BACTERIAL CONTAMINATION OF PLANT IN VITRO CULTURES IN COMMERCIAL PRODUCTION DETECTED BY HIGH-THROUGHPUT AMPICLON SEQUENCING

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Abstract
The study overviews results of bacterial incidence in in vitro plant tissue cultures obtained from commercial laboratory dealing with plants micropropagation. For the exploration, the 454 pyrosequencing of the 16S rRNA gene was used. Three samples of plant in vitro cultures with visual bacterial contamination were subjected for identification of present bacteria. Eleven genera as Acinetobacter, Lactobacillus, Methylobacterium, Roseomonas, Microbacterium, Mycobacterium, Curtobacterium, Acidovorax, Magnetospirillum, Chryseobacterium and Ralstonia were detected. Obtained results confirmed the advantages of high-throughput amplicon sequencing analysis for identification of bacterial communities in contaminated plant in vitro cultures what provides an important information for applying appropriate measures to eliminate bacterial contamination.

Keywords: bacteria, tissue cultures, metagenomics, high-throughput amplicon sequencing

INTRODUCTION
The ability to establish and produce aseptic plant cell, organ, and tissue cultures is an important tool in basic and applied research and for massive production of plants – micropropagation (Cassells, 2012). It is extensively employed in commercial production of plant metabolites, biotransformation of pharmaceuticals, production of proteins including antibiotics, in elimination of plant pathogens, preservation of important plant species or in in vitro multiplication of plants for horticultural industry (Tsoktouridis et al., 2014; Leifert et al., 1991a). Regardless of whether the application is for (research or commerce), it is essential that the cultures are established free of...
microbial contamination and are maintained as aseptic cultures during whole growing process and storage (Cassells, 2012; Reed and Tanprasert, 1995). In spite of aseptic conditions usually implied, plant tissue cultures can be affected by fungi including yeasts, bacteria and bacteria-like organisms, virus and viroids that may be pathogenic to in vitro plants or stay latent (Cassells, 2001). The danger of contaminants is well known in the majority of commercial and scientific laboratories but losses of propagation material still average between 3 and 15 % at every subculture. Roughly 20–55% of contamination losses to in vitro plant cultures arise because of bacteria (Tsoktouridis et al., 2014; Ganen et al., 2009). These bacteria may cause serious problems for in vitro gene banks, prevent the safe exchange of germplasm and may result in misleading conclusions in research studies (Moreno-Vázquez et al., 2014; Wojtania et al., 2005). Due to massive propagation, they can dominate over plants explants, limit oxygen and nutrition accessibility (Orlikowska et al., 2017). In addition, bacterial contaminations may cause a latent explant infections which result in unstable growth, tissue necrosis or decreased shoot proliferation (Klayraung et al., 2017). Presence of bacteria may also affect culture media pH and thereby deteriorate plant cells growth conditions (Leifert and Cassells, 2001). Bacterial contamination may originate from infected plants, inappropriate laboratory conditions or erroneous in vitro techniques (Fang and Hsu, 2012). Moreover, it was observed that the scale of bacterial contamination depends on plant species as well as origin, development stage and explant type (Pirttila et al., 2008; Cassells and Tahmatsidou, 1996; Boxus and Terzi, 1988; Cornu and Michel, 1987).

The character of bacteria introduced to tissue cultures with plant explants can be epiphytic or endophytic. Epiphytic bacteria inhabit a surface of plants, therefore they are in the main removable by various disinfectants (Bhadrawale et al., 2018; Arab et al., 2014, Tomaszewska-Sowa and Figas, 2011; Moutia and Dookun, 1999). Bacteria living within healthy plant tissues, i.e. without causing apparent symptoms of disease all through the entire, or a part of the life cycle, are recognized as plant endophytes (Liaquat and Eltem, 2007). Presence of endophytic bacteria, although sometimes posing beneficial effect on tissue cultures of woody plants (Seker et al., 2017, Quambusch et al., 2014; Lata et al., 2006; Cankar et al., 2005; Thomas, 2004), may be in many cases harmful and cause reduction of culture growth, multiplication and rooting rates (Sarmast, 2018; Fang and Hsu, 2012; Thomas, 2011; Pirttila et al., 2008; Marino et al., 1995).

Characterization of bacterial colonies provides an important information about sources of contaminations and how to prevent or eliminate them (Reed and Tanprasert, 1995). Traditional detection techniques involved isolation and cultivation of bacteria. In some conditions bacterial growth may be slow and impeded by plant culture environment. Molecular detection based on direct DNA analysis makes these time-consuming methods unnecessary and is sensitive enough for most groups of bacteria. The 16S ribosomal RNA (rRNA) gene is the most widely used target for bacterial identification at the genus level (Moreno-Vázquez et al., 2014). Additionally, the development of new determination techniques based on DNA sequencing brought broader opportunities for studies of biological systems. The advantages of techniques using high-throughput sequencing (HTS) of amplicons are numerous, e.g. generating millions of sequences simultaneously from one sample, detection and characterization of pathogens without prior knowledge of their existence (latent presence or unknown cultures) and without the requirement of specific primers (Marston et al., 2013).

Molecular methods using 16S rRNA gene have been applied for identification of bacteria in plant tissue cultures, e.g. in micropropagated Prunus avium (Quambusch et al., 2014), Vitis vinifera (Thomas, 2004), Echinacea species (Lata et al., 2006), artichoke (Navacchi et al., 2013), and in the number of in vitro propagated ornamental plants (Ranunculus Zantedeschia and Paeonia (Barberini et al., 2012), Rosa, Pelargonium, Spatiphyllum, Dendranthema, Ficus and Dahlia (Moreno-Vázquez et al., 2014). In all mentioned studies the Sanger sequencing was used to obtain sequences of 16S rRNA amplicons, whereas in our study we use high-throughput amplicon sequencing.

The aim of this study was to identify bacteria contaminating in vitro cultures produced by commercial laboratory, the subsidiary company of Vivai Piante Battistini. For this purpose, 454 pyrosequencing of 16S rRNA gene have been used.

**MATERIALS AND METHODS**

**Collecting of bacteria**

Contaminated plant in vitro cultures were obtained from Vitrotree by Battistini in Uhřice (Czech Republic), a subsidiary company of Vivai
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Plante Battistini in Cesena (Italy). Micropropagated plants with the highest economic importance for Vitrotree by Battistini were screened and samples were selected by company workers on the basis of visible bacterial colonies present on growth medium (1 contaminated in vitro culture represented 1 sample) (Tab. I). Selected samples were sent to the Enviroinvest Corporation for the detection of present bacteria by the HTS.

### High-throughput amplicon sequencing

**Amplicon library preparation**

20 ± 1 g of micro plants with media were transferred into 50 ml conical tube containing 10 ml of physiological saline. After vortexing, physiological saline was added to a final volume of 50 ml. The mixtures were centrifuged at 1000 × g for 5 min. The supernatants were filtered through a 0.45 μm pore size filter disc. Total genomic DNA was extracted using MetaGNome DNA isolation kit (Epicenter Biotechnologies, Madison, WI, USA).

To generate a partial 16S rRNA gene amplicons, approximately 100 ng of isolated DNA was amplified by PCR using fusion primers and Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Lithuania). Fusion primers (Tab. II) were designed to target mainly the domains of *Proteobacteria, Firmicutes* and *Actinobacteria*. PCR amplification was performed using Thermal Cycler 2720 (Applied Biosystems, Singapore). Annealing temperature was 50 °C.

PCR products were checked on 1.5% agarose gel electrophoresis and purified by the Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) to remove small DNA fragments. Samples were then quantified using the Quant-iT PicoGreen dsDNA reagent (Invitrogen Corporation, Oregon, USA) and LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). All amplicons were then pooled together at an equimolar ratio and diluted to the final concentration of 1 × 10^7 PCR fragment molecules × μl⁻¹.

### 454 pyrosequencing

The metagenome was sequenced using the GS Junior 454 system platform (Roche Diagnostics, Branford, CT, USA). The amplicon library was added to the emulsion PCR at a ratio of 0.2 molecules per bead. emPCR amplification was performed as described in the emPCR Amplification Method Manual – Lib-L of the producer. After the bead recovery and enrichment procedure, the picotiter plate was prepared and approximately 500,000 enriched beads were loaded and sequenced according to the Sequencing Method Manual (http://454.com/downloads/my454/documentation/gs-junior/method-manuals/GSJuniorSequencingManual_Jan2013.pdf).

**Bioinformatics of HTS data**

The obtained file in sff format was transferred into fastq format and after quality checking (FASTQC), the data were transferred into fasta format. Subsequently, tagged sequences were separated into 3 files according to assigned barcodes. The reads were trimmed according to quality – Q30 (FASTX-Toolkit). Total numbers of the evaluated reads were 2115 reads of GF677, 1245 reads of Gisela 5 and 3062 reads of M29C. The reads were assigned to genera using blastn, e-value e-5 (Altschul et al., 1990; Camacho et al., 2009) and GenBank/NCBI database (http://ncbi.nlm.nih.gov/). Resulting hits are shown in Tab. III and numbers of reads per barcode are showed in Fig. 1.

### RESULTS

The HTS approach was applied for plants commercially micropropagated by Vitrotree by Battistini company to detect spectrum of bacteria present in in vitro cultures. For the pyrosequencing, three stone fruit rootstock tissue cultures were

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**Tab. I: In vitro plant tissue cultures selected for identification of bacterial contamination.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Latin name</th>
<th>English name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gisela 5</td>
<td><em>Prunus canescens</em> x <em>P. cerasus</em></td>
<td>Rootstock Gisela 5</td>
</tr>
<tr>
<td>GF677</td>
<td><em>Prunus amygdalus</em> x <em>P. persica</em></td>
<td><em>Prunus amygdalus</em> x <em>P. persica</em> GF677</td>
</tr>
<tr>
<td>M29C</td>
<td><em>Prunus cerasifera</em></td>
<td>Myrobalan plum M29C</td>
</tr>
</tbody>
</table>
selected – Gisela 5, GF677 and M29C (Tab. I). Results of pyrosequencing are shown in Tab. III. The range of bacterial communities varied depending on the rootstock culture and only one genus – Acinetobacter – was present in all tested samples. The broadest spectrum of bacteria was determined in Gisela 5 culture, where eight genera were identified: Methylobacterium, Acinetobacter, Lactobacillus, Roseomonas, Microbacterium, Acidovorax, Chryseobacterium and Ralstonia. Four of them (Acinetobacter, Lactobacillus, Roseomonas, Microbacterium) were common to GF677 culture as well. In Gisela 5 sample, bacteria of genus Methylobacterium were strongly represented (1857 hits), together with Acinetobacter (903 hits) and Lactobacillus (694 hits). The most numerous in GF677 sample were bacteria identified as Acinetobacter (6284 hits), Lactobacillus (2670 hits) and Roseomonas (1116 hits). Sequences obtained for rootstock M29C culture did not show a lot of hits and were assigned to genus Curtobacterium (20 hits) and Acinetobacter (5 hits). This result is in contrast with the number of reads regarding the MID adaptors for M29C, which was 32 % from the whole ssf pyrosequencing data file (Tab. II). Genera distinctive for only one from three examined samples were: Methylobacterium, Acidovorax, Chryseobacterium, Ralstonia (Gisela 5) as well as Mycobacterium and Magnetospirillum (GF677).

II: Fusion primer sequences, resulting number of reads and % of reads according to 454 pyrosequencing. Summary of the last column (% of reads) is not 100% because 2% of reads were not grouped with MID adaptors.

<table>
<thead>
<tr>
<th>Primer</th>
<th>MID per sample</th>
<th>Primer sequence</th>
<th>Number of reads</th>
<th>% of reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>E350-364/1F</td>
<td>Forward</td>
<td>Gisela 5 5'-CCATCTCATCCCTTGCTGCGTGGACTCA GACGAGTGCGTGACGAGTGAGGGGA-3'</td>
<td>1248</td>
<td>19</td>
</tr>
<tr>
<td>E350-364/2F</td>
<td>Forward</td>
<td>GF677 5'-CCATCTCATCCCTTGCTGCGTGGACTCA GACGCTCGACAGGCAGTGGGGGA-3'</td>
<td>3063</td>
<td>47</td>
</tr>
<tr>
<td>E350-364/4F</td>
<td>Forward</td>
<td>M29C 5'-CCATCTCATCCCTTGCTGCGTGGACTCA GACGCTCGACAGGCAGTGGGGGA-3'</td>
<td>2118</td>
<td>32</td>
</tr>
<tr>
<td>E505-524/R</td>
<td>Reverse</td>
<td>- 5'-CCTATCCCCTGTGTGTGGGTCAGTGACGTA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1: Reads per barcode (calculated in hundreds), in case of pyrosequencing MIDs. 1st column represent reads obtained for GF677, 2nd for Gisela 5 and 3rd for M29C.
DISCUSSION

Three selected in vitro cultures of different stone fruit rootstocks were visibly infected by bacteria with heterogeneous bacterial culture appearance. Pyrosequencing of samples collected from contaminated stone fruit rootstock tissue cultures revealed presence of 11 bacterial genera (Acidovorax, Acinetobacter, Chryseobacterium, Curtobacterium, Lactobacillus, Magnetospirillum, Methylobacterium, Mycobacterium, Ralstonia and Roseomonas).

Acinetobacter was the only genus detected in all three analyzed samples as well as the most strongly represented contaminant of GF677 culture (6284 hits). Acinetobacter are prevalent bacteria in clinical environment (Towner, 2009; Beggs et al., 2006), however several species have been found in environmental samples as soil, water and sediments (Kim et al., 2008; Carr et al., 2003; Bouvet and Grimont, 1986; Nishimura et al., 1988; Baumann; 1968). Acinetobacter is also cited in the literature as genus isolated from different plants (Leifert et al., 1991a). Likewise,
there are studies which classify *Acinetobacter* isolates within bacteria contaminating plant tissue cultures what agrees with our results. The other study investigating *Prunus* rootstock tissue cultures revealed *Acinetobacter* sp. in explants (Quambusch et al., 2014). Brunner et al. (1995) characterized isolates *Acinetobacter* from *Diffenbachia* sp. and *Spathiphyllum* sp. cultured in vitro. Moreover, *Acinetobacter* spp. were associated with aseptically micropropagated *Echinacea* plantlets (Lata et al., 2006), and in vitro cultures of *Astillbe* (Leifert et al., 1991b).

The genus *Lactobacillus*, highly present in GF677 and Gisela 5 cultures, has been described as in vitro contaminant (Moreno-Vázquez, 2014; Ganen et al., 2009) which could easily enter samples as inhabitant of the human skin (Leifert et al., 1991a). From plant cultures, it was detected in *Pelargonium* (Wojtania, 2005) and *Hemerocallis* (Leifert et al. 1991b, Leifert et al., 1989) cultures, wherein it proved to be deleterious to last ones.

Members of genus *Methylobacterium*, which in our results was the most frequent in Gisela 5 sample, are distributed in a wide range of natural environments and are very often associated with plants (Van Aken et al., 2004). Thomas et al. (2008) revealed genera *Methylobacterium* among isolates present after tenth passage of banana shoot tips culture. The bacterial strain isolated from *Sambucus nigra* shoot culture was identified as *Methylobacterium* sp. and characterized by Kaluzna et al. (2013).

*Mycobacterium* genus was relatively low represented in the analyzed samples, it was identified only in the sample from GF677 rootstock culture (274 hits). Although it is not discussed as major bacterial contaminant, there are several reports disclosing *Mycobacterium* in relation to plant in vitro cultures. In the study on six genotypes of *Prunus avium* tissue culture conducted by Quambusch et al. (2014) culture-independent and – dependent approaches were used to identify endophytic bacterial communities and *Mycobacterium* sp. as well as *Microbacterium* sp. were described among endophyte isolates obtained from three of six *Prunus* genotypes. Furthermore, *Mycobacterium* spp. were the most numerous from nonculturable bacteria fraction and identified in the clone libraries of all analyzed *P. avium* genotypes. Authors suggested a detrimental effect of those bacteria on in vitro cultures as they were dominant in cultures of two difficult to propagate genotypes. *Mycobacterium* strain was also isolated and identified as bacterial contaminant of *Synginium* and *Spathiphyllum* shoot tips cultures (Taber et al., 1991). Following authors, it was the first report of *Mycobacterium* detected in bacterial contamination of plant in vitro cultures. Fang and Hsu (2012) reported presence of genera *Mycobacterium* and *Magnetospirillum* in *Aglaonema* tissue cultures. *Mycobacterium* sp. was also detected in *Pinus sylvestris* tissue culture where it was likely to cause growth retardation (Laukkanen et al., 2000).

*Microbacterium* genus, detected in two of three analyzed samples– Gisela 5 and GF677- was also described as pathogen of in vitro cultures of *Alysum bortolonii* (Barzanti et al., 2007) and *Billbergia magnifica* (Tsoktouridis et al., 2014). The bacterium *Acidovorax* sp. identified as slightly present in Gisela 5 causes disease in a wide range of economically important monocotyledonous and dicotyledonous plants including corn, rice, watermelon, anthurium, and orchids (Schaad et al., 2008). *Chryseobacterium* sp., again slightly represented in Gisela 5, is often present in tissues of *Beta vulgaris* L. (Shi et al., 2010) and *Ralstonia* sp. is a serious plant pathogen that was also described in tissue cultures (Isenegger et al., 2003).

Moreover, in our study we detected *Roseomonas* sp. in two examined cultures – Gisela 5 and GF677. *Roseomonas* spp. have been frequently isolated from various human clinical samples, including blood samples, samples obtained from wounds, exudates, abscesses or genitourinary sites (Rihs et al., 1993). However, members of genus *Roseomonas* are described in the literature also in relation to plants as well. Ghorbani and Harighi (2017) isolated endophytic *Roseomonas rosea* strain from walnut trees samples and confirmed ability of those strain to inhibit the growth of *Pseudomonas syringae* pv. *syringae*. *Roseomonas mucosa* was detected among endophytic bacteria associated with *Curcuma zedoaria* (Sulistiyani et al., 2014), and *Roseomonas* species were found in banana (Nutaratat et al., 2013) and olive (Damtab et al., 2016) phyllosphere. To our knowledge, so far there are only two reports revealing a direct linkage of bacteria from genus *Roseomonas* to plant in vitro cultures (Pham et al., 2017; Barberini et al. 2012). *Roseomonas gilardii*, which is a human pathogen, was isolated from contaminated culture of *Peonia* (Barberini et al. 2012), while Pham et al. (2017) identified genus *Roseomonas* in endophytic bacterial communities in shoot cultures derived from embryonic tissue of hybrid walnut. In order to isolate endophytic bacteria, authors transferred three leaf or stem pieces of *in vitro* shoot cultures of *Juglans × intermedia* on plates with bacterial medium (R2A). Whereas in our study, growth of
bacteria identified as *Roseomonas* was present on plant tissue culture medium.

Wide spectrum of bacteria detected in three samples of stone fruit rootstocks confirmed advantages of pyrosequencing, or more generally HTS, in determination of occurring contaminations. It is likely to recover higher number of taxa and provide a less biased qualitative picture of the bacterial community composition by high-throughput sequencing platforms in comparison with classical culture-dependent methods (Evers *et al.* 2016) and traditional DNA analysing methods as those based on Sanger sequencing (Tedersoo *et al.*, 2010). High-throughput pyrosequencing was a feasible approach for detecting diverse bacteria associated with micropropagated *Artiplex* species (Lucero *et al.*, 2011). Other high-throughput sequencing platform, based on sequencing-by-synthesis technology, was involved in revealing bacteria in *in vitro* shoot cultures of *Juglans × intermedia* (Pham *et al.*, 2017).

Thomas *et al.* (2017) used the same method for deep amplicon sequencing of 16S rRNA region to identify bacteria in *Vitis vinifera* shoot-tips and callus tissues cultured *in vitro*.

Most bacterial contaminations of *in vitro* plant cultures are introduced by the inefficient sterilization of explants, culture vessels, laboratory equipment or media. They occur when handling the plant material or are transmitted by mites and thrips (Moreno-Vázquez *et al.*, 2014; Reed and Tanprasert, 1995; Van den houwe *et al.*, 1998). The use of microbiological quality assurance (HACCP) systems, training of workers in sterile technique and use of autoclaves and laminar flowboxes have to be the first steps to avoid environmental contaminants and contamination by humans (Reed and Tanprasert, 1995). But when they occur, the high-throughput sequencing approaches allow to determine wide spectrum of present pathogens and could help to choose targeted strategy for their elimination.

**CONCLUSION**

The study confirms bacterial contamination events in plant tissue cultures as highly possible even in the case of cultures treated aseptically. In fact, the HTS approach involving 454 pyrosequencing of 16S rRNA gene detected spectrum of bacteria contaminating stone fruit *in vitro* cultures. All detected bacterial genera were already described as *in vitro* culture contaminants. This precisely identified spectrum of present pathogens could support selection of appropriate direct strategy for their elimination.

To eliminate negative impact of bacteria contaminating tissue cultures, the crucial step is primarily to minimalize the risk of contamination in the workflow and adherence of the disinfection rules. It is considerably evident that advantageous for precise identification of bacterial contaminations within plant micropropagation process are highly perspective methods based on HTS.

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