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BACTERIAL CONTAMINATION OF PLANT *IN VITRO* CULTURES IN COMMERCIAL PRODUCTION DETECTED BY HIGH-THROUGHPUT AMPLICON 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 (Pirtilla *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, Spathiphyllum, 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 Uherčice (Czech Republic), a subsidiary company of Vivai

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

Sample	Latin name	English name
Gisela 5	*Prunus canescens* x *P. cerasus*	Rootstock Gisela 5
GF677	*Prunus amygdalus* x *P. persica*	*Prunus amygdalus* x *P. persica* GF677
M29C	*Prunus cerasifera*	Myrobalan plum M29C

Piante 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.

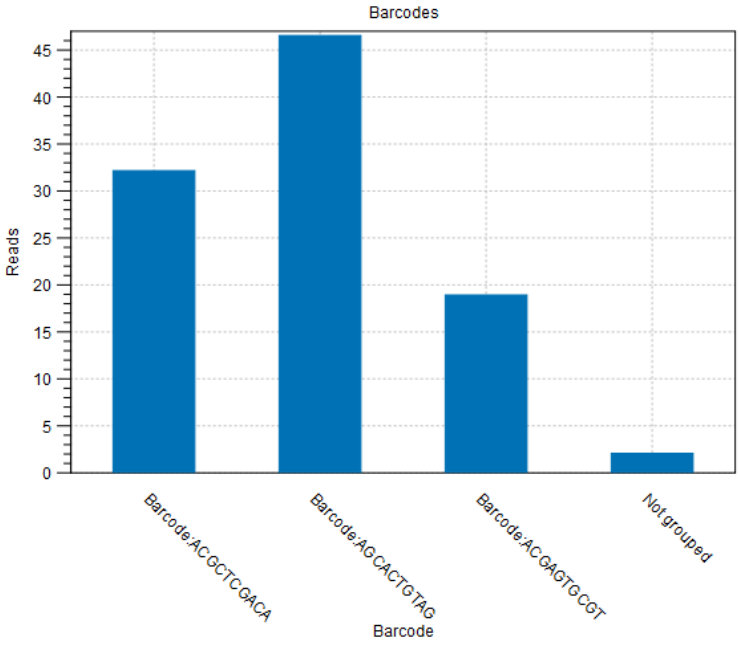
RESULTS

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

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* nad *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, Chrysobacterium, 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.*

Primer		MID per sample	Primer sequence	Number of reads	% of reads
Name	Direction				
E350-364/1F	Forward	Gisela 5	5'-CCATCTCATCCCTGCGTGTCTCCGACTCA GACGAGTGCGTGGCAGCAGTGGGGAA-3'	1248	19
E350-364/2F	Forward	GF677	5'-CCATCTCATCCCTGCGTGTCTCCGACTCA GACGCTCGACAGGCAGCAGTGGGGAA-3'	3063	47
E350-364/4F	Forward	M29C	5'-CCATCTCATCCCTGCGTGTCTCCGACTCA GAGCACTGTAGGGCAGCAGTGGGGAA-3'	2118	32
E505-524/R	Reverse	-	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCA GCTGCTGGCACGTAGTTAGCC-3'		



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.*

III: *Spectrum of bacterial contaminants detected by HTS analysis in stone fruit rootstocks in vitro cultures. Results of 16S blastN analysis against GenBank/NCBI.*

Species of detected bacteria	Hits-blastN (with e-value lower than 0.00146492)		
	Gisela 5	GF677	M29C
Methylobacterium	1857	0	0
Acinetobacter	903	6284	5
Lactobacillus	694	2670	0
Roseomonas	168	1116	0
Microbacterium	55	477	0
Acidovorax	41	0	0
Chryseobacterium	28	0	0
Ralstonia	15	0	0
Mycobacterium	0	274	0
Curtobacterium	0	63	20
Magnetospirillum	0	28	0

2: *Analysed sample of the rootstock GF677 culture with evident bacterial contamination where demonstrably detected genera were Acinetobacter, Lactobacillus and Roseomonas.*

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, Microbacterium, 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 *Astilbe* (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 dominative 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 *Alyssum bertolonii* (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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