LITHUANIAN RESEARCH CENTRE FOR AGRICULTURE AND FORESTRY
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ENDOPHYTIC BACTERIA POPULATION STRUCTURE OF DOMESTIC APPLE AND INTERACTION WITH APPLE CELLS AND SHOOTS *IN VITRO*

Summary of Doctoral Dissertation
Physical Sciences, Biochemistry (04 P)

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Daktaro disertacija bus ginama viešame posėdyje, kuris vyks 2017 m. lapkričio 21 d. 10 val. Atviros prieigos žemės ir miškų jungtinio tyrimų centro J356 auditorijoje.
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INTRODUCTION

Domestic apple (*Malus* × *domestica* Borkh.) is one of the most economically important plants of the *Rosaceae* family and its fruits constitute the largest part of horticultural production in temperate regions (Brown, 2017). Apple is one of the model plant species of the *Rosaceae* (Shulaev et al., 2008) and it is used in genetic studies on cold and disease resistance as well as propagation and genetic transformation *in vitro*.

In vitro technology is used to prepare high quality germplasm for apple propagation, to preserve genetic resources and biodiversity. The main disadvantage of the method is associated with the plant stress induced under *in vitro* conditions that leads to plant growth suppression and somaclonal variation. Imbalance of intracellular production of reactive oxygen and nitrogen species (ROS/RNS) leads to oxidative stress (Cassells and Curry, 2001). It has been established that endophytic microorganisms regulate plant physiology and endophytic microbiota has attracted an increasing attention of science community. However, potential of application of the endophytic microorganisms to reduce plant stress has been under-explored.

Endophytic microorganisms colonize different plant tissues without apparent symptoms of disease. Endophytes are present basically in all plants; they are involved in non-pathogenic interaction and often have positive effect on plant growth. Bacterial endophytes produce a broad spectrum of biologically active compounds that regulate plant physiology (Ryan et al., 2008). It has been established that microorganisms could stimulate plant growth, viability and have effect on development. Meanwhile beneficial effect of endophytic bacteria on plant growth is well established, mechanisms involved are largely unknown. Often endophytic bacteria have a negative effect on plant pathogens and could be used as biocontrol agents. On the other hand, direct plant interaction with endophytes may lead to stress reduction and stimulation of the plant growth (Rosenblueth and Martinez-Romero, 2006).

Information about diversity of endophytic microorganisms of plant species of the *Rosaceae* family, including the domestic apple, is scarce. Understanding the effect of endophytes on redox balance in plant cells and reduction of oxidative stress remains vague. There is no information about role of endophytes involved in regulation of stress response under *in vitro* conditions.

Hypothesis. Among the domestic apple leaf endophytic bacteria, there are strains capable to promote growth and to reduce stress of apple shoots *in vitro*.

The aim of the research. To characterize the taxonomic composition of endophytic bacteria of apple phyllosphere; to evaluate the regulatory effect of the endophytes on the intracellular production of oxygen and nitrogen compounds and on the growth of shoots *in vitro*.

Specific aims:

1. To characterize the endophytic bacteria population of the apple phyllosphere using the 16S metagenomic analysis method and biochemical properties of cultivated isolates.
2. To evaluate the effects of endophytic bacteria on the growth of domestic apple shoots and response to stress under *in vitro* conditions.
3. To investigate the interaction between endophytic bacteria and apple cells, properties of ROS/RNS accumulation using a model of plant cell suspension culture *in vitro*.
4. To determine gene expression patterns characteristic to the interactions between ROS/RNS production regulating endophytic bacteria and apple cells.

Statement to be defended:

1. The domestic apple phyllosphere has large taxonomic diversity of endophytic bacteria, and part of them has significant properties for plant growth.
2. Endophytic bacteria regulate growth of apple shoots *in vitro* and balance of the phytohormonal signaling pathways associated with stress response.
3. Endophytic bacteria associates with apple cells *in vitro* and regulates the production of ROS/RNS in apple cells.
4. Different gene expression patterns are induced by the endophytic isolates of *Bacillus* spp. that has different ROS/RNS regulatory properties.

Practical value and scientific novelty of research. For the first time, the population structure of endophytic bacteria of domestic apple cv. Gala phyllosphere was evaluated using the metagenomic analysis method.

The collection of endophytic bacteria of domestic apple provides important basis for further studies on interaction between apple and endophytes and for development of plant growth promoting regulators or screening for bioactive substances.

An endophytic bacteria strain that stimulates growth of the apple shoots and suppresses oxidative stress has potential application for development of plant stress-reducing means.

A comparative genomics study revealed differentially expressed genes involved in apple and endophytic bacteria interaction.

Publications. The main research data are presented in 3 publications in the journals included in „Clarivate Analytics Web of Science“ database.

Approval of the research. The main results of the study were presented and approved at three international conference abroad: „Plant Biology Europe EPSO/FESPB Congress“ (Prague, Czech Republic, 2016), „XVI International Congress on Molecular Plant-Microbe Interactions” (Rhodes, Greece, 2014), „12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field” (Italy, Verona, 2015), and in five international conference in Lithuania: „9th International Scientific Conference “The Vital Nature Sign” (Kaunas, 2015), „10th International Scientific Conference “The Vital Nature Sign” (Vilnius, 2016), „XIIIth International Conference of Lithuanian Biochemical Society“ (Birštonas, 2014), „1st International Conference on Scientific Actualities and Innovations in Horticulture” (Kaunas, 2016), SmartBIO (Kaunas, 2017), and one in COST FA1306 conference: “Diving into integrative cell phenotyping through-omics” (Versailles, France, 2016).

Content and volume of the dissertation. The doctoral dissertation is written in Lithuanian language. It consists of 122 pages, 10 tables and 26 figures. The dissertation contains introduction, literature review, materials and methods, results and discussion, conclusion, reference and list of publications.

THE OBJECTIVE, MATERIALS AND METHODS

The research was carried out at the Institute of Horticulture, Lithuanian Research Center for Agriculture and Forestry and at the Laboratory of Biological markers of the Open access Joint Research Centre of Agriculture and Forestry during 2013-2017.

Plant material. For analysis of endophytic bacteria, samples were collected from the plants of domestic apple cv. Gala that was maintained under standard cultivation conditions at the field collection of genetic resources. Apple leaves and buds were used for metagenomic analysis and isolate cultivation, respectively.

Apple shoot *in vitro* cultivation experiments included apple genotypes of cv. Gala, Golden delicious, Orlovim and hybrid No. 7 of Noris×Paprastasis antaninis that had different growth characteristics under *in vitro* conditions.

Cell suspension culture of cv. Gala was used in experiments on interaction of endophytic bacteria with plant cells, ROS/RNS production and gene expression analysis.

Apple shoots and cell suspension growth conditions *in vitro*. Apple shoots were maintained on solid Murashige-Skoog (MS) medium (pH 5.8) (Murashige and Skoog, 1962) supplemented with 0.8 % agar at 25±3 ºC under illumination of 16 h photoperiod.

Cell suspension of cv. Gala was initiated from the callus culture that was maintained on Schenk and Hilderbrandt (SH) medium (Schenk and Hildebrandt, 1972), 25 °C, and 4 weeks. Fragments of the callus of approx. 1 g weight were transferred to 25 ml of MS medium. The resulting cell suspension was maintained with shaking at 100 rpm at 24 °C in dark. At two week period, the culture was diluted with fresh medium. Cell viability was estimated using FDA staining (Saruyama et al., 2013) and microscopic analysis or using Evans Blue dye and spectrophotometric detection as described by Baker and Mock (1994).

Sample preparation for metagenomic analysis. Surface sterilization of the apple leaves was carried out as described by (Hata et al., 2002; Mendes et al., 2007). For sterilization control, apple leaves were incubated on Lysogeny Broth (LB) medium (Bertani, 1951).

Eight different bacterial enrichment and DNA extraction methods were employed. Samples were prepared using three bacterial DNA purification methods as

described by (Ding et al., 2013; Li et al., 2001) and PureLink™ Microbiome DNA Purification Kit (Fermentas, Thermo Scientific) was used. For bacterial DNA enrichment, two methods employed enzymatic extraction using macerozyme and cellulase enzymes as described by (Jiao et al., 2006). Bacterial DNA enrichment using SDS detergent extraction was carried out as described by (Wang et al., 2008). Mechanical homogenization and bacterial cell enrichment using overnight incubation was performed as described by (Nikolic et al., 2011).

Metagenomic analysis using next-generation sequencing. Semiconductor based Ion Torrent technology using Personal Genome Machine sequencer (Applied Biosystems) was used for metagenomic DNA analysis. The hypervariable regions of *16S rRNA* gene were amplified using two V2‑4‑8 and V3‑6, 7–9 specific primer sets of the Ion 16S Metagenomics kit (Thermo Fisher Scientific). Cycling conditions were as follows: 25 cycle of 30 s denaturation at 95 ^{o}C, 30 s annealing at 58 ^{o}C and 20 s extension at 72 ^{o}C. In addition, two combinations of DNA primers 799F and 1492R/1391R were used as described by (Arjun and Harikrishnan, 2011).

Amplicons were analysed by MCE-202 MultiNA microchip electrophoresis system using DNA-1000 Reagent kit (Shimadzu). The two samples obtained with V2‑4‑8 and V3‑6, 7–9 primer sets were combined and purified on the magnetic rack (DynaMag-2) using Agentcourt AMPure XP (Beckman-Coulter Genomics), and DNA content was estimated using the MCE-202 MultiNA system.

Libraries were prepared from fifty nanograms of the combined amplicons using the Ion Xpress Barcode Adapters 1-16 kit and Ion Plus Fragment Library kit (Thermo Fisher Scientific) that included ligation of adapters, nick-repair and purification. Each step was followed by purification using the Agentcourt AMPure XP beads. Libraries were quantified with Ion Universal Library Quantification kit (Thermo Fisher Scientific) and each sample was adjusted to 10 pM concentration. Equal volumes of samples were combined and emulsion PCR was carried out using Ion OneTouch 2 System and Ion PGM Template OT2 400 kit (Thermo Fisher Scientific). The amplified clonal libraries were enriched using Ion PGM Enrichment Beads on Ion OneTouch ES instrument (Thermo Fisher Scientific). The enrichment efficiency was assessed using Ion Sphere Quality Control kit (Thermo Fisher Scientific). Prepared template spheres were loaded on Ion 316 v2 chip and sequencing was performed on the Ion Personal Genome

Machine (PGM) using Ion PGM Hi-Q Sequencing kit (Thermo Fisher Scientific). Base calling and run demultiplexing were performed by Torrent Suite v.5.0.5 with default parameters.

Sequencing data was processed using Ion Reporter Software v.4.0 (Thermo Fisher Scientific) using 16S Metagenomic workflow. Reads were trimmed by primers at both ends. Threshold for unique reads was set to 10. Taxonomic identification was performed using MicroSEQ 16S Reference Library v2013.1 and Greengenes v13.5 databases. Threshold value for percentage identity for genus and species ID was 97 %.

Isolation and identification of endophytic bacteria. Culturable endophytic bacteria were isolated from surface sterilized apple buds as described by (Hata et al., 2002) and maintained on LB or actinomycetes agar medium. DNA from bacterial cells was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) and sequence of the D1 region was amplified using universal bacterial primers *FD1* and *RD1* (Weisburg et al., 1991). PCR product was separated by electrophoresis on agarose gel, extracted using Mini DNA Purification Kit (Thermo Fisher Scientific) and sequenced. The obtained sequences were queried against the *16S rRNA* gene sequences at the NCBI Gene database.

Metabolic tests. Reduction of nitrates was assessed on medium supplemented with nitrate as described by (Johnson et al., 2007). Semisolid nitrogen-free medium was used for screening bacteria capable of fixing nitrogen and was prepared as described by (Elbeltagy et al., 2001). Siderophore production was detected by bacterial colony growth on the Chrome azurol S medium prepared as described by (Vellore, 2001). Synthesis of indole-*3*-acetic acid (IAA) was estimated colorimetrically using the ferric chloride-perchloric acid reagent (Gordon and Weber, 1950). Assimilation of ACC was assessed using Dworkin and Foster (DF) medium supplemented with ACC (Akhgar et al., 2014; Dworkin and Foster, 1958).

Histochemical staining of apple shoots. Nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining was used to detect the production of superoxide anion radicals and hydrogen peroxide in apple shoot tissues and were carried out according to (Thordal-Christensen et al., 1997) and (Wohlgemuth et al., 2002), respectively.

Analysis of oxidative injury of cellular membranes. A quantitative analysis of the lipid peroxidation product malondialdehyde (MDA) was carried out as described by (Jagendorf and Takabe, 2001).

Assessment of effect of endophytes on shoot morphology and growth. At the exponential growth phase (~10^7 cfu/ml) endophytic bacteria were sedimented by centrifugation and resuspended in MS medium. Three microliter of the bacterial suspension was inoculated at several nodes of leaf petiole of the apple shoots that had been transferred to fresh medium one day prior inoculation. MS medium without bacteria was used for control. The inoculated shoots were maintained under standard conditions. An assay of oxidative injury of cellular membranes and gene expression analysis were carried out after one week, weight and propagation coefficient of the shoots were assessed after 3 weeks of co-cultivation.

Plant cell and endophytic bacteria association assays. Apple cell suspension was inoculated with endophytic bacteria at ~10^7 cfu/ml resuspended in MS medium and cultivated under standard conditions. After 1 and 16 h of incubation, association of bacteria to plant cells was assessed microscopically as described by (Bordiec et al., 2011), and quantified using serial dilution after 6 h of incubation.

Assessment of endophytic bacteria effect on ROS/RNS production in apple cells. Plant cells and endophytic bacteria were used at the exponential growth phase. Bacteria were sedimented by centrifugation, resuspended in MS medium and were used to inoculate plant cells at ~10^7 cfu/ml final concentration. After 2 and 6 h incubation under standard conditions, intracellular accumulation of ROS/RNS in apple cells was estimated using dichlorofluorescein diacetate (H_2DCFDA) staining, as described by (Joo et al., 2005). Fluorometer LS55 (Parkin-Elmer) with Ex=485 nm, Em=525 nm wavelengths and 5 and 2.5 nm slit setting, respectively, was used for detection. Epifluorescent microscope Eclipse 80i (Nikon) was used for microscopic visualization of cell fluorescence.

Real time PCR gene expression analysis. Total cell RNA was purified using GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific). RNA concentration was estimated using spectrophotometer and RNA quality was assessed based on 18S and 25S ribosomal RNA ratio using the MCE-202 MultiNA system and RNA analysis kit (Shimadzu). Genomic DNA was removed using DNase I (Thermo

Fisher Scientific) and complementary DNA (cDNA) was synthesized using RevertAid reverse transcriptase (Thermo Fisher Scientific) according to manufacturer instructions.

Primers specific to the NOX homologous genes of apple (*rbohD1, rbohD2, rbohD3, rbohF*) developed using Primer3web software (Untergasser et al., 2012) and previously published primers of genes involved in plant response to stress signaling pathways (*COI1, JAR, PLD*, *LOX2, AOS, ACS, ERF, PR-1, WRKY*) (Shin et al., 2014; De Bernonville et al., 2012; Rosen and Skaletsky, 2000) were used. The RT-PCR analysis was performed using Realplex thermocycler (Eppendorf) and cycling conditions were as follows: primary denaturation at 95 ºC, 2min; 40 cycle of 30 s denaturation at 95 ^{o}C, 45 s annealing at 60 ^{o}C and 45 s extension at 72 ^{o}C. To determine PCR product melting temperature, denaturation at 95 ºC, 15 s and 60-95 ºC, 20 min was used. Expression of house-keeping gene glyceraldehyde 3-phosphate dehydrogenase was used for data normalization and relative expression was estimated using method described by (Livak and Schmittgen, 2001). PCR product size was estimated using the MCE-202 MultiNA system and DNA-1000 analysis kit.

Apple cell proteome analysis using two dimensional electrophoresis.

Proteins were extracted using phenol extraction and ammonium acetate precipitation method described by Isaacson et al. (2006). Protein samples were solubilised in DIGE lysis buffer and protein concentration was measured using Bradford assay (Bradford, 1976).

Protein sample aliquots of 50 µg were labeled with Cy3 and Cy5 fluorescent dyes (Lumiprobe). Internal standard was prepared from an equimolecular mixture of all protein extracts and was labeled with Cy2 dye. In total, four biological repeats were prepared. After labeling and quenching with 1 mM lysine, protein samples were mixed to include two samples and one internal standard. For preparative gel, 500 µg of unlabeled internal standard was mixed with 50 µg of the labeled internal standard. Rehydration solution was added to the mixed samples to the final volume of 450 µl. Proteins were applied to 24 cm IPG strips pH 4-7 linear gradient and isoelectric focusing was performed on Ettan IPGphor (GE Healthcare). After the isoelectric focusing, strips were stored frozen at -20 ^{o}C. After two step equilibration with buffer containing 2 % dithiothreitol and then 4 % iodoacetamide, the proteins were separated on 1-mm thick 12.5 % polyacrylamide gels in Ettan DALTsix chamber (GE Healthcare).

Gels were scanned at 50 μm resolutions with FLA 9000 fluorescence scanner (GE Healthcare). Relative protein quantification across the experiment was performed using DeCyder 2-D Differential Analysis Software, v. 7.0 (GE Healthcare).

Preparative gel was fixed in 50 % methanol, 10 % acetic acid. Protein spots were excised manually and subjected to protein digestion with trypsin according to Shevchenko et al. (2006). Protein digests were separated on 75μm x 150mm Acclaim PepMap C18 column using Ultimate3000 RSLC system (Thermo-Scientific) coupled to Maxis G4 Q-TOF mass spectrometer detector with Captive Spray nano-electrospray ionization source (Bruker Daltonics). Peptide identification was performed using MASCOT server (Matrix Science) against *Malus* sp. genome database v.1.0 (Velasco et al., 2010). The threshold value for the identification of the proteins was Mascot score >50 and at least 2 peptides.

Blast2GO software (Conesa et al., 2005) was used for the annotation and gene ontology analysis of the identified protein sequences using the NCBI Protein database. The obtained GO terms were summarized using REVIGO server (Pesquita et al., 2009) and *A. thaliana* database, SimRel semantic similarity method with level set at 0.7 values. *A. thaliana* homologues of the identified proteins were obtained from the GDR Cyc Pathways Database v. 1.0.2-w (http://pathways.rosaceae.org) and their interaction was assessed using the String database (Szklarczyk et al., 2015).

RESULTS AND DISCUSSION

Characterization of structure of the apple endophytic bacteria population

Metagenomic analysis and isolation of culturable bacteria was used to characterize population of endophytic bacteria of the domestic apple. For the metagenomic analysis sequencing of *16S rRNA* gene was used. Semiconductor based next-generation sequencing method is capable to determine sequence of approx. 200 bp DNA fragments therefore eight regions corresponding to variable domains of the gene were employed in the analysis (Fig. 1). Six of the regions include hypervariable domains V2, V3, V4, V6-7, V8 and V9 and were amplified using commercially available set of primers dedicated for microbial metagenomic analysis. In addition, two regions partially corresponding to the hypervariable domains V5, V6, V7, V8 and V9 were amplified using two combinations of forward primer 799F and reverse primers 1492R and 1391R (Beckers et al., 2016; Ghyselinck et al., 2013; Munter, 2014). For microorganism identification, all regions were used independently.

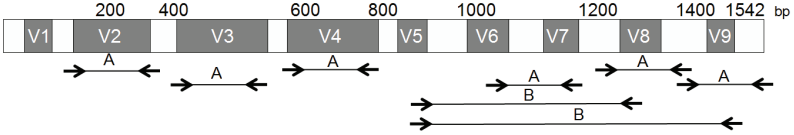

Figure 1. Nine variable domains (V1-V9) of bacterial 16S rRNA gene. The arrows indicate eight areas amplified using a metagenomic analysis PCR primer set (A) or two 799F and 1492R, 1391R primer combinations (B)

Application of the DNA isolated from apple leaves using commercial microbial DNA purification kit for *16S rRNA* gene sequencing resulted in large proportion of *16S rRNA* gene sequences of plant plastids and mitochondria due to low specificity of the primers used in the study. Large proportion of bacterial sequences (79 %) was obtained using combinations of the 799F and 1492R/1391R primers. Specificity of these primers to bacterial *16S rRNA* gene had been demonstrated and they do not amplify plant genomic and plastid sequences of the gene (Beckers et al., 2016; Ghyselinck et al., 2013; Munter, 2014). Most of primers included in the metagenomic analysis kit had lower specificity and combined proportion of bacterial sequences constituted only 9 % of all

sequences. Bacterial DNA enrichment would be required to improve efficiency of the metagenomic analysis of plant endophytic bacteria using these primers. Therefore, application of different bacterial DNA enrichment methods for characterization of apple endophyte population had been assessed.

Addition DNA samples were prepared from apple leaves using five different previously published methods. Two approaches were used to purify DNA that employ different cell lysis and DNA precipitation methods but do not include additional steps for bacterial DNA enrichment (Ding et al., 2013; Li at al., 2001). In addition, bacterial DNA enrichment was carried out using methods based on enzymatic hydrolysis, detergent or mechanical extraction. Method described by (Jiao et al., 2006) includes steps of enzymatic hydrolysis of apple leave tissue and bacterial cell separation using centrifugation. Two different conditions of this approach using different concentrations of macerozyme and cellulase enzymes were tested. SDS detergent extraction method destroys membranes of plant organelles (Wang et al., 2008). Using mechanical extraction approach, bacterial cells were enriched by bacterial cultivation in physiological solution after mechanical disintegration of leaf tissue (Nikolic et al., 2011).

Samples prepared using the different DNA preparation methods were used as DNA template to amplify regions of the *16S rRNA* gene using the metagenomic analysis primer set. The results of sequencing analysis presented in Table 1. Results corresponding to hypervariable domains V2, V3, V4, V6-7 and V9 are shown. The results obtained using primer set corresponding to the region V8 were excluded from the analysis as the majority (99 %) of the obtained 781,997 sequences were assigned to *Firmicutes* group and *Bacillaceae* family. None of these sequences were assigned at the level of genus or species, therefore suggesting their low similarity to the sequences of *Firmicutes* group microorganism. Additional analysis revealed similarity of the sequences to plastid *16S rRNA* sequence. The database used for sequence analysis does not include sequences of plastid *16S rRNA* gene; it could be presumed that the sequences were erroneously assigned to relatively closely related *Firmicutes* group.

Overall, the metagenomic analysis resulted in 8.301.187 sequences. After sequence quality assessments, the number was reduced to 2.864.817 (34.5 %) and included sequences with length from 159 to 226 bp. Due to 10 copy threshold used to

remove unique sequences, the number of sequences was further reduced and average number of sequences included in data analysis was approx. 37 % of total number of sequences (Table 1.). The largest proportion of sequences (44.7 %) included in the analysis was characteristic to the DNA samples prepared one of the enzymatic extraction methods. On the other hand, meanwhile total number of sequences obtained using combination of the 799F and 1492R/1391R primers was approx. three fold larger as compared to other methods, only 0.5 % of the sequences were included in data analysis. These primers amplify two long regions (V5-V7 and V5-V9 domains) and obtained sequences correspond to non-overlapping terminal 159 bp segments of these regions that might have effect on sequence quality assessment.

Table 1. Statistical summary of metagenomic data obtained from the bacterial community of domestic apple leaves

	799F and 1492R/ 1391R	**Metagenomic analysis primers**					
	Bacterial DNA preparation methods						
	DNA kit		**Li et al., 2001**	**Ding et al., 2013**	**Jiao et al., 2006a**	**Jiao et al., 2006b**	**Wang et al., 2008**	**Nikolic et al., 2011**
Sequence length (bp)	159	176	226	222	218	217	224	219
Number of sequences	2.935.724	2.046,449	714.088	590.828	506.384	496.827	473.642	537.236
Valid sequences [a]	600,627 20.5 %	693,052 33.9 %	294,925 41.3%	267,351 2.3 %	273,928 54.1 %	257,950 51.9 %	211,203 44.6 %	265,781 49.5 %
Analyzed sequences [a]	15,316 0.5 %	550,182 26.9 %	245,541 34.4 %	213,729 36.2 %	226,344 44.7 %	213,709 43 %	172,799 36.5 %	225,678 42 %
Bacterial sequences [b]	12,131 79.2 %	47,711 8.6 %	48,243 19.6 %	39,795 18.6 %	47,985 21.2 %	36,780 17.2 %	18,359 10.6 %	25,088 11.1 %

The brackets indicate the percentage of sequences calculated from the total number of sequences [a] and the number of sequences analyzed [b]

Set of sequences used for taxonomic assignment included 2.041.810 sequences and the proportion of the sequences assigned to bacterial taxonomic units constituted from 9 to 79 % depending on method used for DNA preparation or amplification of the *16S rRNA* sequences. The results obtained using DNA preparation methods that included mechanical or SDS detergent extraction were similar to initial results (shown in second column of Table 1.), meanwhile another four DNA preparation methods provided approx. two fold higher proportion of bacterial sequences.

The results showed that samples prepared with different DNA preparation methods had different taxonomic diversity of bacteria. The results of diversity assessment using Simpson (S) (Simpson, 1949) and Shannon-Wiener (H) (Shannon, 1948) indexes are shown in Table 2. These indexes were applied to all the analyzed sequences.

Table 2. Species richness and diversity of endophytic bacteria of apple cv. Gala leaves

	799F and 1492R/1391R	**Metagenomic analysis primers**						
	Bacterial DNA preparation methods							
	DNA kit		**Li ir et al., 2001**	**Ding et al., 2013**	**Jiao et al., 2006**	**Jiao et al., 2006**	**Wang et al., 2008**	**Nikolic et al., 2011**
H	1.986	1.335	1.751	2.836	1.277	1.382	1.602	1.126
S	0.6218	0.4957	0.6398	0.8353	0.4679	0.5043	0.6032	0.3552

Based on the S index, the highest diversity of endophytic bacterial families was identified using one of the cell lysis – 0.8353 (Ding et al., 2013). Thus, the endophytic bacterial families are distributed in this population evenly. The smallest variety of bacterial families was determined by method of mechanical extraction of bacterial DNA (Nikolic et al., 2011). In this case the species richness and variety index was 0.3552. Simpson index evaluates the number of bacterial families in accordance with the overall number of bacterial sequences. This means that diversity of population is determined by one or more dominant endophytic bacteria families and the proportion of other families in population is not significant. Our research showed that only family of

Rhodobacteraceae was dominant. The bacterial DNA extraction based on SDS detergent (Wang et al., 2008), cell lysis (Li et al., 2001) and commercial kit (amplified with specific 799F-1492R/1391R primer pairs) revealed approx. 0.6216 varieties, thus indicating that the endophytic bacteria populations harbor similar bacteria species structure.

The Shannon diversity index (H) is another index that is commonly used to characterize species diversity in a community. Evaluating endophytic bacteria species richness and diversity by H index, the results are in accordance with those obtained with S index. The highest diversity was identified by cell lysis method as described in Ding et al. (2013). The smallest diversity was established by mechanical extraction method (Nikolic et al., 2011).

Taxonomic composition of endophytic bacteria population in apple leaves was inferred from combined results obtained using different DNA preparation methods and *16S rRNA* regions. It was established that endophytes of *Proteobacteria* group were dominant (~97 %) in the apple endophyte population. Other bacterial groups included *Firmicutes* (2.7 %)*, Bacteroidetes* (0.2 %) and *Actinobacteria* (0.04 %). Total of 0.1 % was represented by rare endophytic bacteria classes of *Nitrospirae, Aquificae* and *Deinococcus-Thermus*. Among the *Proteobacteria* group, largest proportion was assigned to subgroup of *α-Proteobacteria* (89 %) and *γ-Proteobacteria* (11 %), meanwhile *β* and *δ-Proteobacteria* constituted only 0.05 %.

Among the 27 bacterial families identified in the analysis, dominant were *Rhodobacteraceae* (63 %), *Rhodobiaceae* (19 %), *Methylobacteriaceae* (8 %), *Enterobacteriaceae* (7 %) and *Pseudomonadaceae* (1.3 %) (Fig. 2). Other families constituted 2.1 %, and the remaining 0.01 % of sequences was not identified at the family level. Similar taxonomic distribution of endophytic bacteria had been previously described for other plant species, such as maize, sugarcane, rice, poplar, grape or sunflower (Ambrosinie et al., 2012; Compant et al., 2011; Magnani et al., 2010; Maropola et al., 2015; Pereira et al., 2011; Sun et al., 2008; Ulrich et al., 2008).

Approx. 15 % of the sequences were assigned at the level of genus. Total of 17 bacterial genera were identified. Among the dominant genera were agronomically important bacteria such as *Pseudomonas* (1.3 %), *Paracoccus* (1.6 %), *Bacillus* (0.4 %), and *Pantoea* (0.2 %). Previously it had been established that related bacteria species

colonize plant without adverse effect on plant growth (Maropola et al., 2015; Sun et al., 2008).

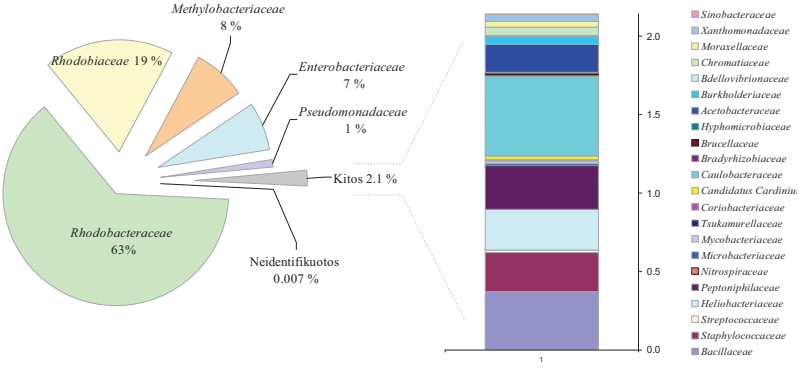

Figure 2. The composition and relative abundance of major bacterial families of the leaves-associated microbiome in domestic apple cv. Gala

Thirty-eight culturable bacterial strains were isolated from sterilized apple buds. The majority of endophytic bacteria isolates were identified at species level based on the sequence of *16S rRNA* gene (Table. 3.). Phylogenetic analysis revealed that isolates belong to three taxonomic clusters including *Proteobacteria, Actinobacteria* and *Firmicutes*. The isolates were assigned to six bacterial families: *Pseudomonadaceae, Enterobacteriaceae, Microbacteriaceae, Bacillaceae, Staphylococcaceae* and *Micrococcaceae. Pseudomonadaceae* family was dominant and included 18 isolates. Ten of these isolates were identified as *Pseudomonas fluorescens*, two – *Pseudomonas orientalis* and one for each – *Pseudomonas putida* and *Pseudomonas stutzeri*.

Seven and six *Curtobacterium* spp. and *Pantoea* spp. isolated bacteria belong to *Microbacteriaceae* and *Enterobacteriaceae* family, respectively. Six isolates were identified as *Curtobacterium flaccumfaciens*. Among the *Pantea* spp. isolates, *Pantoea vagans* and *Pantoea agglomerans* were identified. Also, five isolates were assigned to genus of *Bacillus*; one was identified as *Staphylococcus* sp. One isolate was identified as *Micrococcus luteus.*

Table 3. Identified endophytic bacterial *isolates* of domestic apple bud

Isolate	Number of aligned base	Taxon	Accession number	Sequence maching, %	Reference
Oa_1	1374	*Pseudomonas fluorescens*	418807	99.7	(Behrendt et al., 2003)
Oa_2	1442	*Pseudomonas fluorescens*	028986.1, 025586.1	99.7	(Behrendt et al., 2003)
Oa_3	1436	*Curtobacterium pusillum*	018783.1	97	(Niu et al., 2017)
Oa_4	1340	*Bacillus* sp.	566847.1	99.8	(Rae et al., 2010)
Oa_5	806	*Pseudomonas* sp.	969316.1	97	(Walke et al., 2015)
Oa_6	1445	*Pseudomonas putida*	744357.1	99	(Behrendt et al., 2003)
Oa_7	1496	*Pseudomonas* sp.	187248.1	99	(Walke et al., 2015)
Oa_8	962	*Curtobacterium flaccumfaciens*	970145.1	97.9	(Behrendt et al., 2003)
Oa_9	1361	*Staphylococcus* sp.	359407.1	98	(AlDisi et al., 2017)
O_10	1421	*Pseudomonas fluorescens*	028986.1, 025586.1	99.6	(Behrendt et al., 2003)
O_11	1269	*Curtobacterium flaccumfaciens*	025467.1	99.1	(Behrendt et al., 2003)
O_12	1449	*Pantoea* sp.	102966.1, 041978.1	98.2	(Smits et al., 2010)
O_13	1011	*Curtobacterium* sp.	595334.1	99.9	(Behrendt et al., 2003)
O_14	1441	*Pseudomonas fluorescens*	028986.1, 025586.1	99.6	(Behrendt et al., 2003)
O_15	1298	*Pseudomonas orientalis*	638095.1	98	(Behrendt et al., 2003)
O_16	1433	*Pseudomonas stutzeri*	074829.1	100	(Yan et al., 2008)
Da_1	1217	*Bacillus* sp.	476262.1	99.9	(Rae et al., 2010)
Da_2	1101	*Curtobacterium flaccumfaciens*	025467.1	99.2	(Behrendt et al., 2003)
Da_3	1436	*Bacillus* sp.	045082.1	99	(Rae et al., 2010)
Da_4	1335	*Bacillus* sp.	566850.1	99.9	(Rae et al., 2010)
Da_5	1419	*Bacillus* sp.	077842.1	99.8	(Rae et al., 2010)
D_6	1140	*Pantoea agglomerans*	765839.1	99	(Kim et al., 2013)

Isolate	Number of aligned base	Taxon	Accession number	Sequence maching, %	Reference
D_7	1419	*Pseudomonas fluorescens*	028986.1, 025586.1	99.7	(Behrendt et al., 2003)
D_8	1439	*Pantoea* sp.	102966.1, 041978.1	98.2	(Smits et al., 2010)
D_9	1454	*Pantoea agglomerans*	584289.1	99.9	(Kim et al., 2013)
D_10	985	*Pantoea vagans*	102966.1	98.7	(Smits et al., 2010)
Ga_1	1418	*Pseudomonas fluorescens*	102514.1, 028987.1	99.8	(Behrendt et al., 2003)
Ga_2	1396	*Pseudomonas* sp.	187248.1	99	(Walke et al., 2015)
Ga_3	1394	*Pseudomonas fluorescens*	028986.1, 025586.1	99.6	(Behrendt et al., 2003)
Ga_4	998	*Pseudomonas fluorescens*	025103.1	99.8	(Baida et al., 2001)
Ga_5	1413	*Pantoea vagans*	102966.1	99.6	(Smits et al., 2010))
Ga_6	1130	*Curtobacterium* sp.	450473.1	99	(Manirajan et al., 2016)
Ga_7	1431	*Curtobacterium flaccumfaciens*	605725.1	99	(Behrendt et al., 2003)
G_8	1446	*Pseudomonas fluorescens*	028986.1, 025586.1	99.7	(Behrendt et al., 2003)
G_9	1374	*Pseudomonas fluorescens*	418807.1	99.9	(Behrendt et al., 2003)
G_10	1126	*Micrococcus luteus*	884071.1	99.8	(Vacchini et al., 2017)
G_11	1388	*Pseudomonas* sp.	187335.1	98	(Walke et al., 2015)
G_12	1488	*Pseudomonas orientalis*	233760.1	98.9	(Behrendt et al., 2003)

Endophytic bacteria among genera of *Pseudomonas, Pantoea, Brevundimonas, Pseudoxanthomonas,* and *Sphingomonas* have been described by Hallmann et al. (1997). Later studies included more bacterial genera to the list (Bacon and Hinton, 2007; Rosenblueth and Martinez-Romero, 2006; Ryan et al., 2008), however, it is not complete as new endophytic bacterial species and genera are being described.

To date, the diversity of the domestic apple endophytome remains largely unexplored. There is only few research related with cultivable endophyte of rizosphere

and endosphere using culture dependent techniques (Dos Passos et al., 2014; Miliute and Buzaite, 2011). It was found that members of *Enterobacter, Bacillus, Pseudomonas, Burkholderia, Pantoea, Cedecia, Leclercia, Stenotrophomonas, Rhanella* and *Ewingella* genus are always dominant. Only analyzing apple phyllosphere endophytes using direct culture dependent method and culture-independent technique were carried out (Yashiro et al., 2011). The comparison revealed that among cultivable isolates the only one bacteria group of *Actinomycetales* was found. Meanwhile, metagenomic analysis identified endophytic bacteria groups of *Bacteroidales, Enterobacteriales,* and *Myxococcales* and *Sphingobacteriales* species.

In this study, we evaluated the endophytic bacterial diversity of apple retrieved using both culture dependent and metagenomic techniques. The population structure diversity revealed that genus of *Pseudomonas, Bacillus, Pantoea* and *Micrococcus* were the most dominant. Studies that has done the similar investigations concluded that genus of *Pseudomonas, Bacillus, Enterobacter, Klebsiella, Rhizobium, Sphingomonas, Pantoea, Microbacterium, Acinetobacterium* and *Arthrobacter* are the main bacteria groups in rizosphere of apple (Dos Passos et al., 2014). The differences between the groups of identified bacteria are partly determined by the niche (rhizosphere, endosphere and phyllosphere), that is colonized by endophytes (Hardoim et al., 2011).

Only the cultivation method identified endophytic bacteria of *Curtobacterium* genus. Thus, isolates of this genus form a small part of the all population, that is not reflect in metagenomic analysis due to the excessive number of amplified sequences or the inadequate application of PCR primers for the sequence of the *16S rRNA* gene of actinobacteria. To our knowledge, there is no published study that has assessed the endophytic bacteria of *Curtobacterium* genus in domestic apple phyllosphere. However, the endophytes of *Curtobacterium* spp. have been identified in citrus plants, clovers, potatoes (Elbeltagy et al., 2001; Lacava et al., 2007; Sturz et al., 1998).

Morphological and biochemical activity of endophytic isolates

The current study of cultivable endophytes showed that among gram-positive bacteria (66 %) only few isolates (21 %) were able to produce endospores, the structures that are resistant to unfavorable environmental conditions. Previous studies have shown

that endophytes are able to produce not only endospores, but also other defensive structures in the absence of nutrients (Yang et al., 2008). Nitrate reduction test revealed that more than half of the isolated endophytic isolates (53 %) reduced nitrate to nitrite, thus bacteria were able to grow in oxygen free environment and use nitrates as an alternative electron acceptor (Mbai et al., 2015).

Studies related with bacterial siderophores started six decades ago. The most common objects of these investigations are economically valuable agricultural crops, including maize, rice and wheat.

Verma et al. (2010) defined that siderophore synthesis is a characteristic for all endophytes; however our study found that this property was characterized by major part (71 %) of apple endophytic bacteria. It is known that bacterial siderophores play crucial role in plant growth promotion and protection against pathogen (Kruasuwan et al., 2017). Our study revealed that all endophytes of *Pantoea* spp. (*P. agglomerans* ir *P. vagans*) produced siderophores. Previous studies on bacterial siderophores have indicated that all endophyte isolates from cotton roots produce siderophores; however, siderophores originating from *Pantoea* spp. have the strongest antagonistic properties (Li et al., 2008; Yang et al., 2008). Due to this feature *Pantoea agglomerans* endophyte is classified as a group of plant growth promoting bacteria (Quecine et al., 2012). Siderophore production was observed in ten *Pseudomonas* genus (*P. fluorescens, P. putida, P. stutzeri,* and *P. baetica*) strains, similar to the previous report by Jasim et al. (2014). Earlier studies found that endophytes of *Pseudomonas* spp. secrete different types of siderophores that functions as antagonistic agents against pathogens. Six endophytic strains of *Curtobacterium* spp. produced siderophores, therefore acts as indirect factors of biocontrol (Abbamondi et al., 2016). The iron chelation by apple endophytes makes them better competitor for the available iron, so that protects host plant from pathogens attack.

More than half endophytes (66 %) were obtained on N-free Rennie medium. Since nitrogen is one of the factors limiting the growth of plant, the ability of endophytic bacteria to fix nitrogen is an important process for normal plant growth. Previous data shown that nitrogen fixing bacteria are widespread not only in legume plants (Santi et al., 2013). In this study we revealed, that endophytes of *Pseudomonas* spp. (82 %), *Bacillus* spp. (60 %), *Curtobacterium* spp. (29 %) and *Pantoea* spp. (67 %)

grew in N-free media and had nitrogenase activity. Many species of endophytic nitrogen fixing bacteria have since been isolated from apple rizosphere (Dos Passos et al., 2014).

One of the most important endophytic bacterial properties associated with the growth of plants is the ability to synthesize phytohormones. It is known that the plant hormones indole-*3*-acetic acid (IAA) not only stimulate plant cell division and roots formation, but is associated with the maintenance of homeostasis during a unfavourable environment (Bianco et al., 2009). To determine the amounts of IAA produced by each bacterial isolate a colorimetric technique was performed with ferric perchlorate reagent. It was determined that 84 % of all isolates were able to produce IAA. The amount of produced IAA in suspension varied from 6 to 23 of μg ml^{-1} bacterial protein. The greater producer of IAA were *Bacillus* sp. Da_1, *Micrococcus* sp. D_9, *Pantoea* sp. D_8, *Pantoea vagans* Ga_5 ir *Pseudomonas fluorescens* Oa_1 (20-23 μg ml^{-1}). Our date is in accordance with studies that were made recently by Kumar et al. (2016). Authors have published that endophytes, isolated from turmeric plants synthesized 14 – 23 μg ml^{-1}. Moreover, it was found that endophytic *Bacillus subtilis* LK14 isolate significantly increase host plant biomass due to IAA production (Khan and Doty, 2009).

It is know that 1-aminocyclopropane-1-carboxylate (ACC) deaminase is synthesized mostly by free living soil bacteria, however recently this property has been identified in the endophytic bacteria. According to Khan et al. (2016) ACC deaminase producing endophytes promote plant growth under various stress conditions. ACC deaminase producing endophytic bacteria reduces various environment stresses by lowering plant ethylene levels (Onofre-Lemus et al., 2009). ACC deaminase activity was evaluated in apple phyllosphere endophytes by their ability to grow in Dworkin and Foster minimal medium containing ACC. We have found that only twelve of all isolates (31 %) ACC used as the main source of nitrogen and grew in minimal medium. It should be noted that all ACC deaminase positive strains were able to produce significant amount of IAA. These data are in accordance with Akhgar et al. (2014) studies, which states that ACC deaminase and IAA producing endophytes stimulate growth of host plant. Most of the bacteria that synthesize ACC deaminase belong to *Pseudomonas* spp., which is one of the most prevalent bacteria genus (Akhgar et al., 2014). Similarly, in our results the four strain of *Bacillus* spp. produced ACC deaminase.

According to Khan et al. (2016) *Bacillus subtilis* LDR2 strain induces plant growth in drought stress conditions due to stress induced ethylene levels.

Characteristics of apple shoot stress and interaction with endophytes

Recalcitrance of plant species or individual genotypes to adapt and to grow *in vitro* is the most limiting stage in the application of plant *in vitro* technology. For preparation of plant explant and propagation of plants *in vitro*, the antimicrobial agents, synthetic media and other means that induce plant stress are often used. The slow growth or death of explants might be associated with the stress induced by cultivation conditions that leads to oxidative-reductive disequilibrium and damage of plant cell components due ROS accumulation (Bairu and Kane, 2011; Cassells and Curry, 2001; Sen, 2012). In this study, the *in vitro* oxidative stress of apple shoots was characterized by assessing the damage induced by stress and quantitative estimation of ROS accumulation and localization in shoot tissues. Four apple genotypes that showed different growth traits under *in vitro* conditions, including cv. Gala, Golden delicious, Orlovim and Noris × Paprastasis antaninis, were used in the study.

The oxidative stress symptoms of domestic apple shoots acclimated and maintained *in vitro* were assessed in five weeks after replanting. Histochemical detection of superoxide (by Nitro blue Tetrazolium Chloride (NBT) staining) and hydrogen peroxide (by 3,3'-Diaminobenzidine (DAB) staining) accumulation in apple shoots under *in vitro* conditions revealed that $O_2^{\bullet -}$ were accumulated in leaves and mechanically damaged tissues of all genotypes. Although DAB staining was distributed evenly in shoot tissues, higher concentration of H_2O_2 was characteristic to upper part of the shoots and leaf tissues. It has been established, that formation of $O_2^{\bullet -}$, H_2O_2 and their derivatives in the plant tissues is associated with oxidative stress (Apel and Hirt, 2004). Plant stress response *in vitro* has been investigated in *Malus* sp., which belongs to *Rosaceae* family (Sotiropolous et al., 2006), however the mechanism of stress has not been characterized. Therefore, based on our results, it could be presumed that accumulation of $O_2^{\bullet -}$, H_2O_2 and related derivatives in apple shoots under *in vitro* conditions was related to oxidative stress.

The accumulation of ROS is capable to cause damage to the tissues of the explants therefore accumulation of lipid peroxidation product malondialdehyde (MDA) was assessed. The shoots of four apple genotypes were transferred to fresh medium and cultivated for six weeks. The analysis revealed that average concentration of MDA in apple shoots varied from 16.6±0.6 to 31.2±1.5 nmol/g of fresh weight (F.W.). The increase of MDA concentration for genotypes of cv. Gala and Noris×Paprastasis antaninis was on average 25.6±0.8 and 22.4±1.5 nmol/g F.W. during the first week after transfer on fresh medium. The MDA levels in shoots of cv. Golden delicious genotype remained comparable to the control shoots. Decrease MDA concentration (from 24.2±0.9 to 18.5±0.9 nmol/g F.W.) as compared to control was observed in cv. Orlovim. All studied genotypes demonstrated statistically significant reduction in oxidative stress symptoms during the first three weeks of growth (MDA concentration decreases to a similar level: 17.7±1.2 – 18.5±0.9 nmol/g F.W.). During later growth period (4-6 weeks), the MDA concentration increased (approx. 2 fold), thus indicating the oxidative stress symptoms were associated with the aging of the apple shoot culture.

The increase in MDA levels is associated with increase in cellular ROS production (Thompson et al., 1987). Therefore measurement of MDA has been often used as a marker for oxidative lipid damage (Hasan et al., 2015; Kong et al., 2016). Previous studies have shown that plant exposed to stressful conditions accumulate higher concentration of MDA (Barnawal et al., 2013).

Among the four apple genotypes involved in the study, only cv. Gala demonstrated increase in the lipid peroxidation during first week after transfer to fresh medium followed by statistically significant decrease during following 3 weeks of growth. This suggested that peroxidation of lipid membranes is a consequence of the oxidative stress associated with adaptation of the shoots of this apple genotype on the fresh medium. Due to the most prominent symptoms of the oxidative stress damage, this genotype was selected for the study on interaction with endophytic microorganisms.

The influence of endophyte on apple shoot morphology and growth properties

Understanding plant oxidative stress mechanism *in vitro* and structure of population of endophytic microorganisms provides potential to manipulate the plant

microbiome to reduce the stress caused by *in vitro* conditions and to improve efficiency of micropropagation of plants. Therefore in this study the effect of endophytic bacteria strains on apple shoots biomass and additive shoot growth was evaluated. The results suggested, that co-cultivation of apple shoots with endophytic bacteria strains of *Bacillus* spp. induced the highest accumulation of biomass and additive shoot number (Fig. 3). Among the five *Bacillus* spp. strains, Da_4 and Da_5 demonstrated shoot growth enhancing properties, as the plant biomass and additive shoot number were increased 1.9 and 1.7 fold, respectively. Another *Bacillus* sp. strain Da_1 demonstrated statistically significant increase in shoot biomass and additive shoot number compared to control. Incubation with *Bacillus* sp. strain Oa_4 resulted in approx. 2.6 fold reductions in apple shoot biomass and additive shoots number compared with control. It is notable, that in this case, no adverse effect on shoot morphology was observed, despite the reduced shoot biomass.

Our study revealed that three isolates of *Bacillus* spp. Da_1, Da_4 and Da_5 had stimulating effect not only on shoot biomass accumulation, but also on additive shoot number, whih increased 1.6-1.9 fold compared to control (Fig. 3).

Our results are in agreement with previously published date, which indicate that endophytic *Bacillus* sp. UCMB5113 induce accumulation of leaf and root biomass of host plant. The authors constitute, that the increase was associated with the metabolites produced by the endophytic bacteria that had regulating effect on plant growth (Asari, 2015). Stimulation of shoot biomass accumulation was dependent on size of bacterial inoculum, and the phyla of *Firmicutes*, including *Bacillus* spp., formed the largest group of plant growth promoting bacteria (Xia et al., 2015). Our results revealed that different strains of the same species had different effect on shoot growth; therefore it could be presumed that the mechanism of shoot growth stimulating effect was common among the different bacterial genera.

It is established that plant growth promoting bacteria induce plant host growth, however the specific mechanism of process is still unknown (Zhao et al., 2016). Plant hormones production or modulation of the phytohormone balance is one of the major properties of endophytes that are involved in stimulation of plant growth. In our study, tested *Bacillus* spp. strains demonstrated different apple shoot growth promoting properties, despite all of them being IAA and ACC deaminase positive. Similar results

were obtained for potato, tomato and grape plant inoculated with endophytic *Bulholderia phytofirmans* PsJN strain (Mei and Flinn, 2010).

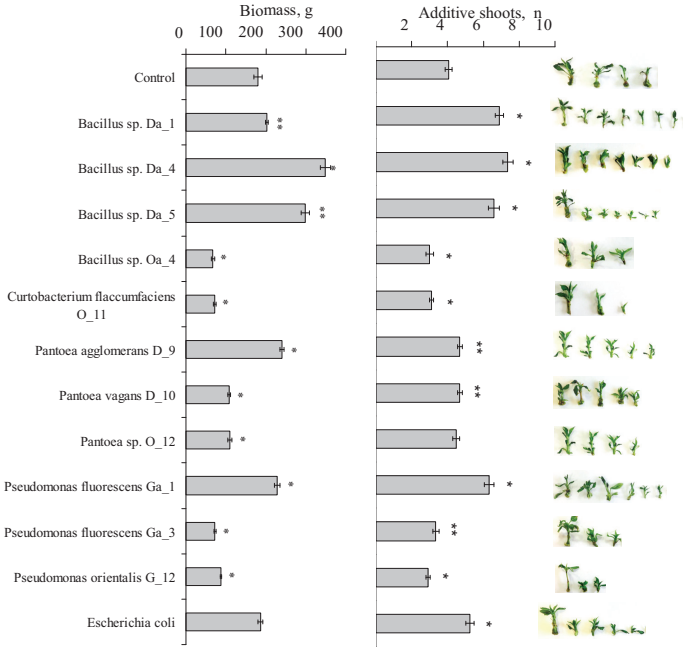

*Figure 3. The biomass (left panel) and additive shoots (right panel) of domestic apple shoots after three weeks of inoculation with endophytic bacteria isolates. Stars indicate statistically significant differences as compared to control (**<0.05; * <0.01)*

Apple shoot growth promoting properties were demonstrated by *Pantoea agglomerans* D_9 and *Pseudomonas fluorescens* Ga_1 endophytes and approx. 1.3 fold increase in shoot biomass and additive shoot number was observed. Interestingly, both of the isolates were able to produce IAA and fix N. The amount of IAA produced by *Pantoea agglomerans* D_9 and *Pseudomonas fluorescens* Ga_1 strains was 5 and 7 µg ml^{-1} of total protein, respectively. Thus, based on these results, the increase of shoot biomass and additive shoot number could be associated with ability of the endophytes to provide essential nutrients and phytohormones to plant. According to Knoth et al.

(2013), plants inoculated with bacterial diazotrophs accumulated larger biomass and approx. 25 % fold higher amount of N.

Co-cultivation with another five strains, including *Curtobacterium flaccumfaciens* O_11, *Pantoea vagans* D_10, *Pantoea* sp. O_12, *Pseudomonas fluoresens* Ga_3 and *Pseudomonas orientalis* G_12, resulted in 1.6-2.5 fold lower additive shoot number. Interestingly, co-cultivation with *Pantoea vagans* D_10 resulted in approx. 1.6 fold reduced biomass of apple shoots, however the shoots had one additional shoot compared to control. Also, it is notable that all these five strains demonstrated plant growth promoting properties assessed in the study. This could be due to disturbed interaction between plant and endophytic bacteria under *in vitro* conditions. It has been previously established, that endophytic bacteria could attain pathogenic traits and have adverse effect on plant growth under *in vitro* conditions (Bailey et al., 2006; Partida-Martinez and Heil, 2011).

Oxidative stress symptoms of apple shoots inoculated with the endophytic bacteria strains were assessed using MDA assay. The concentration of MDA was established in stems and leaves of shoots, separately. The results revealed that more prominent oxidative processes took place in leaves of shoots as MDA concentration in stems in all cases complied with control (8.9±0.2 to 15.9±0.67 nmol/g F.W.). Similar results were obtained by Jbir-Koura et al. (2015). Authors established that MDA was mainly accumulated in plant leaves under oxidative stress conditions induced by drought stress. In our study, the strains of *Bacillus* spp. Da_1, Da_4, Da_5, Oa_4; *Pantoea agglomerans* D_9; *Pantoea vagans* D_10; *Pantoea* sp. O_12; *Pseudomonas fluorescens* D_7, Ga_1, Ga_3, Oa_2, O_10; *Pseudomonas stutzeri* O_16 demonstrated statistically significant suppression of the lipid peroxidation product accumulation (1.5-3 fold). These results indicate that the strains of endophytic bacteria could have oxidative stress suppressing properties.

Analysis of gene expression associated with apple shoot and endophytic bacteria interaction

Endophytic bacteria strains *Bacillus* spp. Da_1, Da_4 ir Da_5, *Pantoea* D_9 and *Pseudomonas* Ga_1 had a stimulating effect on apple shoot growth that could be a

consequence of oxidative stress suppressing effect of the bacteria. In order to confirm this hypothesis the effect of bacteria on expression of salycilic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways in apple shoots was assessed. SA, JR and ET are plant phytohormones, involved in plant cell response to abiotic and biotic stress and mediate induced systemic resistance response (ISR).

RT-PCR was used to assess expression of genes involved in SA, JR and ET signaling pathways in apple shoots of cv. Gala after seven days of co-cultivation with different endophytic strains, including pathogenesis-related gene 1(*PR-1*) (Bonasera et al., 2006), coronatine-insensitive gene 1 (*COI1*), allene oxide synthase gene (*AOS*), jasmonic acid-amido synthetase gene (*JAR*), lipoxygenase 2 (*LOX2*), WRKY transcription factor (*WRKY*) (De Bernonville et al., 2012) and ethylene transcription factors (*ERF, ERF1*) (Shin et al., 2014).

The expression of phospholipase D (*PLD*) and ACC synthetase (*ACS*) gene was not detectable.

The expression of *AOS* gene, involved in the JA signaling pathway, increased significantly after co-cultivation with *Bacillus* sp. Oa_4, *Pantoea* spp. (D_9, D_10 and O_12) and *Pseudomonas* sp. Ga_3 endophytic bacteria (Fig. 4.). Expression of the *AOS* increased 200 and 50 fold compared to control after incubation with *Bacillus* sp. Oa_4 and *Bacillus* sp. Da_1, respectively. All three tested *Pantoea* spp. strains similarly effected expression of the *AOS* gene, resulting to approx. 100-150 fold increase. Previous studies had shown that the AOS enzyme is involved in the first step of jasmonic acid biosynthesis from lipoxygenase-derived hydroperoxides of free fatty acids. Moreover, the expression of *AOS* gene can be induced by ET, which is believed to have synergistic effect with jasmonates (Sivasankar et al., 2000).

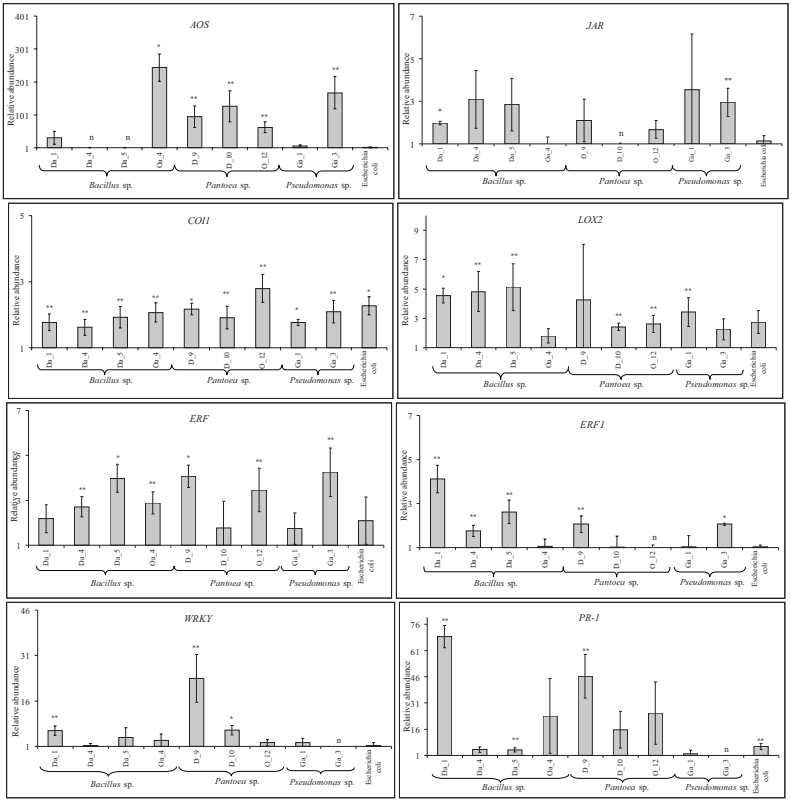

Figure 4. The relative expression of apple shoot genes, involved in JA (COI1, JAR, LOX2 ir AOS), SA (PR-1, WRKY) signaling pathway and ET synthesis (ERF and ERF1) after seven days inoculation with endophytic isolates. The average of all genes is equivalent to a unit. Data is presented as mean and SEM. Stars indicate statistically significant differences as compared to E. coli control assessed by t-test (p<0.05, ** p<0.01)*

It is known that *LOX2* gene catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides, leading to formation of JA synthesis precursor. Previous studies show lipoxygenase genes are downregulated by plant pathogens, and JA synthesis is inhibited. Our results revealed that, co-cultivation resulted in statistically significant upregulation of *LOX2* for majority of endophytic strains (except *Bacillus* sp. Oa_4,

Pantoea sp. D_9 and *Pseudomonas* sp. Ga_3), suggesting that endophytes might activate the systemic resistance repose in apple shoots.

Co-cultivation with the endophytic bacteria resulted not only in upregulated expression of *AOS* and *LOX2* genes involved in the initial stage of the JA signaling pathway, but also *COI1* gene responsible for subsequent signal perception. For two of the strains (*Bacillus* sp. Da_1 and *Pseudomonas* sp. Ga_3) *JAR* involved in JA conjugation was also significantly upregulated. However, it is notable that high level of expression of *JAR* was observed in control shoots as well.

It is known that *COI1* mediates JA and ET mediated signal transduction that regulates expression of the ethylene transcription factors (ERF). The family of *ERF* genes encodes transcription regulators involved in various functions related to development and physiological processes. *Bacillus* spp. (Da_4, Da_5 and Oa_4), *Pantoea* spp. (D_9, O_12) and *Pseudomonas* sp. Ga_3 significantly upregulated expression of *ERF,* while the expression of *ERF1* was isolate specific. The results suggested that co-cultivation with the endophytic bacteria leads to JA and ET mediated activation of systemic resistance response in apple shoots.

The study showed that the endophytic bacteria strongly induced the JA biosynthesis initial phase (*AOS*), related to the JA signal transmission pathway (*COI1*) and the ethylene responsive transcription factor (*ERF*). Thus, activated ET/JA signal transduction synergistic induces *ERF* genes via ET/JA hormone receptor signals.

It is known that the SA or JA signaling pathways could mediate activation plant defense response against pathogens. Gene expression analysis revealed that three endophytic strains, including *Bacillus* spp. Da_1 and Da_5, *Pantoea* sp. D_9, upregulated *PR-1* expression. *Bacillus* sp. Da_1 and *Pantoea* spp. D_9 and D_10 bacterial strains also upregulated expression of the gene of *WRKY* transcription factor, although the intensity of expression was different compared to the *PR-1* gene. *WRKY* transcription factor is involved in regulation of SA signaling pathway mediated gene expression (De Bernonville et al., 2012). Upregulation of SA signaling pathway (*PR-1* and *WRKY* genes) implied that the bacterial strains were involved in pathogenic interaction with the apple cells. However, all isolates except *Pantoea* sp. D_10 had a stimulating effect on apple shoot growth and additive shoot number. In such a case, upregulation of the genes involved in the SA signaling pathway was not related to pathogenesis.

The obtained results revealed that different patterns of expression of the SA and JA/ET signaling pathway genes were characteristic to endophytic bacterial isolates. Endophytic bacteria isolates demonstrated JA/ET mediated response that activates systemic inducible resistance that ensures priming of adaptation response to abiotic and biotic stress of plant cells. In addition, for several of bacterial strains, systemic inducible resistance could be activated through SA signaling pathway.

It is well established that plant response to stress activates the plasma membrane enzyme – NADPH-oxidase (Apel and Hirt, 2004). In this study, we evaluated effect of the endophytic bacteria on expression of the NADPH-oxidase homologs *MdRboh* D1, *MdRboh* D2, *MdRboh* D3 and *MdRboh* F. The genes were selected based on the study on the gene expression of apple shoots under *in vitro* conditions that showed differential expression of the *Mdrboh* D 1-3 and *Mdrboh* F genes (Cepauskas et al., 2015).

The analysis revealed that expression of the *Mdrboh* D1 gene was downregulated bellow the detection level after co-cultivation with *Bacillus* sp. Oa_4, *Pantoea* spp. D_10 and O_12, *Pseudomonas* spp. Ga_1 and Ga_3. Statistically significant upregulation of the *Mdrboh* D2 gene was characteristic to all endophytic strains, no expression of the *Mdrboh* D3 was detectable. Statistically significant downregulation of the *Mdrboh* F was detected only for *Bacillus* sp. Oa_4.

Different RBOH homologues have a distinct function. Differential expression of *Atrboh* D gene has been shown for various organs and tissues of *Arabidopsis thaliana*. *Atrboh* D and *Atrboh* F is activated and play important role in incompatible plant and pathogen interaction (Torres and Dangl, 2005). Our results imply that upregulation of *Mdrboh* D2 may be important for the interaction between the plant and the endophytic microorganism. In this regard, the most distinctive was the *Pantoea agglomerans* D_9 isolate, that resulted in more than 10 fold higher expression of *Mdtrboh* D2 gene expression. This strain also upregulated *PR-1* and *WRKY* gene expression, therefore it could be presumed that regulation of *Mdtrboh* D2 gene expression could be associated with SA signaling pathway.

Analysis of apple cell and endophytic bacteria interaction

To study effect of endophytic bacteria on apple shoot growth, the suspension of endophytic bacteria has been inoculated at the base of petiole of apple shoots. It could

be expected that within hours the bacteria form local interaction with shoot tissues lead to activation and systematic spreading of the ISR in the shoot tissues. Since endophytic bacteria only gradually colonize the shoot tissues and are involve in localized interaction with plant cells, the plant response is not homogeneous and the shoot inoculation with endophytic bacteria approach is not optimal to study initiation stage of plant and endophyte interaction. Therefore model system of co-cultivation of apple cell suspension with endophytic bacteria was employed to study formation of the interaction between apple cells with the endophytic bacteria strains. The apple cell culture is a homogeneous population of undifferentiated cells that maintains its biological properties and enables to track molecular events that are involved in interaction between the cells and bacterial endophytes (Bordiec et al., 2011; Garcia-Brugger et al., 2006).

Little is known about initial phase of the interaction between the plant and the endophytic bacterium. In this study, we assessed qualitative and quantitative traits of association of endophytic bacteria to apple cells. The physical interaction was observed by fluorescent microscopy over 16 h. The apple plant cells were stained with acridine orange dyes according to Bordiec et al. (2011). Quantitative proportion of the endophytic bacteria associated with the apple cells were determined by serial dilution and plating on LB medium. *E. coli* was used as negative control according to method described by Bordiec et al. (2011).

A qualitative assessment by microscopic analysis revealed that endophytic bacteria interact with apple cells and, after 16 h incubation proportion of the associated bacterial cells appeared similar for all of the endophytic bacterial strains. The lower proportion of associated bacteria was detected in the case of *E. coli* negative control. These results are in agreement with the results observed by Bordiec et al. (2011) where *E. coli* did not associate to the plant cells even after 24 h incubation. Our analysis of cell viability using Evans blue staining demonstrated that the treatment with different endophytic isolates had little effect on viability of the apple cells after 16 h incubation and the cell viability varied from 77 to 97 %. Therefore it could be concluded that the endophytes did not had adverse effect on apple cell viability.

Based on the microscopic analysis of endophytic bacteria association to apple cells it was difficult to estimate intensity of the interaction, therefore quantitative assay was employed. Application of serial dilution method revealed that the endophytic

bacteria associated to apple cells and proportion of associated bacteria cells comprised from 9.3 ± 0.43 to 46.5 ± 2.3 % after 6 hours of incubation (Fig. 5.).

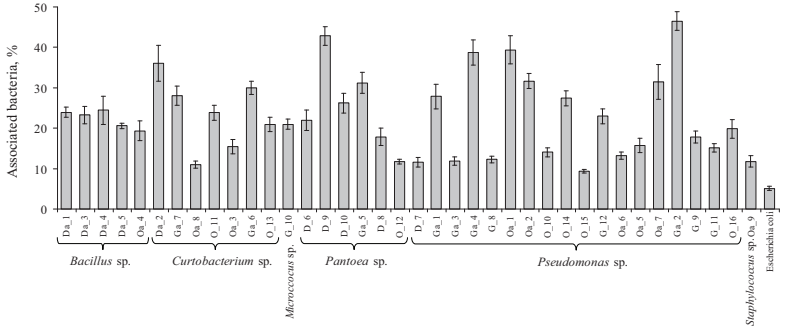

Figure 5. Quantitative association of endophytic isolates and apple cells cv. Gala. Data is presented as mean and SEM. All isolates are statistically significant from E. coli control ($p<0.01$)

The lowest proportion of associated cells was estimated for the *E. coli* control – 5.1±0.48 %. Moreover, all *Bacillus* spp. strains demonstrated similar level of association, which varied from 19.4±2.4 to 24.5±3.5 %. The largest proportion of associated cells was characteristic to five strains including *Pseudomonas* spp. Ga_4, Oa_1, Ga_2, Pantoea sp. Ga_5 and *Curtobacterium* sp. Da_2 genus.

ROS/RNS production of apple cell and endophytic bacteria interaction

The early events involved in formation of plant-microorganism interaction stimulate signaling processes such as the synthesis of active oxygen and nitrogen compounds (ROS/RNS) (Bordiec et al., 2011; Garcia-Brugger et al., 2006). Using model system of apple cell suspension culture and specific 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) dyes, the accumulation of ROS/RNS during apple cell and endophytic association were estimated after 2 and 6 hour co-incubation (Fig. 6.). H_2DCFDA is non-fluorescent compound that is oxidized in the presence of H_2O_2 and other ROS/RNS and becomes strongly fluorescent fluorescein compound.

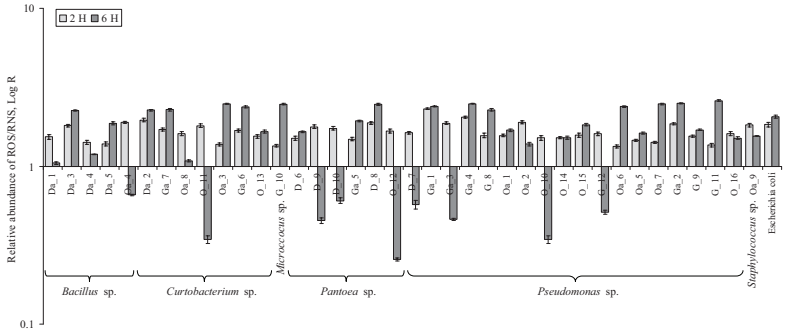

Figure 6. Influence of endophytic bacteria on the accumulation of ROS/RNS in domestic apple plant cells. Data is presented as mean and SEM. Difference from E. coli control was statistically significant for all strains ($p<0.05$)

The apple cell suspension incubated with endophytic bacteria demonstrated strain specific ROS/RNS accumulation. It was found that all 38 bacterial strains included in the study induce ROS/RNS accumulation after 2 hour of incubation. After 6 hours, the intensity of ROS/RNS accumulation increased for majority of the strains, however, nine strains (*Bacillus* spp. Oa_4, Da_4; *Curtobacterium* sp. O_11; *Pantoea* spp. D_9, D_10, O_12; *Pseudomonas* spp. D_7, Ga_3, O_10, G_12) reduced ROS/RNS accumulation level as compared to control. After incubation with the nine bacterial strains, ROS/RNS concentration decreased from 2.5 to 3.0 folds.

Evans blue assay demonstrated that the treatment with different endophytic isolates had little effect on viability of the apple cells and it varied from approx. 76 to 86 %. Such results show a specific effect of bacterial isolates on the accumulation of ROS/RNS in apple cell culture. Therefore variations of ROS/RNS concentration could be associated with signaling processes induced by microbial compounds as well as systemic resistance response activated by endophytic bacteria. The results suggested that interaction with endophytic microorganisms could have inhibiting effect on ROS/RNS producing enzymes directly or it induced changes in the expression of cellular gene expression that resulted in alterations in production or metabolism of ROS/RNS.

Gene expression analysis of apple cell and endophytic bacteria interaction

Expression of genes involved in the SA, JA and ET regulated signaling pathways in apple shoots incubated with the endophytic bacteria strains was evaluated using RT-PCR.

The expression of *LOX2, ACS,* and *WRKY, ERF1, ERF2* transcription factors was not detected. An intense RT-PGR signal was observed for *PR-1* gene. This suggested that *PR-1* is highly upregulated in apple cell suspension due to response to stress induced by cultivation under *in vitro* conditions. This would provide explanation for only slight changes in gene expression in the cells incubated with the endophytic bacteria strains.

The expression of the *PLD* gene was downregulated significantly (except *Bacillus* spp. Da_4, Oa_4, *Pantoea* sp. D_9 and *Pseudomonas* sp. G_12) after incubation with the endophytic bacteria. It is well established that *PLD* is a constituent of pathogenesis signaling pathway (Singh et al., 2012) and during response to pathogens *PLD* gene expression is a highly upregulated and it leads to suppression of JA synthesis (Yamaguchi et al., 2009). Therefore in our study, suppressed expression of *PLD* was favorable for JA synthesis and activation of induced systemic resistance response.

The expression of *AOS* gene involved in JA signaling was highly upregulated after incubation with the endophytic bacteria (approx. from 50 to 100 fold). It is known that the expression of the *AOS* gene is necessary for the synthesis of biologically active jasmonates (Park et al., 2002). However, the treatment had little effect or even slightly reduced expression of other genes involved in JA signal transduction (*COI1*) and conjugation (*JAR*). A high biological variance was characteristic to these results and no significant difference was detected as compared to control.

The majority of the bacterial strains (*Bacillus* Da_1, *Curtobacterium* sp. Da_6, *Pantoea* spp. D_9, D_10 and *Pseudomonas* spp. Oa_1, Oa_10) had enhancing effect on expression of the ERF transcription factor that mediates JA and ET signaling. The result indicates that activation of the JA and ET mediated apple cell response was bacterial strain specific.

The results of gene expression analysis suggest that endophytic bacteria interaction with apple cells lead to JA and ET mediated response that is associated with

downregulated expression of *PLD* gene and upregulated of *AOS* gene and ERF transcriptional factor. Overall, it appears that differential expression of phytohormone signaling pathways is less prominent at the initial stage of endophytic bacteria interaction with apple cells as compared to the results observed for leaves of apple shoots co-cultivated with the endophytic bacteria for 7 days. This leads to suggestion that modulation of the phytohormone signaling pathways plays more important role at the later stages of the interaction. However, the differences in gene expression could also be a consequence of different growth conditions or tissue specific response.

As for the experiments with apple shoot culture, expression levels of *MdRboh* D1-D3, and *MdRboh* F were assessed for apple cell suspension incubated with the endophytic bacteria. Expression of *MdRboh* D1 and D3 was not detected. *MdRboh* D2 was downregulated after incubation with majority of the strains, except for *Bacillus* sp. Da_1, *Pantoea* sp. D_8, *Pseudomonas* sp. G_12 and *Curtobacterium* sp. Da_2 that significantly upregulated expression of this gene. Larger effect was observed for *MdRboh* F that was downregulated by incubation with the endophytic bacteria up to approx. 16 fold compared to control. The suppressing effect of the endophytic bacteria strains *Pantoea* spp. O_12, D_9*, Bacillus* sp. Oa_4, *Curtobacterium* sp. O_11, *Pseudomonas* spp. D_7 and Ga_3 on expression of *MdRboh* D2 and F genes showed similar trends as compared to the bacteria inhibiting effect on ROS/RNS accumulation in the apple cells.

Proteomic analysis of apple cell and endophytic bacteria interaction

Regulation of ROS/RNS production by endophytic bacteria is related to specific changes in expression of the genes involved in phytohormone JA, ET and SA signaling pathways. However, role of these pathways in initiation of interaction of plant cells with endophytic bacteria remains ambiguous. Activation of the pathways might be crucial for initiation of the interaction or the pathways might be activated as part of long-term physiological changes induced by the endophyte. To establish gene expression differences associated with contrasting effect of endophytic isolates on ROS/RNS production, a proteomic analysis of apple cells was carried out.

Previously, several studies described proteome analysis of plant – endophytic bacteria interaction. Kandasamy et al. (2009) identified differential expression of

proteins involved in processes of defence response and metabolism. Faleiro et al. (2015) identified group of proteins (46) that were upregulated in maize inoculated with endophytic strain *Azospirillum brasiilense* FP2. The study established that part of the proteins was specifically involved in compatible interaction of the plant and *Azospirillum brasiilense* FP2. Mercado-Blanco et al. (2016) identified a group of plant cell proteins involved in metabolism that were essential for successful plant colonization by *Pseudomonas fluorescens* PICF7 strain.

In this study, proteomics analysis was used to assess differential gene expression in apple cv. Gala cell suspension incubated for 6 hours with the two different endophytic strains of *Bacillus* spp. (Oa_4 and Da_4) that had contrasting effect on ROS/RNS production in the cells and growth of apple shoots *in vitro*. 2D electrophoresis in acidic gradient (pH 4-7) was used for protein fractionation. Average number of detected protein spots was 2246±119 per gel. After gel alignment using internal standard, the number of protein spots decreased to 1975±197. Results of quantitative analysis are presented in Table 4. Overall, 65 proteoforms were identified that had significant and more than two-fold difference in abundance between the experimental groups (Table 4.).

Table 4. Number of differentially expressed proteoforms identified by two-dimensional gel electrophoresis analysis in the apple cell suspension after incubation with Bacillus spp. Oa_4 or Da_4

Differential expression	Oa_4 / Control[a]	Da_4 / Control[a]	Oa_4 / Da_4
Increased	32 (29)	10 (7)	30
Decreased	5 (0)	25 (20)	2
In total	37 (29)	35 (27)	32

[a] brackets indicates number of proteins, which differential expression is characteristic only to this bacterial strain. The results include statistically significant ($p<0.01$) and higher than two fold changes

Samples of differentially expressed protein spots were digested with trypsin and identified using LC-MS/MS analysis. Forty-six proteoforms were unequivocally identified as 36 unique genes of the domestic apple. Eight of the genes matched two proteoforms each and one gene matched three proteoforms. Thirty-five of the genes derived from the apple genome database were annotated, and one of the genes was

described as protein of unknown function. Hierarchical cluster analysis was used to compare differential protein abundance of the 65 proteoforms and four distinct groups of the proteoforms were identified (Fig. 7).

The largest group included 28 proteoforms that showed large increase in abundance in the apple cells incubated with strain Oa_4 compared to control, meanwhile incubation with Da_4 had no significant effect on the protein abundance (with exception of Mal d 1 like protein). It is notable, that protein abundance varied from 2.4 to 69 fold in this group and it was the largest differences observed in the experiment. Among the highly up-regulated proteins were PLAT1 (~69 fold increase) and two different proteoforms of HSC 70-1 (~46 and 58 fold), and abundance of another 19 proteoforms increased more than 10 fold.

Meanwhile increase in abundance of Mal d 1 like protein was observed for samples incubated with Da_4, the difference was 7.5 fold larger in case on Oa_4 (protein abundance increased 19.3 and 2.6 fold compared to control for Oa_4 and Da_4, respectively). Therefore, it appeared that the change in abundance of proteins that belong to this group was largely specific to response induced by the Oa_4 strain.

Among the 11 proteoforms included in the second group, abundance of 9 and 4 proteoforms increased more than two fold after incubation with the strains Da_4 and Oa_4, respectively. The differences were moderate and varied from 2 to 4 fold. It is notable, that less than two fold (1.5-1.9 fold) but statistically significant difference was detected for another 7 proteins in this group, except for RidA and unidentified proteoforms No. 35. Therefore, this group includes proteins that had moderate change in abundance but were responsive to incubation with either strain, and it might reflect gene expression patterns universal to cell interaction with endophytic bacteria or at least *Bacillus* spp.

Only one protein, GSTL3, was assigned to the third group. Abundance of the protein varied significantly but only slightly as compared to control (decreased and increased 1.4 fold after incubation with Oa_4 and Da_4, respectively). However, the difference between the two treatments was more than two fold.

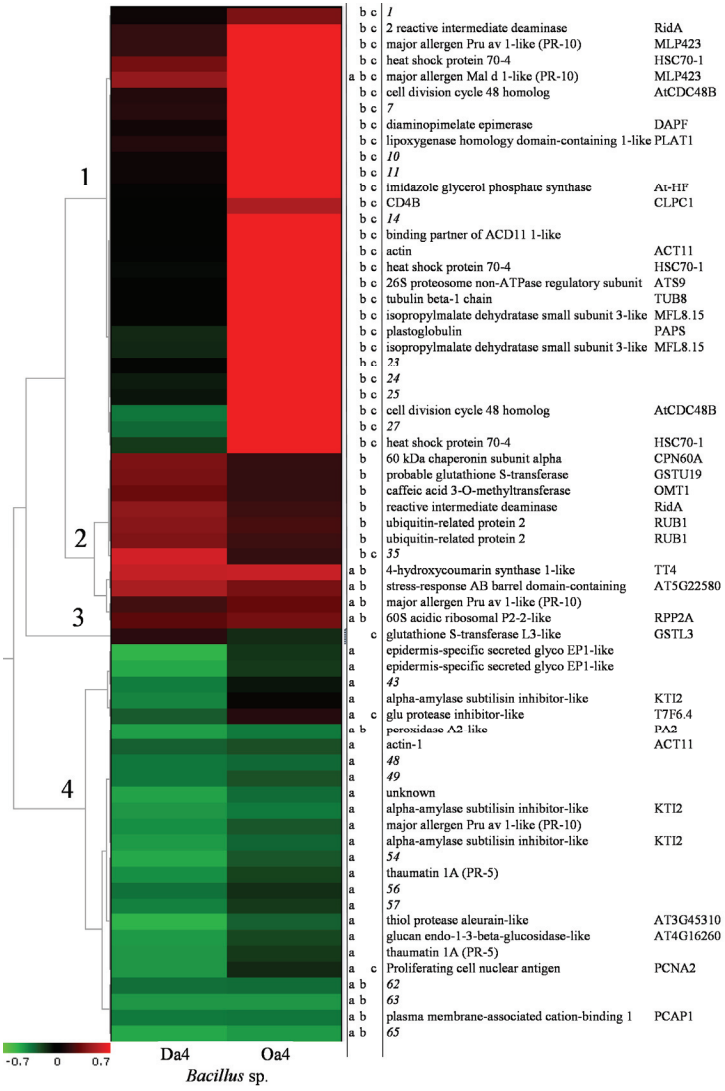

Figure 7. The hierarchical cluster analysis of the differently expressed proteins in apple cells. The four main groups are indicated by the numbers on the left. The color of the chart indicates decrease (green) or increase (red) in protein abundance. Letters in 1-3 columns indicates statistically significant ($p<0.01$) differences after inoculation with Da_4 (a) and Oa_4 (b) as compared to control an in between (c). Column 3 contains protein names

Lastly, 25 proteoforms were assigned to the fourth protein group that had reduced abundance after incubation with Da_4 and five of them had reduced abundance after incubation with Oa_4 as well. The differences were moderate (from 2 to 3.2 fold). It is noteworthy that for most of the proteoforms there were no significant difference between the two treatments, and only Ser protease inhibitor and PCNA2 had Da_4 specific response. This suggested that among the samples incubated with Oa_4 moderate down-regulation of the proteoforms included in this group could be partially similar to the treatment with Da_4, but the difference was not prominent due to larger biological variation. Therefore it leads to conclusion that this group of proteins was similar to the second group as it reflected more universal mechanism involved in cell interaction with endophytes.

The results of cluster analysis revealed traits that appeared common or specific for the two related strains of *Bacillus* spp. Further analysis was aimed to define functions associated with the groups of differentially expressed proteins and would provide clues about potential mechanisms involved in the plant cell and endophytic bacteria interaction. Two different methods were used to assess function of the identified genes. Biological processes were summarized based on gene ontology (GO) and interactions among the genes was assessed using the String database.

To assess biological function of proteins included in different protein groups identified by cluster analysis, annotation of GO term was performed separately for the three of the groups (1, 2 and 4) that included more than one protein. The analysis resulted in 37, 33 and 38 unique terms of biological process assigned to these groups, respectively. The terms were summarized based on semantic similarity using REVIGO algorithm (Pesquita et al., 2009).

As compared to other groups, the first group had the largest number of unique proteins identified (15) and associated GO terms (37). However, the later were summarized by the lowest number of biological processes – the most dominant were metabolism (56 %), protein metabolism (19 %), and also part of the proteins were associated with defence response processes (5 and 6 %). The processes of oxidation-reduction (7.5 %) and cell cycle (2.5 %) had the lowest score of uniqueness.

In contrast, only 9 identified proteins included in the second group were associated with the largest diversity of GO terms (33) that were summarized to include 11

processes. Biosynthesis was the most dominant process (24.4 %). Processes related to defence response had similar frequency as in the first group (5 and 6 %). Overall, the uniqueness score was high (>0.8 for three of the terms and >0.6 for all of the terms) and that suggested that all processes are closely related.

The fourth group included 13 identified proteins and 38 GO terms were assigned. This result was comparable to the first group, but number of summarized processes was larger (13). More than half of the processes were closely related in the semantic space and were associated with various aspects of defence response, such as response to stimulus (12.6 %), defence response (6 %), response to biotic stimulus (5.2 %), immune response (1.4 %), response to wounding (0.8 %) and neutralization of cellular oxidants (0.9 %). Other processes characteristic to this group included oxidation-reduction (7.5 %) and carbohydrate metabolism (4.7 %).

The String database did not include information about apple genes, therefore genes of *A. thaliana* homologous to 45 of the proteins identified this study were used for the analysis of protein interaction. Protein homologue in *A. thaliana* genome was not available for apple genome peptide MDP0000942516 that was annotated as Pru av 1 like protein.

The analysis identified significant interaction for three genes, including RNR binding motif protein, thaumatin 1A and curculin related lectin. Other links among the proteins revealed interactions involved in processes of amino acid biosynthesis and protein anabolism, cytoskeleton, gene expression and cell development, as well as functions associated with cell signaling and response to stress.

CONCLUSIONS

1. Metagenomic analysis method identified 27 endophytic bacteria families in the apple phyllosphere. Among them, the bacteria of *Proteobacteria* group (97 %) are dominant: *Rhodobacteraceae* (63 %), *Rhodobiaceae* (19 %), *Methylobacteriaceae* (8 %), *Enterobacteriaceae* (7 %) and *Pseudomonadaceae* (1.3 %). Other bacterial groups include *Firmicutes* (2.7 %), *Bacteroidetes* (0.2 %) and *Actinobacteria* (0.04 %).

2. Plant growth promoting properties are common among the culturable endophytic bacteria strains isolated from domestic apple phyllosphere: 53 % reduce nitrate to nitrite, 71 % produce siderophores, 32 % assimilate 1-aminocyclopropane-1-carboxylic acid as the main source of nitrogen; 66 % fix atmospheric nitrogen and 84 % synthesize plant growth hormone – indole-*3*-acetic acid.

3. The effect of endophytic bacteria on *in vitro* parameters of domestic apple shoot growth is not universal at the genus level. Three isolates of *Bacillus* sp. Da_1, Da_4 and Da_5, *Pantoea* sp. D_9 and *Pseudomonas* sp. Ga_1 had stimulating effect on shoot biomass accumulation and additive shoot number.

4. The endophytic bacteria that promote growth of domestic apple shoots demonstrate statistically significant suppression of lipid peroxidation from 1.5 to 3 fold in apple shoots. These bacteria upregulate jasmonic acid and ethylene phytohormone mediated gene expression, while *Bacillus* sp. Da_1, *Pantoea vagans* D_10 and *Pantoea agglomerans* D_9 strains upregulate cell responce mediated by the salicylic acid signalling pathway. Both of these signalling pathways could activate systemic induced resistance and improve adaptability of the apple shoots.

5. At the initial phase of interaction, the endophytic bacteria and apple cells form an association, which is characterized by a distinct changes in accumulation of reactive oxygen and nitrogen species – among the 38 strains used in the study, nine strains (including *Bacillus, Curtobacterium, Pantoea* and *Pseudomonas* spp.) significantly suppress accumulation of reactive oxygen and nitrogen species. For most strains, these characteristics correspond to shoots growth-enhancing properties.

6. Proteomic analysis revealed that *Bacillus* sp. Da_4 isolate inhibits the expression of genes, involved in defence response, oxidative stress regulation, as well as the catabolism of hydrogen peroxide. This corresponds to the characteristic of this strain to

stimulate the accumulation of active oxygen and nitrogen species in apple cells *in vitro*. *Bacillus* sp. Oa_4 isolate, which has inhibiting effect on accumulation of reactive oxygen and nitrogen species compounds has specific and strong stimulating effect on expression of the genes involved in amino acid biosynthesis, protein metabolism, cell cytoskeleton and has profound effect on cell development.

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1. **Miliute I**., Buzaite O., Gelvonauskiene D., Sasnauskas A., Stanys V., Baniulis D. Plant growth promoting and antagonistic properties of endophytic bacteria isolated from domestic apple. Zemdirbyste - Agriculture. 2016. 103(1):77-82
2. **Miliute I**., Buzaite O., Baniulis D., Stanys V. Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. Zemdirbyste - Agriculture. 2015. 102(4):465–478
3. Cepauskas D., **Miliute I**., Staniene G., Gelvonauskiene D., Stanys V., Jesaitis A. J., Baniulis D. Characterization of apple NADPH oxidase genes and their expression associated with oxidative stress in shoot culture *in vitro*. Plant Cell, Tissue and Organ Culture (PCTOC): Journal of Plant Biotechnology. 2015. 124: 621–633

LIST OF POSTER PRESENTATIONS

1. **Miliute I**., Cepauskas D., Haimi P., Staniene G., Baniulis D. Gel based proteome analysis of oxidative stress response in *Malus* sp. WG2 meeting of the COST FA1306. Versailles, France, 2016
2. **Miliute I**., Frercks B., Buzaite O., Staniene G., Baniulis D. Composition of endophytic bacteria in phyllosphere of domestic apple and their effect on expression of stress response genes. Plant Biology Europe EPSO/FESPB Congress. Prague, Czech Republic, 2016
3. **Miliute I**., Staniene G., Gelvonauskiene D., Baniulis D., Stanys V. Stress regulating properties of bacterial endophytes in apple (*Malus* × *domestica* Borkh.) culture *in vitro*. 1st International Conference on Scientific Actualities and Innovations in Horticulture. Kaunas, 2016
4. **Miliute I**., Cepauskas D., Haimi P., Staniene G., Baniulis D. Gel Based Proteome Analysis of Oxidative Stress Response in *Malus* sp. 10th International Scientific Conference “The Vital Nature Sign” Vilnius, 2016
5. **Miliute I**., Buzaite O., Staniene G., Stanys V., Baniulis D. Effect of bacterial endophytes on ROS/RNS production and regulation of stress response in apple (*Malus* × *domestica* Borkh.) cell suspension. 12th International Conference on

Reactive Oxygen and Nitrogen Species in Plants: from model systems to field. Italy, Verona, 2015

6. **Miliute I**., Buzaite O., Stepulaitiene I., Staniene G., Sikorskaite-Gudziuniene S., Stanys V., Baniulis D. Assessment of biochemical growth promoting and plant cell association aspects of apple endophytic microbiome. XVI International Congress on Molecular Plant-Microbe Interactions. Greece, 2014
7. **Miliute I**., Staniene G., Gelvonauskiene D., Stanys V., Baniulis D. Oxidative stress injury in apple shoot culture *in vitro*. XIII-oji Tarptautinė Lietuvos biochemikų draugijos konferencija – „50-asis FEBS jubiliejus" Birštonas, 2014
8. Cepauskas D., Baniulis D., Staniene G., **Miliute I**., Stanys V. Identification and Characterization of NADPH-oxidase Genes in Domestic Apple (*Malus* × *domestica* Borkh.). 9th International Scientific Conference "The Vital Nature Sign" Kaunas, 2015
9. Buzaite O., **Miliute I**., Gelvonauskiene D., Baniulis D. Microbial antagonism of putative bacterial endophytes from apple (*Malus x domestica* Borkh.). XIII-oji Tarptautinė Lietuvos biochemikų draugijos konferencija – „50-asis FEBS jubiliejus" Birštonas, 2014
10. Buzaite O., **Miliute I**., Gelvonauskiene D., Baniulis D. Microbial antagonism of putative bacterial endophytes from apple (*Malus x domestica* Borkh.). XVI International Congress on Molecular Plant-Microbe Interactions. Greece, 2014 July

SANTRAUKA

Tyrimų hipotezė. Tarp naminės obels lapų endofitinių bakterijų yra kamienų, pasižyminčių obels ūglių augimą reguliuojančiomis ir stresą slopinančiomis savybėmis *in vitro.*

Tyrimų tikslas – charakterizuoti naminės obels filosferos endofitinių bakterijų sistematinę sudėtį, įvertinti endofitų reguliuojantį poveikį ląstelės aktyvių deguonies bei azoto junginių gamybai ir ūglių augimui *in vitro.*

Tyrimų uždaviniai:

2. *16S rRNR* metagenominės analizės metodu charakterizuoti naminės obels filosferos endofitinių bakterijų populiaciją ir įvertinti biochemines kultivuojamų kamienų savybes.
3. Įvertinti endofitinių bakterijų poveikį naminės obels ūglių augimui ir atsakui į *in vitro* sąlygų sukeltą stresą.
4. Ištirti endofitinių bakterijų ir obels ląstelių sąveiką, būdingus ADJ/AAJ gamybos dėsningumus panaudojant *in vitro* augalų ląstelių kultūros modelį.
5. Nustatyti ADJ/AAJ gamybą reguliuojančių endofitinių bakterijų ir obels ląstelių sąveikai būdingus genų raiškos dėsningumus.

Disertacijos ginamieji teiginiai:

1. Naminės obels filosferai būdinga endofitinių bakterijų sistematinė įvairovė, ir dalis endofitų pasižymi augalų augimui reikšmingomis savybėmis.
2. Endofitinės bakterijos reguliuoja obels ūglių augimą *in vitro* ir atsaką į stresą, kuris yra susijęs su signalinių kelių tarpininkaujant fitohormonams pusiausvyra.
3. Endofitinės bakterijos susijungia su obels ląstelėmis *in vitro* ir reguliuoja ADJ/AAJ gamybą ląstelėse.
4. Skirtingomis ADJ/AAJ reguliacijos obels ląstelių suspensijoje savybėmis pasižymintys *Bacillus* spp. kamienai lemia skirtingus genų raiškos dėsningumus obels ląstelėse.

Mokslinio darbo naujumas ir praktinė reikšmė

Pirmą kartą ištirta naminės obels veislės 'Gala' filosferos endofitinių bakterijų populiacijos sistematinė sudėtis panaudojant metagenominės analizės metodą.

Išskirta ir charakterizuota naminės obels kultivuojamų endofitinių bakterijų kolekcija yra vertinga obels ir endofitų sąveikos tyrimams, perspektyvi augalų augimą reguliuojančių preparatų gamybai ar biokatyvių medžiagų paieškai.

Identifikuoti bakterinių endofitų kamienai, kurie stimuliuoja obels ūglių augimą bei slopiną oksidacinio streso pažaidą turi perspektyvą augalų stresą mažinančių priemonių kūrimui.

Lyginamosios genomikos metodu identifikuoti obels ir endofitinių bakterijų sąveikos iniciacijai reikšmingi genų raiškos pakitimai.

Tyrimo rezultatų aprobavimas

Disertacijos tyrimų rezultatai paskelbti 3 leidiniuose, referuojamuose ir turinčiuose citavimo indeksą duomenų bazėje „Clarivate Analytics Web of Science“, ir 8 tarptautinių konferencijų tezėse.

Pagrindiniai disertacijos rezultatai pristatyti trijose tarptautinėse konferencijose užsienyje: „Plant Biology Europe EPSO/FESPB Congress“ (Praha, Čekija, 2016), „XVI International Congress on Molecular Plant-Microbe Interactions“ (Rodas, Graikija, 2014), „12th International Conference on Reactive Oxygen and Nitrogen Species in Plants: from model systems to field“ (Italija, Verona, 2015), penkiose tarpautinėse konferencijose Lietuvoje: „9th International Scientific Conference “The Vital Nature Sign” (Kaunas, 2015), „10th International Scientific Conference “The Vital Nature Sign” (Vilnius, 2016), „XIIIth International Conference of Lithuanian Biochemical Society“ (Birštonas, 2014), „1st International Conference on Scientific Actualities and Innovations in Horticulture” (Kaunas, 2016), SmartBIO (Kaunas, 2017), COST FA1306 veiklos konferencijoje „Diving into integrative cell phenotyping through-omics“ (Versalis, Prancūzija, 2016).

IŠVADOS

1. Metagenominiu analizės metodu naminės obels filosferos endofitų populiacijoje identifikuotos 27 šeimoms priklausančios bakterijos, tarp kurių dominuoja *Proteobacteria* grupės bakterijos (97 %): *Rhodobacteraceae* (63 %), *Rhodobiaceae* (19 %), *Methylobacteriaceae* (8 %), *Enterobacteriaceae* (7 %) ir *Pseudomonadaceae* (1,3 %). Taip pat identifikuotos *Firmicutes* (2,7 %), *Bacteroidetes* (0,2 %) bei *Actinobacteria* (0,04 %) grupių bakterijos.

2. Daugumai kultivuojamų naminės obels filosferos endofitų būdingos augalų augimą skatinančios savybės: 53 % kamienų redukuoja nitratą; 71 % kamienų išskiria sideroforus – netiesiogiai slopina patogenus; 32 % bakterijų 1-aminociklopropano-1-karboksilatą naudoja kaip pagrindinį azoto šaltinį; 66 % kamienų fiksuoja atmosferos azotą ir 84 % bakterijų sintetina augalų augimo hormoną – 3-indolilacto rūgštį.

3. Endofitinių bakterijų poveikis naminės obels ūglių augimo *in vitro* parametrams genties lygmenyje nėra vienodas. Trys iš keturių tirtų *Bacillus* spp. Da_1, Da_4, Da_5 kamienų ir vienas *Pantoea* sp. D_9 bei vienas *Pseudomonas* sp. Ga_1 kamienai didino naminės obels ūglių biomasės prieaugį ir pridėtinių ūglių skaičių.

4. Naminės obels ūglių augimą ar pridėtinių ūglių formavimąsi *in vitro* skatinančios endofitinės bakterijos mažina oksidacinę membranos lipidų pažaidą ūglių lapuose nuo 1,5 iki 3 kartų. Šios bakterijos didina jazmonato ir etileno tarpininkaujamų signalinių kelių genų raišką, o *Bacillus* sp. Da_1, *Pantoea vagans* D_10 ir *Pantoea agglomerans* D_9 kamienai didina ir salicilato tarpininkaujamo signalinio kelio genų raišką. Abu šie keliai skatina augalų ląstelių sisteminį indukuotą atsparumą ir gali pagerinti ūglių adaptyvumą.

5. Pradiniu sąveikos etapu endofitinės bakterijos ir obels ląstelės sudaro asociaciją. Jai yra būdinga specifiška aktyvių deguonies bei azoto junginių gamybos dinamika – iš 38 tirtų kamienų, devyni (*Bacillus, Curtobacterium, Pantoea* ir *Pseudomonas* genčių) slopina aktyvių deguonies bei azoto junginių gamybą. Daugumai kamienų šie dėsningumai atitinka ūglių augimą skatinančias savybes.

6. Visuminės genų raiškos tyrimu nustatyta, kad *Bacillus* sp. Da_4 kamienas slopina su apsauginiu atsaku, oksidacinio streso reguliacija, taip pat ir vandenilio peroksido katabolizmu susijusių genų raišką, ir tai atitinka šiam kamienui būdingą savybę skatinti aktyvių deguonies ir azoto junginių kaupimą obels ląstelėse *in vitro*. Aktyvių deguonies bei azoto junginių kaupimą slopinantis *Bacillus* sp. Oa_4 kamienas didina genų, dalyvaujančių ląstelės vystymosi, baltymų apykaitos ir citoskeleto procesuose, raišką ir lemia reikšmingus obels ląstelių vystymosi pokyčius

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ENDOPHYTIC BACTERIA POPULATION STRUCTURE OF DOMESTIC APPLE AND INTERACTION WITH APPLE CELLS AND SHOOTS *IN VITRO*

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