shoots transferred to propagation agar medium appropriate for each plant species. Explants showing symptoms of bacteria presence in the form of visible leakage into agar medium or halo around shoot base were aseptically divided into three segments: I – at the base, II – middle part and, III – top. Bulk samples of 100 mg of fragmented tissues from several explants of the same segment of each plant species were shaken in 3 ml of PBS buffer (0.27% Na_2HPO_4, 0.04% NaH_2PO_4, 0.8% NaCl) at 26 °C for 1h. Two ml of obtained liquids were used for isolation of DNA by boiling or using Genomic Mini kit as described above.

Additionally, to compare detection sensitivity by standard PCR and BIO-PCR (bacteria pre-incubation followed by DNA isolation and amplification) 100 µl of liquid obtained from each plant segment was placed on appropriate medium for each isolate, as described above. After incubation at 26°C for up to 5 days, the colonies were counted and then washed off with 3 ml of sterile water; 2 ml of the resulting suspension were used for DNA isolation using the same two methods as described above. Detection of bacterial DNA in plant material was conducted using PCR with the genus specific primers and according to conditions described above.

Bacteria detection in apparently healthy *in vitro* plant explants. Total DNA was individually isolated by Qiagen Plant Mini kit from bulk samples of *in vitro* shoots of *Chrysanthemum* x *hybrida* 'Ludo', *Kalanchoë blossfeldiana* 'Debbie', *Gerbera* x *hybrida* 'Kormoran' and *Sorbus aucuparia*, which never have shown contamination symptoms at visual inspections. DNA was used for detection of possible bacterial contamination using standard PCR. Universal primers fD1/rP2 for 16S rDNA of bacteria [17] and genus specific primers for *Bacillus*, *Methylobacterium* and *Pseudomonas* (as listed above) were used. The following PCR conditions for primers fD1/rP2 were applied: 94°C 4 min., 35 cycles: 45 s at 95°C, 45 s at 55°C, 90 s 72°C and 10 min at 72°C. 25 µl of reaction mixture contained: 25 ng DNA, 0.5 U *Tag* polymerase (Fermentas), 0.5 µM of each primer, 50 µM each of dNTP and 1.5 mM $MgCl_2$. For genus specific primers, the same PCR conditions were applied, as described above.

RESULTS

Growth and colony morphology of bacteria on different media., The most efficient among six media tested in terms of rate of bacteria growth was medium 523. The IM medium proved to be useful for growth three of the four bacterial strains, although at the slowest rate (colonies were visible 3 – 5 days later in comparison to other media used). However, single colonies of *Bacillus* spp. have not been observed on this medium within 7 days (Table 1; Figs 1–4).

All the five media used allowed growth of 14 bacterial strains representing different genera. NBY was the most favourable medium for growth of *Bacillus*, *Curtobacterium*, *Lactobacillus*, *Methylobacterium, Plantibacterium*, *Xanthomonas* and *Serratia marcescens* strains. The King B medium appeared to be most appropriate for *Stenotrophomonas* because of secretion of characteristic inclusions and for *Pseudomonas* because of fluorescent pigment production (Table 2). *Lactobacillus* growth was possible under mineral oil on four media but not on KB.

Sensitivity of bacteria detection/identification in pure cultures using PCR with genus-specific primers. The sensitivity depended on DNA isolation method (boiling or Genomic Mini kit) and applying pre-incubation of bacteria on bacteriological medium (BIO-PCR). The use of Genomic Mini kit for DNA extraction let to increase sensitivity 100 times for *Methylobacterium* and 1000 times for *Bacillus*, as compared to extraction of DNA by boiling. Only the sensitivity of detection/identification of *Pseudomonas* isolate did not depend on the method of DNA isolation used. Application of BIO-PCR increased the sensitivity by 10^2 to 10^5 times over the standard PCR, independently on the method of DNA isolation used (Table 3, Figs 5–7).

Detection of bacteria using PCR with genus-specific primers in plant explants artificially contaminated. The analysis of bacteria presence in all three segments of *Anthurium* x *andreanum* 'Bolero', *Gerbera*

x *hybrida* 'Kormoran' and *Sambucus nigra* explants, intentionally contaminated with bacteria, showed decrease in their number in the shoots going from bottom to top (Table 4). The highest differences between segments were found in *Bacillus* spp./*Anthurium* complex where in the top segments 125 times less bacteria was found than in a bottom part. However, in case of *Pseudomonas putida/Gerbera* there was only 8 times less bacteria in the top segments and in *Methylobacterium lusitanum/ Sambucus* only 3 times less than in a bottom segments, respectively.

Independently of the method of DNA extraction – Genomic Mini kit or boiling, plating of liquids after shaking of tissues in PBS buffer on bacteriological media (BIO-PCR) allowed detection/identification of one cell of *Pseudomonas* and *Methylobacterium* isolates. Moreover, applying pre-incubation of bacteria increased the sensitivity by 100 to 1000 times in comparison to standard PCR (data not shown). However, in case of *Bacillus* strain the detection was possible only with pre--incubation.

Bacteria detection in apparently healthy *in vitro* plant explants using PCR with genus-specific and universal bacterial primers. None of specific DNA fragments of *Pseudomonas*, *Bacillus,* and *Methylobacterium* were detected in total DNA isolated using the Qiagen Plant Mini Kit from explants of *Kalanchoë*, *Gerbera*, *Sorbus* and *Chrysanthemum* that did not exhibit bacterial contamination. On the other hand, PCR analyses of plant DNA with primers fD1/rP2, universal for bacterial 16S rDNA, revealed the presence of bacterial DNA in each sample of plant explants.

Fig. 1. The growth of *Bacillus* spp. (strain 87 isolated from shoot cultures of *Phalenopsis*) on 6 media after 48 h: a. KB, b. NA + 1% sucrose, c. R2A, d. 523; after 120 h: e. K medium, f. IM (½ MS medium + 0.025% milk albumine).

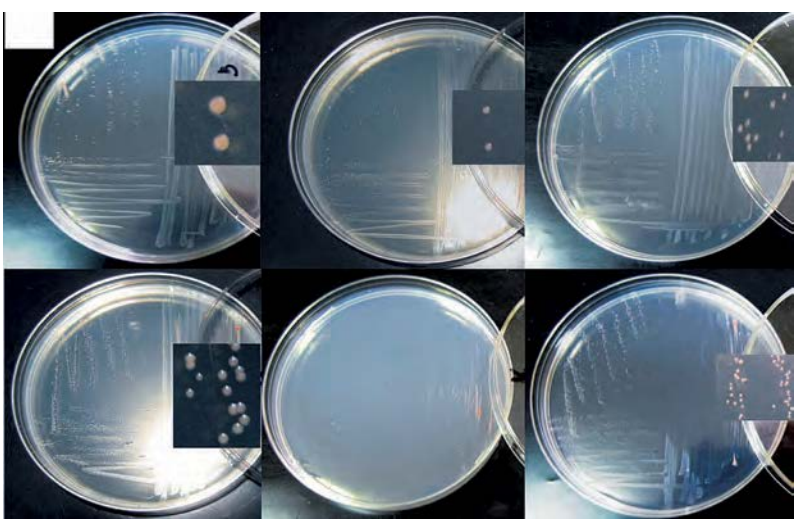

Fig. 2. The growth of *Methylobacterium lusitanum* (strain 81b isolated from shoot cultures of *Sambucus nigra*) on 6 media after 96 h: a. KB, b. NA + 1% sucrose, c. R2A, d. 523; after 120 h: e. K medium, f. IM (½ MS medium + 0.025% milk albumine).

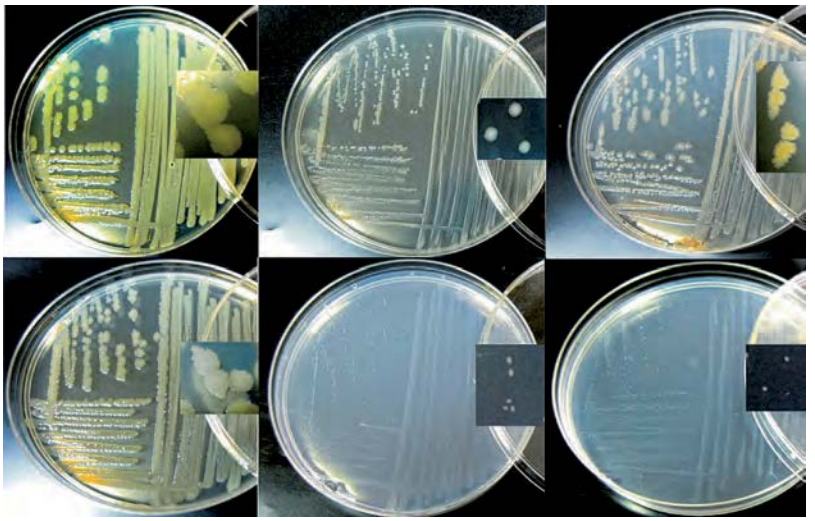

Fig. 3. The growth of *Pseudomonas putida* (strain E isolated from shoot cultures of *Rubus ideaus*) on 6 media after 48 h: a. KB, b. NA + 1% sucrose., c. R2A, d. 523; e. after 72 h on K medium, f. after 96 h on IM (½ MS medium + 0.025% milk albumine).

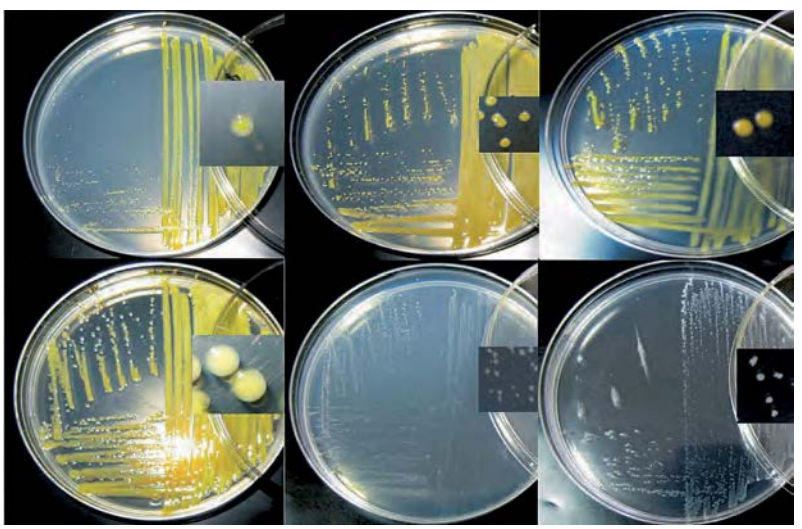

Fig. 4. The growth of *Xanthomonas* spp. (strain 78 isolated from shoot cultures of *Sambucus nigra*) on 6 media after 48 h: a. KB, b. NA + 1% sucrose, c. R2A, d. 523; e. after 96 h: K medium, f. on IM (½ MS medium + 0.025% milk albumin)

DISCUSSION

Our survey of bacteria contaminated plant tissue cultures originating from eight laboratories confirmed earlier reports about the presence of multitude of bacteria associated with plant tissue cultures [4, 5]. The range of bacterial taxa varies depending on the geographical zone, plant species, conditions in which donor plants grew and on the source of contamination [18]. Nevertheless, some bacteria, such as those belonging to genera: *Bacillus*, *Pseudomonas*, *Methylobacterium*, *Corynebacterium*, *Staphylococcus*, and *Agrobacterium* are ubiquitous.

Although disinfection of donor plant materials is always of special importance in the process of micropropagation, usually contaminations are introduced with the initial explants. Bacteria live in the plant vessels, intercellular spaces but also inside plant cells [19], which is the reason of unsuccessfulness of surface sterilization. To overcome this problem initiation of *in vitro* cultures from as small explants (very short shoot tips or meristems) as possible and detection of cultivable bacteria presence in these small explants as early as possible, is recommended. For this purpose, a shaking of fragmented bases of explants in sterile water or PBS buffer and plating of obtained liquid on microbiological medium, as it was performed in our study, is advisable. It is most effective when bacteria can be detected already on/in the initial medium, as IM, typical medium for plant culture initiation, with the addition of 0.025% of milk

albumin, which, in our experiment, enabled detection at least strains of *Methylobacterium*, *Pseudomonas* and *Xanthomonas*. On this medium, colonies of *Pseudomonas* were visible after 3 days, *Xanthomonas* after 4 days, and *Methylobacterium* after 5 days. Only in case of *Bacillus* strain single colonies were not observed after streaking on Initiation Medium until 7th day but weak growth of bacteria only at first lines of streaking was visible. All microbiological media used enabled to observe growth of bacteria but for not all of them single colonies were present. K medium, recommended for Methylobacteriaceae [7] was not useful for our *Methylobacterium* strain. However, we can underline usefulness of the 523 medium [9], which stimulated growth of colonies of all strains used more than KB or NA media. Nevertheless, a lack of bacteria growth on the bacteriological media does not guarantee the absence of any bacteria in the surveyed tissue because some bacteria can be uncultivable permanently or temporarily. For example, *Xanthomonas campestris* pv. *dieffenbachiae* (anthurium pathogen) could persist in the latent stage in *in vitro* shoot explants remaining undetected for one year [20]. The bacteriological media used in our experiment were not relevant to complete characterization and identification of bacteria. The exception was King B, which enables visualization of two features – fluorescent pigment characteristic to most *Pseudomonas* species and secretion of inclusions characteristic to *Stenotrophomonas*. Although the color of bacterial colonies as the only feature, can be not used for bacteria identification, nevertheless pink color of colonies and their slow growth may suggest the presence of one of the *Methylobacterium*, claret color and very rapid growth – *Serratia marcescens*, and yellow color – *Xanthomonas* or *Flavobacterium*.

Table 1
Morphology of bacterial colonies on different media

KB	NA + 1% sucrose	R2A	523	K	IM (1/2 MS) + 0.025% milk albumin
		***Bacillus* spp.** (87 from *Phalenopsis*)			
* 24 h, 1-2 mm in diameter, light beige, flat, irregular shaped in the form of elongated tear drops (Fig. 1a)	24 h, 1-2 mm, gray-white, flat ,circular, irregular shaped (Fig. 1b)	24 h, 1 mm, gray-white, elongated, flat, matt, circular, irregular shaped slightly jagged edges (Fig. 1c)	24 h, 2-3 mm creamy, circular shaped with a distinctive shiny hallo around convex, flat rough (Fig. 1d)	120 h lack of single colonies, growth only in the first line of a smear (Fig. 1e and f)	
		Methylobacterium lusitanum (81b from *Sambucus nigra*)			
96 h 1 mm, bright pink, regular in shape, round and slightly convex with a full edge (Fig. 2a)	96 h, 0.5 mm, small bright pink, regular in shape round and slightly convex with a full edge (Fig. 2b)	96 h, 0.5-1 mm, bright pink, regular in shape, round and slightly convex with a full edge (Fig. 2c)	96 h, 0.5-1 mm, pink, regular in shape, round and slightly convex with a full edge (Fig. 2d)	120 h lack of single colonies growth only in the first line of a smear (Fig. 2e)	120 h, 0.2 mm pink, regular in shape, round and slightly convex (Fig. 2f)
		Pseudomonas putida (E from *Rubus ideaus*)			
24 – 48 h, 2 mm, yellow-green, shiny, flat, round fluorescent under UV light (Fig. 3a)	24-48 h, 0.5 mm, circular, regular in shape, shiny, lightly convex (Fig. 3b)	24 – 48 h, 1 mm, single, irregular, tear-shape, more intensive color in the middle, little frayed at the margins (Fig. 3c)	24- 48 h, 3-4 mm, candle-flame in shape, creamy with more intensive color in the middle, little frayed at the margins (Fig. 3d)	72- 96 h 0.2 mm, very small, round, creamy, single, round (Fig. 3e and f)	
		***Xanthomonas* spp.** (78 from *Sambucus nigra*)			
48 h, 0.5-1 mm yellow, round, in shape shiny, little convex (Fig. 4a)	48 h, 0.5-1 mm, small, light yellow, shiny, regular in shape (Fig. 4b)	48 h, 1-2 mm, single, yellow, shiny, circular, regular in shape, little convex (Fig. 4c)	48 h, 2-3 mm bright yellow, shiny, little convex, regular in shape (Fig. 4d)	96 h, 0.5 mm, creamy on K and white on MS medium, flat, regular in shape (Fig. 4e and f)	

*Given is time (h) and diameter of colonies (mm) when they were recognizable

Table 2

Morphology of bacterial colonies on different microbiological media. The shadowed cells show the most appropriate medium.

Morphology of bacterial colonies on microbiological media				
KB	NBY	YDC	YNA	YPGA
***Agrobacterium tumefaciens* (81afrom *Sambucus nigra*)**				
*96 h, 0.1-0.2 mm, white-creamy, small, regular, shiny	96 h, 1 mm, creamy translucent, growth only in the first line of streaking	96 h, 1 mm ,white-creamy, luminescent	96 h, 1 mm, creamy translucent, growth only in the first line of streaking	96 h, 1-1.5 mm, white, round shiny,
***Bacillus* spp. (87 from *Phalenopsis*)**				
24 h, light beige, flat, irregular shaped in the form of elongated tear drops, 1-2 mm	24 h, white to white-creamy; flocky, tear-shape, 2 mm	24 h, white to white-creamy, flocky, tear-shape, 2 mm	24 h, white to white-creamy, flocky, tear-shape, 2 mm	24 h, white to white-creamy, flocky, tear-shape, 2 mm
***Curtobacterium* spp. (38 from *Rubus ideaus*)**				
96 h, creamy, 0.6 mm	96 h, light yellow, 1 mm	96 h, creamy, 0.3 mm	96 h, light yellow, 0.6 mm	96 h, light yellow, 0.3 mm
***Flavobacterium* spp. (4W from *Daucus carota*)**				
48 h, yellow with darker in the centre, flat, 0.4 mm	96 h, dark yellow, flat, 0.4 mm	96 h, pale yellow, flat, 0.5 mm	96 h, pale yellow, flat, 0.5 mm	96 h, yellow, convex, 2 mm
*****Lactobacillus* spp. (90 from *Hosta*)**				
No growth	96 h, light creamy, translucent, 0.2 mm	96 h, light creamy, translucent, 0.2 mm	96 h, light creamy, translucent, 0.2 mm	96 h, light creamy, translucent, 0.2 mm
***Methylobacterium* spp. (55 from *Rubus ideaus*)**				
96 h, light pink, 0.6-0.7 mm	96 h, light pink, 1 mm	96 h, pink 0.3-0.5 mm	168 h, pink, 0.2 mm	168 h, light pink, 0.6 mm
***Methylobacterium* spp. (B from *Rubus ideaus*)**				
96 h, bright pink, regular in shape, round and slightly convex with a full edge, 1 mm	96 h, pink, shiny, 0.1-0.2 mm	96 h, light pink, shiny, 0.1-0.2 mm	96 h, light pink, shiny, 0.1-0.2 mm	96 h, pink, shiny, 0.1-0.2 mm
***Mycobacterium* spp. (39 from *Rubus ideaus* 0**				
96 h, creamy, 0.4-0.5 mm	96 h, light yellow, 0.4-0.5 mm	96 h, creamy, 0.4-0.5 mm	96 h, light yellow, 0.4-0.5 mm	96 h, creamy, 0.4-0.5 mm
***Paenibacillus* spp. (I from *Rubus ideaus*)**				
48 h, white-creamy, non regular- flocking, 0.1 mm, 96 h-0.3 mm	48 h, white, non regular, translucent, 0.1 mm, 96 h - 0.3 mm	48 h, white-creamy, translucent, 0.1 mm; 96 h - 1 mm	48 h, white, 0.1 mm; 96 h- 0.3 mm	96 h, white-crème, translucent, 0.3 mm
***Plantibacterium* spp. (752 from *Sambucus nigra*)**				
48 h, creamy-yellow, translucent, 0.4 mm	48 h, white to yellow, translucent, shiny, flat, 0.4 mm; 168 h - 0.6 mm	96 h, sunny yellow, shiny, 0.3 mm , 168 h - 1-2 mm	48 h, lemon yellow, flat, translucent, shiny, 0.4 mm	48 h, white creamy, shiny, 0.3 mm
***Pseudomonas putida* (E from *Rubus ideaus*)**				
24 – 48 h, yellow-green, shiny, flat, round, fluorescent under UV light, 2 mm	24 h, yellow to creamy, shiny, translucent, 0.4 mm; 48 h - 1 mm, 168 h - 2-2.5 mm	24 h, creamy to white; light shiny, flat, 0.2 mm; 48 h - 0.6 mm, 96 h - 1-1.5 mm, 168 h - 1.5-2 mm	24 h yellow to creamy; 0.4 mm; 96 h 0.7 mm	24 h, white to creamy, 0.2 mm; 96 h-0.5 mm
***Serratia marcescens* (from *Alocasia*)**				
24 h, claret, lightly convex, shiny, 0.7 mm; 96 h 1 mm	24 h, red, lightly convex, shiny, 0.7 mm; 48 h 1 mm	24 h, reddish-pink, lightly in the middle, lightly convex, shiny, 0.7 mm	24 h, light red, lightly convex, shiny, 0.7 mm; 48 h 1 mm	24 h, red, lightly convex, shiny, 0.7 mm; 48 h 1 mm
***Stenotrophomonas* spp. (H from *Rubus ideaus*)**				
24 h, creamy, light flat, shiny, 0.1 mm; small inclusions in the medium. 168 h 1 - 1.2 mm	24 h, creamy, light shiny, translucent, flat, 0.1 mm; 96 h 0.5 mm, 168 h-0.6 mm	24 h, creamy-yellow, shiny, 0.1 mm , 168 h 0.9 mm	24 h, creamy, shiny, translucent, flat 0.1 mm; 96 h 0.3 mm, 168 h 0.6 mm	24 h, creamy, shiny, translucent, flat, 0.1 mm; 168 h 0.5 mm
***Xanthomonas* spp. (78 from *Sambucus nigra*)**				
48 h, yellow, round, in shape shiny, little convex, 0.5-1 mm	48 h, yellow, shiny, translucent, 0.2 mm; 168 h 1–1.5 mm	48 h, yellow, shiny, translucent, darker in the middle, 0.2 mm; 168 h 1-1.5 mm	48 h, yellow, shiny translucent, 0.2 mm; 168 h 0.5 mm	48 h, dark yellow, 0.2 mm; little slimy, 168 h 2 mm

*Given is time (h) when colonies were recognizable and their diameter (mm); **growth under mineral oil.

Table 3
The sensitivity of bacterial DNA detection by PCR depending on technique of DNA isolation and application of biological amplification (BIO-PCR)

Bacteria	Method of DNA isolation	Sensitivity of detection (number of bacterial cells)
Bacillus spp. (87) 1.5×10^7 w 1 ml	Boiling	1.5×10^5
	Boiling + BIO-PCR	1.5×10^0
	Genomic Mini	1.5×10^2
	Genomic Mini + BIO-PCR	1.5×10^0
Pseudomonas putida (E) 5×10^7 w 1 ml	Boiling	5×10^2
	Boiling + BIO-PCR	5×10^0
	Genomic Mini	5×10^2
	Genomic Mini + BIO-PCR	5×10^0
Methylobacterium lusitanum (81b) 3×10^6 w 1 ml	Boiling	3×10^4
	Boiling + BIO-PCR	3×10^0
	Genomic Mini	3×10^2
	Genomic Mini + BIO-PCR	3×10^0

Table 4
The number of bacterial cells detected in different segments per 1 mg of multishoot tissue

Bacteria/plant	Segment of multishoot		
	I (at the base)	II (middle part)	III (top)
Bacillus spp. (87)*/Anthurium andreanum*	1.67×10^5	3×10^4	1.33×10^3
Pseudomonas putida (E)*/Gerbera hybrida*	1.33×10^5	1×10^5	1.67×10^4
Methylobacterium lusitanum (81b)*/Sambucus nigra*	1×10^5	0.66×10^5	0.33×10^5

Fig. 5. Sensitivity of detection of *Bacillus* spp. strain 87 from tenfold dilutions of the bacterial DNA obtained by: Genomic Mini kit + BIO-PCR (lines 1-8) and by Boiling (lines 9-14): M – O'GeneRuler 100-3000bp (ThermoScientific, Life Science, Lithuania), 1 – 3×10^7, 2 – 3×10^6, 3 – 3×10^5, 4 – 3×10^4, 5 – 1.5×10^3, 6 – 1.5×10^2, 7 – 1.5×10^1, 8 – 1.5×10^0, 9 – 1.5×10^7, 10 – 1.5×10^6, 11 – 1.5×10^5, 12 – 1.5×10^4, 13 – 1.5×10^3, 14 – 1.5×10^2

Fig. 6. Sensitivity of detection of *Methylobacterium lusitanum* strain 81b, from tenfold dilutions of the bacterial DNA obtained by: boiling (line 1-7) and by boiling + BIO-PCR (lines 8-14): M – O'GeneRuler 100-3000bp (ThermoScientific, Life Science, Lithuania), 1 – 3×10^6, 2 – 3×10^5, 3 – 3×10^4, 4 – 3×10^3, 5 – 3×10^2, 6 – 3×10^1, 7 – 3×10^0, 8 – 3×10^6, 9 – 3×10^5, 10 – 3×10^4, 11 – 3×10^3, 12 – 3×10^2, 13 – 3×10^1, 14 – 3×10^0, 15 – negative control

Fig. 7. Sensitivity of detection of *Pseudomonas putida* strain E from tenfold dilutions of the bacterial DNA obtained by: Genomic Mini kit (line 1-6), Genomic Mini kit + BIO-PCR (lines 7-14) and by boiling (lines 16-21): M – O'GeneRuler 100-3000bp (ThermoScientific, Life Science, Lithuania), 1 – $5x10^7$, 2 – $5x10^6$, 3 – $5x10^5$, 4 – $5x10^4$, 5 – $5x10^3$, 6 – $5x10^2$, 7 – $5x10^7$, 8 – $5x10^6$, 9 – $5x10^5$, 10 – $5x10^4$, 11 – $5x10^3$, 12 – $5x10^2$, 13 – $5x10^1$, 14 – $5x10^0$, 15 – O'GeneRuler 100-3000bp (ThermoScientific, Life Science, Lithuania), 16 – $5x10^7$, 17 – $5x10^6$, 18 – $5x10^5$, 19 – $5x10^4$, 20 – $5x10^3$, 21 – $5x10^2$

The highly sensitive method for detection of bacteria in contaminated plant tissue cultures is very important especially at the initial stage when only a small number of bacteria can be present without visible symptoms both on/in explants and in/on media. Results of our study can suggest that detection of cultivable bacteria in washing liquid or plant fragment is more effective using microbiological method (streaking on bacteriological medium) than method based on PCR. A molecular detection of bacteria of known genus using PCR with the genus-specific primers confirmed that the BIO-PCR increased detection level of *Bacillus* spp. by 10^2–10^5 times, *Methylobacterium* by 10^2–10^4 times and *Pseudomonas* by 10^2 times, depending on the DNA isolation method. Wang et al. [21] obtained similar results in detection of *Xanthomonas albilineans*, the pathogen of sugar cane, where BIO--PCR combined with the use of the half selective m--XAM medium was more sensitive than ELISA, DIA and PCR. BIO-PCR technique was most sensitive and reliable for *Xanthomonas axonopodis* pv. *phaseoli* and *P. syringe* pv. *phaseolica* detection [22, 23]. Puławska and Sobiczewski [24] recommended this technique in detection of tumorigenic strains of *Agrobacterium tumefaciens* in soil and *Erwinia amylovora* in infected plants [25, 26]. Sensitivity of detection in the above experiments was from 1 to 20 cells per 1 g or 1 ml of a sample. These results suggest that instead of detection specific bacterial DNA, more effective are microbiological methods, including of plating of tissue washings on bacteriological media, because in the molecular method, detectability begins at app. 15 cells whereas on bacteriological medium, it is possible to detect a single bacterium. Moreover, searching for bacterial DNA could give false positive results by amplifying also DNA of bacteria, which do not survive disinfection.

In case of searching for specific bacteria using genus specific DNA primers in specimens of plant body, method of DNA extraction is important. The use Genomic Mini kit enables 10 times more sensitive bacteria detection than the extraction by boiling. However, in our study in case of *Bacillus* isolate the detection was possible only after pre-incubation, which could be related to presence of plant DNA polymerase inhibitors in *Anthurium* tissues and in shaking washings.

The use of primers universal for amplification of bacterial rDNA, recommended by Weisburg et al. [17], which were applied in this work and in others [27] should be probably not advisable in cases of detection of bacterial DNA in a mixture with plant DNA because the primers amplify also fragments of chloroplast DNA [28]. In addition, this method detects all bacterial DNA, also those belonging to uncultivable bacteria and bacteriosomes. To exclude chloroplast DNA amplification other primers, e.g. according to Chelius and Triplett [29] or Bulgari et al. [30], should be used to ensure that obtained PCR products are derived from bacterial templates. On the other hand, there is a question if a plant (*in vitro* explant) tissue could exist as free of bacteria [1].

CONCLUSIONS

1. The bacteriological media – King B, NBY, YDC, YNA, and YPGA enabled growth and morphological characterization of strains of 13 genera occurring as contaminants in plant tissue cultures, with the exception that *Lactobacillus* strain did not grow on KB medium, even under mineral oil. Only King

B medium enabled the appearance of the characteristic traits as fluorescence of *Pseudomonas* or secretion of characteristic inclusions by *Stenotrophomonas* strains. Other strains studied could be not identified even in terms of genus. Pink color of slow growing colonies may eventually imply affiliation to pink *Methylobacterium*, claret color of fast growing ones to *Serratia marcescens* and yellow color to *Xanthomonas* or *Flavobacterium.*

2. The most suitable for visualisation of bacteria present in initial explants or in washings from *in vitro* explants at the initiation stage was 523 medium. The IM medium (1/2 MS salts with milk albumin 0.025%) used routinely for initiation of plant tissue cultures can be useful for isolation of bacteria directly at culture initiation.
3. Medium K, recommended for Methylobacteriaceae, proved to be useless for the growth of *Methylobacterium* strain used in this study.
4. The BIO-PCR with genus-specific primers can be recommended for detecting/identifying single bacterial cells, but the procedure is extended by 2–5 days.
5. At a low bacteria number population, more recommendable for detection of cultivable bacteria is the use of microbiological (placing of explants fragments or washing liquids on bacteriological medium) than molecular method based on DNA markers.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: concept of the study: TO; molecular identification and analyses: MK; microbiological identification: AM; consulting and managing of microbiological study: PS; plant cultures: MZ; providing *Serratia marcescens* for study: EZ; writing of the manuscript: TO, MK, PS.

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Izolacja, wstępna charakterystyka i wykrywanie bakterii zanieczyszczających kultury roślinne *in vitro*

Streszczenie

W pracy określono możliwość wykrywania obecności bakterii najczęściej spotykanych w kulturach roślinnych *in vitro*, należących do różnych rodzajów, przy pomocy technik mikrobiologicznej i molekularnej. Za najbardziej przydatną do izolacji uznano pożywkę 523 a także pożywkę zawierającą ½ soli mineralnych MS stosowaną do inicjacji kultur roślinnych, z dodatkiem 0,025% albuminy mlecznej. Większą czułość wykrywania bakterii przy pomocy markerowego DNA uzyskano przy zastosowaniu techniki polegającej na preinkubacji badanego materiału na pożywkach bakteriologicznych, a następnie na izolacji DNA ze wzrostu (BIO-PCR). Przy porównaniu sposobów izolacji DNA większą czułość uzyskano stosując zestaw Genomic Mini (A&A Biotechnology) niż po zastosowaniu ekstrakcji w temperaturze wrzenia.

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