

Microbial Diversity of *Paulownia* spp. Leaves – A New Source of Green Manure

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This study aimed to analyze the structural and functional diversity of microorganisms inhabiting *Paulownia* spp. leaves. Next Generation Sequencing (NGS) and Biolog EcoPlates were used to determine microbial diversity. The leaves of *Paulownia* spp. were taken from two different plantations. Among all the samples, P112_1 was the most abundantly colonized by plant growth promoting bacteria. Overall, the microbial community of the *P. elongata* × *P. fortunei* (PB) sample characterized the lowest metabolic activity with the utilization of the carbon sources. All communities used carbohydrates abundantly, whereas amines and amides were used the least. The differences observed may have been due to a variety of factors from composition of the chemicals in the leaf, to the soil type, to the climatic conditions.

Keyword: Short-rotation tree; Next generation sequencing (NGS); Biolog EcoPlates; Structural and functional diversity

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INTRODUCTION

In addition to a biomass source for energy production and industrial use, short-rotation tree plantations are used as a promising tool for decreasing the concentration of carbon dioxide in the atmosphere. Currently, one of the most popular trees in these plantations is the *Paulownia* spp., which is a fast growing deciduous tree that is native to China and East Asia (Tu *et al.* 2016).

The *Paulownia* spp. is a fast growing variety of deciduous tree that belongs to the Paulowniaceae family (Yadav *et al.* 2013). This tree comes from China, where it has been cultivated for more than 2000 years (Tu *et al.* 2016). The leaves of young trees are big and can measure 80-cm-wide. The tree varieties from this type of tree are extremely adaptive to wide climate and soil factor changes.

The *Paulownia* spp. is one of the few species of tree that is characterized by the presence of a C4 photosynthesis pathway. These trees are involved in regulating the climate, absorbing significant amounts of carbon dioxide, and releasing a large amount of oxygen. *Paulownia* leaves have high protein, fats, sugars, and nitrogen, phosphorus, potassium (NPK) content (Liu *et al.* 2013). The nitrogen content in the leaves is comparable to the content of legumes leaves. *Paulownia* leaves are used as animal feed and also as a green fertilizer, enriching the soil with organic matter (Popova and Baykov 2013). Due to its large amount of excreted metabolites, *Paulownia* tree species are used in the treatment of a variety of diseases in traditional medicine (Ayan *et al.* 2003).

Microbial communities in soil provide a vast array of benefits to soil fertility and ecological functionality (Łyszcz and Gałązka 2017). Microorganisms take an active part in

the decomposition of organic matter and contribute to the circulation of elements in the soil (Grządziel and Gałązka 2018). Bacteria present on the leaves of trees are also characterized by a function of plant growth and defense promoters by synthesizing phytohormones and producing biosurfactants, phyto-active volatile organic compounds, enzymes, or precursors for secondary plant metabolites. The bacteria also fixes atmospheric nitrogen and controls plant diseases. The structural and functional characteristics of the microorganisms present on *Paulownia* leaves may prove to be valuable information that can be useful for characterizing the transformation of the green leaf mass into a valuable fertilizer fertilizing the soil.

Paulownia leaves are used as green manure, particularly in China (Yadav *et al.* 2013). The 8- to 10-year-old tree produces about 100 kg green compound (leaves), which is about 2.8 to 3% N and 0.4% K (Woods 2008). Due to the constantly growing number of *Paulownia* tree plantations in Europe, it is worth analyzing the possibility of using the leaves as a green fertilizer under these conditions. The analysis should be carried out both for the content of nutritional compounds, as well as for the microorganisms inhabiting these leaves. Both diversity, as well as structure and metabolic activity of microbial communities are of particular importance for soil functioning and maintaining its biological balance (Zak *et al.* 1994). Fallen leaves may be carriers of microorganisms, including pathogenic and plant growth-promoting microorganisms. Therefore, this study aims to investigate the microbial community on leaves in order to better understand their role in the preparation of green manure. The application of green manures to soil is considered an important agricultural practice because it can improve soil physical properties by increasing soil organic matter and nutrient retention. Green manure plants are safe for the environment because they are less toxic and biodegradable compared to agrochemicals and pesticides (Tejada *et al.* 2008).

It is estimated that bacteria are most abundant on the leaves, from 10^6 cells/cm² to 10^7 cells/cm² (Whipps *et al.* 2008). Bacteria located on the leaves interact in such a way to influence host growth and development, specifically the production of phytohormones, the biocontrol of the phytopathogens, or the enhancement of minerals (Kembel *et al.* 2014). The number of studies on the phyllosphere microbial communities has been constantly growing. Contemporary research methods using new molecular tools give novel insights into the microorganisms that inhabit the leaves. It is important to understand the interactions between microorganisms, behavior, colonization, survival, identification, and biodiversity, as well as microorganism metabolic activity. Such information will be important for understanding the microbial ecology of the phyllosphere. The Biolog EcoPlates method allows a way to determine the metabolic diversity of microorganisms in environmental samples (Garland and Mills 1991; Gałązka *et al.* 2017). From the available literature, a number of research studies on the application of this technique to study the microbiome of plant samples were found (Yang *et al.* 2001; Yadav *et al.* 2008). However, there is no study available dealing with the analysis of *Paulownia* leaves using the Biolog EcoPlates method. Furthermore, next generation sequencing (NGS) enables the rapid analysis of the structural biodiversity of microbial communities in several habitats. The combination of these two methods allows for the thorough examination of the microorganisms inhabiting the *Paulownia* leaves. The purpose of this study is to determine bacterial functional and genetic diversity from leaves of the *Paulownia* spp.

EXPERIMENTAL

Materials

Sample collection

Plant samples were collected in autumn, specifically October 2016. Only healthy and mature leaves from 5-month-old trees of *Paulownia* spp. were selected. The four samples were clones of the same *Paulownia elongata* and *Paulownia fortunei* hybrids. To compare results, additional hybrids of *Paulownia tomentosa* and *Paulownia fortunei* were included. The study materials were taken from two plantations of Paulownia: 1) Podkamins, Mazowieckie Voivodeship, Poland (52° 14'N, 20° 27'E), which supplied a hybrid of two species *P. elongata* × *P. fortunei*- Paulownia *in vitro* 112 – (P112_1); 2) Otrębusy, Mazowieckie Voivodeship, Poland, (52° 07'N, 20° 45'E) that supplied the following four types of samples: a) hybrid of two species *P. elongata* × *P. fortunei*- Paulownia *in vitro* 112 – (P112_2), b) hybrid of two species *P. tomentosa* × *P. fortunei*- Paulownia Shandong- (PS), c) hybrid of two species *P. elongata* × *P. fortunei*- Paulownia Cotevisa 2- (PC), and d) a hybrid of two species *P. elongata* × *P. fortunei*- Paulownia Belissima- (PB). The experiment included three replications. The leaves were harvested randomly from several trees on a given experimental combination. Then, for the given clone, leaf samples were pooled. In each pooled sample, three replications were made for analysis. The current experiment is the first research on the assessment of *Paulownia* leaf microbiome on two selected plantations under the same climatic conditions.

All of the samples were transported in sterile plastic bags in an ice box, and they were analyzed in the authors' lab within 24 h.

Sample preparation

Each sample of plants was washed under running tap water to remove soil and dust particles and allow for drainage. The leaf samples were first washed with sterile distilled water and second washed in a phosphate buffered saline (PBS) solution. A total of 10 g of leaves were weighed from each sample and ground with a sterilized mortar and pestle. Macerated plant tissues were transferred to the sterile tubes. Extracts were suspended in 90 mL 0.85% sterile sodium chloride (NaCl) and were shaken with glass balls to break up the leaf cells (30 min, 140 rpm, 20 °C). Then, the extracts were transferred to a NaCl solution of 4 °C for 30 min. Afterwards, the extracts were diluted 10× in 0.85% sterile NaCl (Yang *et al.* 2001; Yadav *et al.* 2008).

Methods

Assessment of the functional diversity of bacteria using Biolog EcoPlate

Each well of the Biolog EcoPlates was inoculated by 120 µL of the clear supernatant. Inoculated plates were incubated in the dark at 28 °C for 160 h. The results were read every 24 h on a MicroStation ID systems by Biolog at 590 nm of absorbance (Biolog Inc., Hayward, CA, USA). The experiment included three replications ($n=3$). The most intensive metabolism of carbon substrates was observed after 72 h of incubation and, as such, this hour was chosen to analyze the results. The activities of soil microorganisms were based on all the carbon source values and on the grouped sources defined as amines and amides, amino acids, carbohydrate, carboxylic acids, and polymers.

The assessment of the diversity of microorganism communities by the NGS method

The total DNA was extracted from the extract of leaves using a FastDNA SPIN Kit (MP Biomedicals, Solon, OH, USA). The 200 µL of extract was used for DNA extraction. Amplicons of variable fragments (V3-V4) were sequenced with the NGS technique (MiSeq by Illumina, Genomed, Warsaw, Poland), which allows for the qualitative and quantitative analysis of the species' composition. Metagenomic analyses of the coding gene 16S rRNA were conducted based on the hypervariable region V3-V4 of the 16S rDNA gene. Specific primers 341F and 785R were used for amplification of chosen region and library preparation. PCR reaction was conducted with the use of Q5 Hotstart High-Fidelity DNA Polymerase kit (NEBNext) with reaction conditions according to manufacturer's specification. Sequencing was conducted on MiSeq sequencer in 2x250 bp paired – end (PE) technology using v2 Illumina chemistry kit. The sequences were subject to bioinformatics analyses, such as clustering and separation of operational taxonomic units (OTU). The 16S rDNA metagenomics protocol provides the classification of readings to the species level, and is based on the GreenGenes version 13_8 reference sequence database (LBNL, Berkeley, CA, USA), (DeSantis *et al.* 2006).

Statistical analysis

The main statistical analyses were performed using the package STATISTICA.PL (StatSoft. Inc., version 10.0, Tulsa, OK, USA). The collected data were subjected to an analysis of variance (ANOVA) for the comparison of means, and significant differences were also calculated according to post-hoc Tukey's HSD test at a $P < 0.05$ significance level. The cluster analysis methods were performed on standardized data from the average absorbance values at 72 h (Biolog EcoPlate). The average well-color development (AWCD) was evaluated according to Garland and Mills (1991). The results were also subjected to the principal component (PC) analysis to determine the common relations between the bacterial metagenome and different leaf samples.

RESULTS AND DISCUSSION**Assessment of the Functional Diversity in Microorganism Communities Using Biolog EcoPlate**

Changes in the catabolic diversity of the microbial community on leaves are presented in Fig. 1. The carbohydrates were the most utilized compound group in all of the leaf samples (Fig. 1a), and the lowest compound groups were the amines and amides (Fig. 1b).

Table 1. Catabolic Diversity of a Microbial Community of Paulownia Leaves

Species	Simpson (D)	Shannon (H')	Richness (R)	Evenness (E)	AWCD ₅₉₀
P112_1	0.982 ^b	2.979 ^a	20.33 ^b	0.990 ^a	0.755 ^b
P112_2	0.992 ^a	3.000 ^a	18.00 ^c	1.039 ^b	0.641 ^b
PB	0.998 ^a	2.890 ^b	14.67 ^c	1.078 ^b	0.441 ^c
PC	0.981 ^b	3.068 ^a	22.67 ^b	0.986 ^a	0.909 ^a
PS	0.987 ^a	3.130 ^c	23.33 ^a	0.995 ^a	0.901 ^a

Note: Evaluated by the Shannon's diversity index (H), Simpson's diversity (D), substrate richness, $OD > 0.25$ (R), substrate evenness (E), and average well-color development (AWCD₅₉₀) after being incubated in a Biolog EcoPlate for 72 h. Each of the treatment means, separated below by different letters, differed significantly (Tukey's mean separation test, $P < 0.05$, $n=3$)

The samples of PC and PS were also characterized by having the most intense metabolic activity of the indexes. The lowest metabolic activity was demonstrated by the PB sample, while the AWCD values of samples P112 were from the same clone of the same hybrid (Table 1).

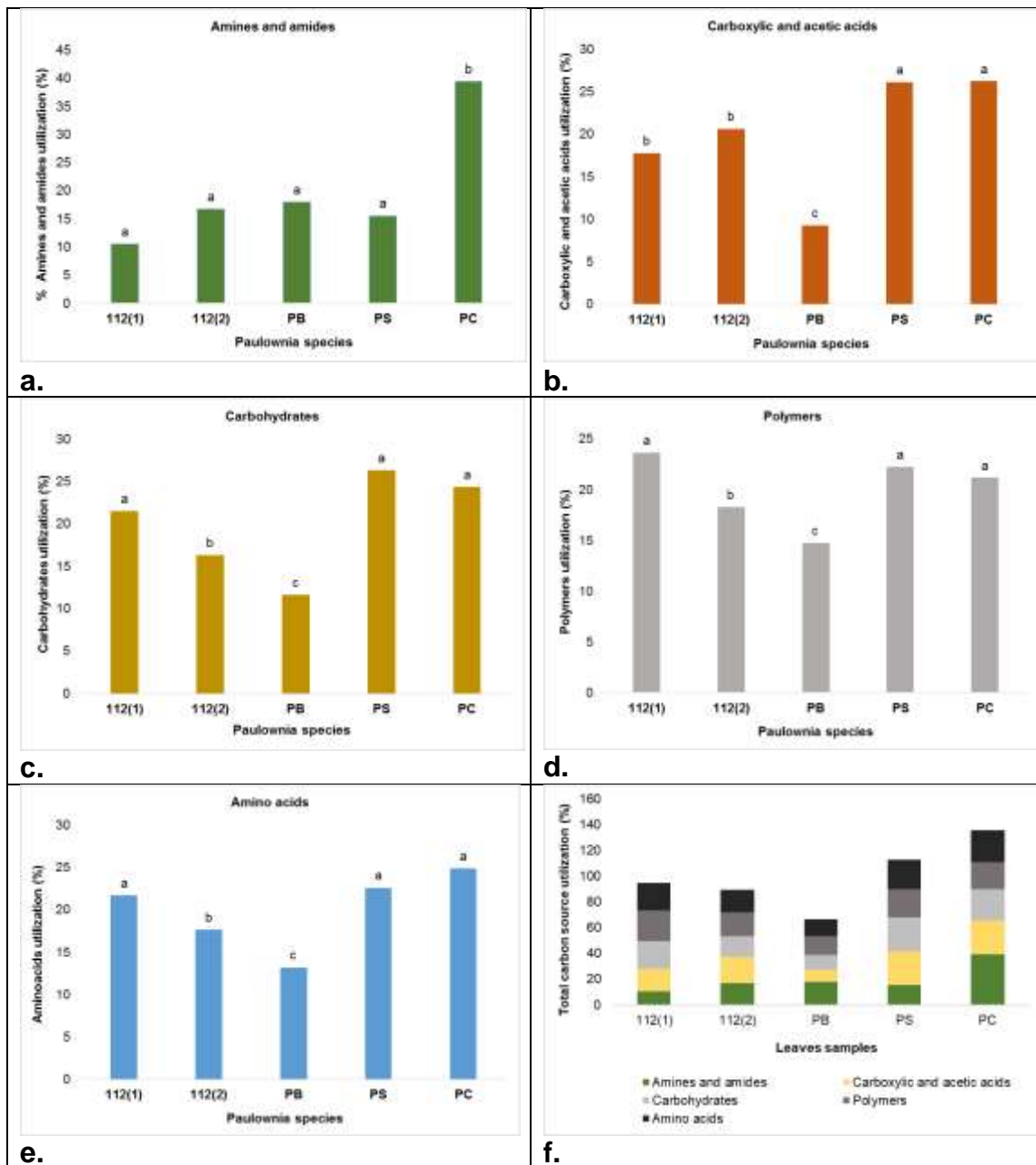


Fig. 1. Effect of different *Paulownia* spp. leaves on the microbial community catabolic diversity as evaluated by substrate utilization in the Biolog EcoPlate incubated for 72 h. The treatment means, separated by different letters were significantly different (Tukey's mean separation test, $P < 0.05$, $n=3$): a) amines and amides, b) carboxylic and acetic acids, c) carbohydrates, d) polymers, e) amino acids, and f) percent of total carbon source utilization of Paulownia leaves

Yadav *et al.* (2008) investigated the metabolic activity of the phyllosphere bacterial communities of the eight perennial species naturally occurring in a Mediterranean climate. The current study revealed that carbohydrates and amines/amides were characterized with the highest metabolic activity, while the lowest were polymers. The samples of PC and PS were characterized by the highest AWCD values and biodiversity indexes (Table 1).

The carbon substrate utilization showed that microbial populations of sample leaves PC, PS, and P112_1 exhibited an intensive and high metabolic potential. Despite the short incubation time (72 h), the microbial populations of these leaves samples showed a relatively high metabolic activity (Fig. 2).

The most intensively used substrates of the PS microbial community included the following: β -methyl D-glucoside, Tween 40, Itaconic Acid, D-Malic, D-Galactonic Acid γ - Lactone, N-acetyl-D-glucosamine, D- Xylose, α -D-Lactose, D-Mannitol, Glycyl-l-glutamic Acid, α -cyclodextrin, 2- hydroxy Benzoic Acid, γ -Hydroxy Butyric Acid, Glucose- 1 -Phosphate, and L- Threonine. The lowest utilization of all carbon sources was seen in the PC sample, where only 2-Hydroxy Benzoic Acid, L-Phenylalanine, and α -Ketobutyric Acid were utilized at the highest level.

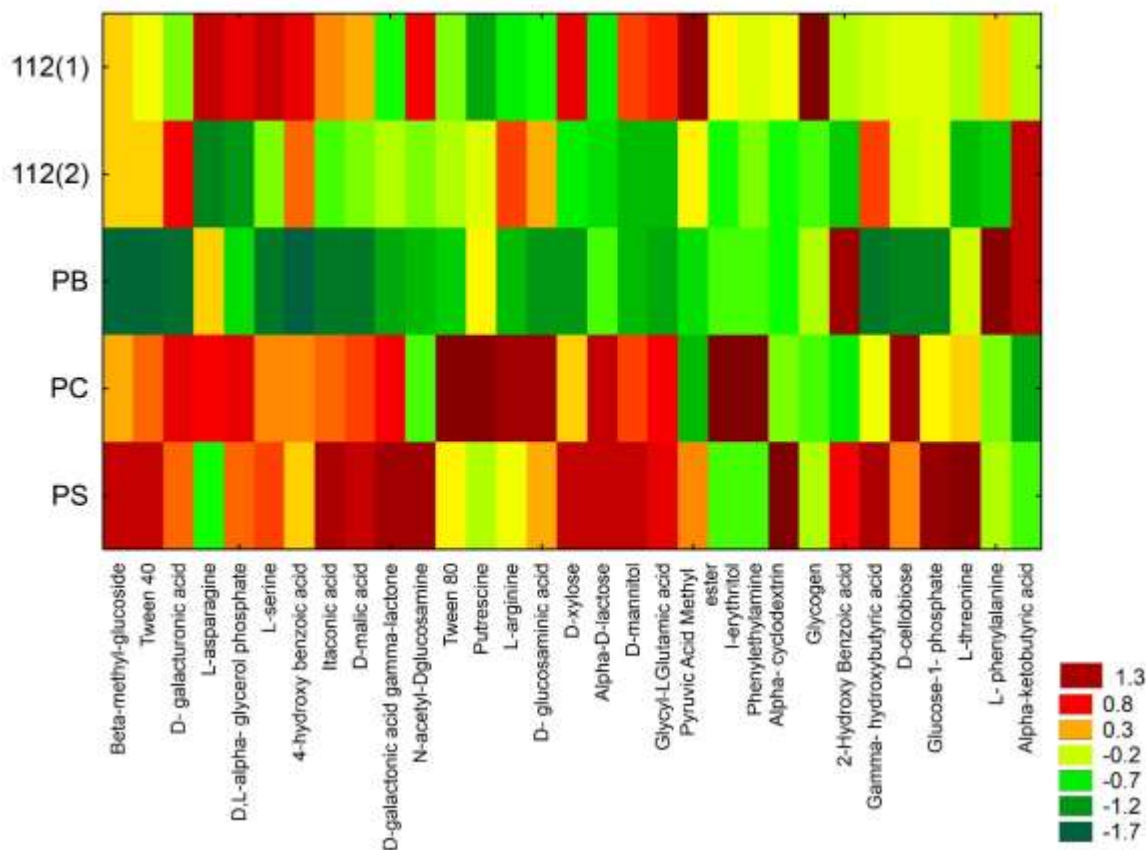


Fig. 2. Heatmap for the carbon utilization patterns of the substrates located only on the Biolog EcoPlates data of leaves samples (incubated for 72 h, n=3)

The principal component of the principal component analysis (PCA) analysis showed strong correlations between the leaf samples and biodiversity indicators. The selected indicators showcased a 97.06% biological variability (Fig. 3). Based on the PCA analysis, two major groups of leaves were indicated.

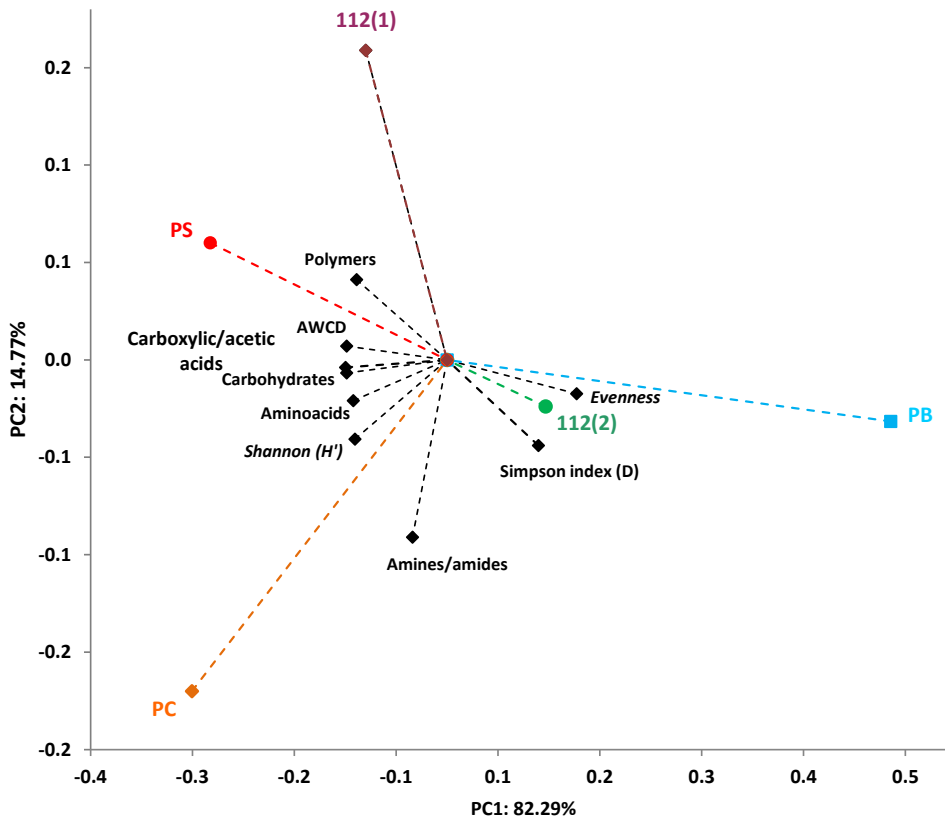


Fig. 3. PCA of microbial parameters, biodiversity indexes, and Biolog EcoPlate data of the leave samples after being incubated for 72 h

Table 2. Correlation of Carbon Source with the First (PCA1) and Second (PCA2) Components in Paulownia Leaves

Selected Carbon Source	PCA1 (53.81%)	PCA2 (21.36%)
Beta-Methyl-DGlucoside	-0.908	
D-Galactonic acid gamma-Lactone	-0.883	
L-Arginine	-0.608	0.729
D-Xylose	-0.838	
D- Galacturonic acid	-0.765	
Tween 40	-0.940	
Tween 80	-0.749	0.633
D-Mannitol	-0.890	
4-Hydroxy benzoic acid	-0.754	
L-Serine	-0.833	
Gamma- Hydroxybutyric acid	-0.779	
D- Glucosaminic acid	-0.783	0.598
Itaconic acid	-0.959	
Glycyl-LGlutamic acid	-0.899	
D-Cellobiose	-0.901	
Glucose-1- phosphate	-0.875	
Alpha-Ketobutyric acid	0.882	
Alpha-D-Lactose	-0.745	
D,L-alpha- glycerol phosphate	-0.764	
D-Malic acid	-0.988	
Putrescine		0.928

The correlations of carbon sources with the first (PCA1) and second (PCA2) components are shown in Table 2. The carbon sources that gave statistically significant correlations could be biochemical marker characteristics for different Paulownia leaves (Table 2).

The bond distances between the tested objects are presented according to the Ward/Euclidean distance method (Fig. 4). The two main groups were created. The samples, P112_1 and P112_2, and the other two samples, PS and PC, presented microbial profiles strictly related to each other. However, the PB sample was clearly different, having microbial profiles at a higher Euclidean distance (approximately 6.4).

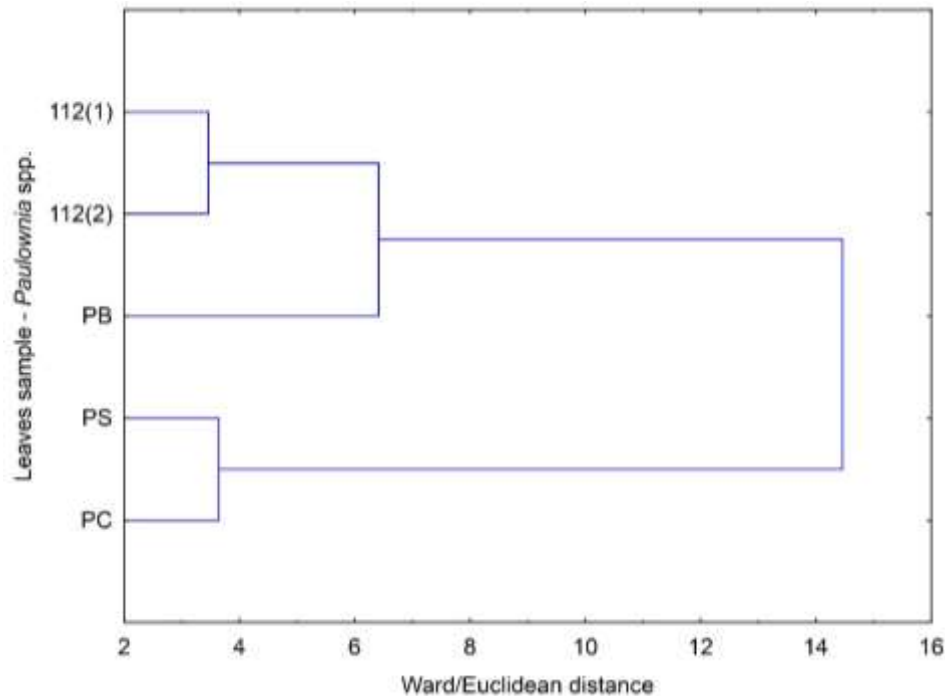


Fig. 4. Dendrogram of the bond distances between the carbon utilization patterns of the substrates located on the Biolog EcoPlates and the biodiversity indicators

Next Generation Sequencing

The Greengenes database led to the successful taxonomic assignment of only 51.99% and 44.18% of the reads to phyla and genera. An analysis of bacterial communities in samples PB, PS, and P112_2 revealed that the most dominant phylum of bacteria was Bacteroidetes (22.68% to 53.77%) (Fig. 5).

Redford *et al.* (2010) characterized the bacterial communities from leaves of 56 tree species in the United States. The study showed that Bacteroidetes represented 21.5% of the OTUs sequenced in the samples of tree species.

In contrast, the dominant phylum in the PC sample was Proteobacteria. The differences were also visible within the same clone of the hybrid P112, but from the other plantations. In the P112_2, the Bacteroidetes was dominant in contrast to the P112_1, where two phyla were dominant: Bacteroidetes and Proteobacteria. Interestingly, the PB was characterized by the absence of Firmicutes bacteria, which in other samples were at a stable level from 1.1% to 3%.

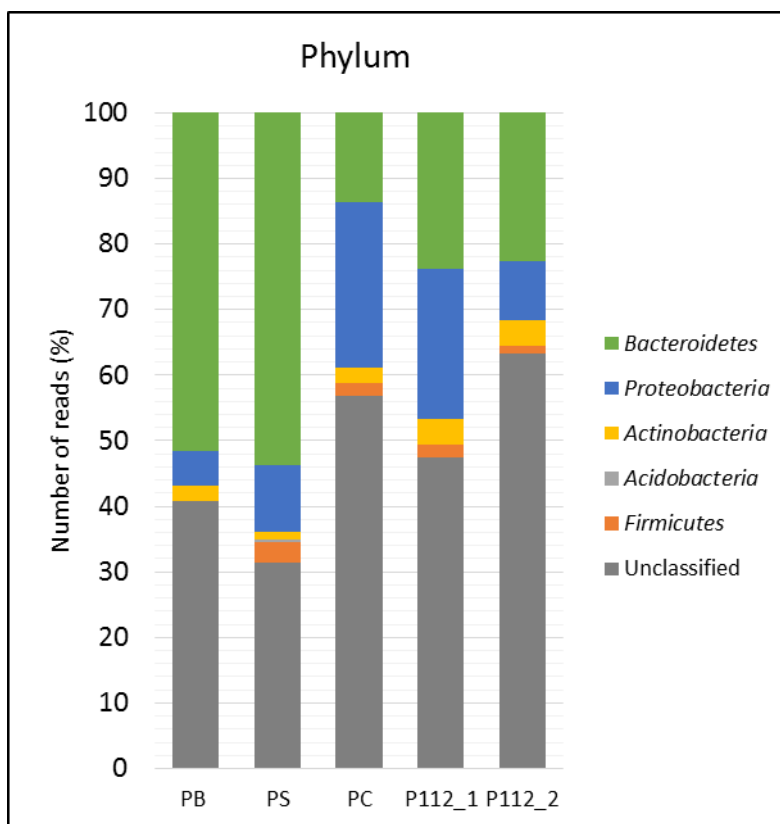
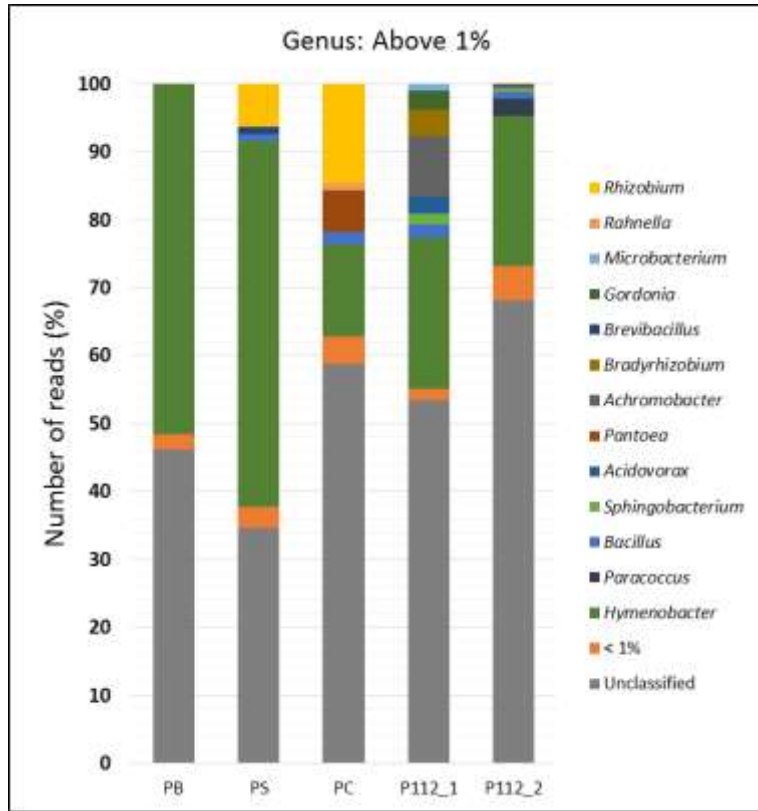
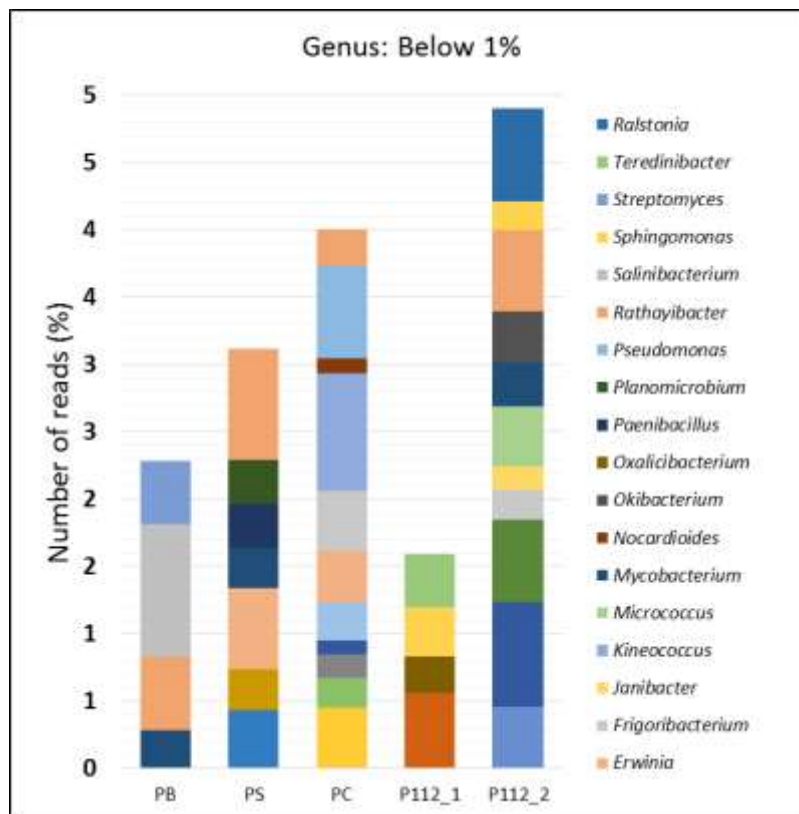


Fig. 5. Phylum-level bacterial community composition in leaves sample

At the genus level, *Hymenobacter* was the dominant genus in all samples (Fig. 6). *Hymenobacter* was isolated from entirely different backgrounds. Kim *et al.* (2008) isolated *Hymenobacter* from grass soil in the Republic of Korea, while Kojima *et al.* (2016) isolated it from a red snow obtained from Antarctica. The genus *Hymenobacter* was identified in each leaf sample independently of the hybrid and the Paulownia clone and independently of the environment. The *Hymenobacter* bacteria are most likely characteristic of the Paulownia leaves and thus represent the core microbiome of the Paulownia leaf. The PS was dominated by the genus: *Hymenobacter* (53.78%), *Rhizobium* (6.31%), and *Brevibacillus* (1.09%) (Fig. 6a). Members of this family cover a highly diverse spectrum of bacteria that are assumed to be involved in the plant growth promoting bacteria (PGPB). This bacterium is known for its capacity for nitrogen fixation, solubilize P, and production of phytohormones, siderophores, and antibiotics. The dominant genera from the PC leaves were: *Rhizobium* (14.69%), *Pantoea* (6.09%), *Bacillus* (1.84%), and *Rahnella* (1.01%). The numbers of previous scientific data show that these bacteria also have the capacity to promote plant growth (Huo *et al.* 2012). The sample P112_1 was characterized by such genera as: *Achromobacter* (8.86%), *Bradyrhizobium* (3.78%), *Gordonia* (2.80%), *Acidovorax* (2.63%), *Bacillus* (1.91%), and *Sphingobacterium* (1.57%) (Fig. 6a). In turn, only two types of bacteria, namely *Paracoccus* (2.6%) and *Bacillus* (1.08%), dominated in the P112_2 sample. The *Paracoccus* species exhibited a biodegradation ability of potentially hazardous compounds (Nisha *et al.* 2016). The highest diversity of genera occurred below 1% of the abundance and was characterized by the sample 112_2 (Fig. 6b).



a



b

Fig. 6. Community composition of bacteria of leaves samples for the genus level: a) the genus classifications with more than 1% abundance, and b) genus classifications below 1% abundance

A metagenomics analysis showed that most bacteria colonizing *Paulownia* leaves were classified to PGPB. These bacteria probably can contribute to the fast growth of the tree and the large size of leaves. Searching for PGPB in *Paulownia* leaves can further increase their potential as a green soil-enriching fertilizer. The chemical composition of the leaves, combined with the microorganisms inhabiting these leaves, allows for the assumption that they will be a good fertilizer (Huo *et al.* 2012). Qualitative features of the leaf chemistry and soil type could contribute in explaining the differences observed. Further research is needed to determine the fungal community inhabiting leaves of *Paulownia* spp. when regarding metagenomics, as well as metabolic analysis. Additionally, it is worth looking closer at the physicochemical and biological properties of the soil. All of the analyses are included in the project and will be performed by the authors' research team.

CONCLUSIONS

1. The carbon substrate utilization showed that microbial populations of sample leaves PC, PS, and P112_1 exhibited an intensive and high metabolic potential. The carbohydrates were the most utilized compound group in all of the leaves samples and the lowest compound groups were the amines and amides.
2. The samples of PC and PS were characterized by the highest AWCD values and biodiversity and were also characterized by having the most intense metabolic activity of the indexes. The lowest metabolic activity was demonstrated by the PB sample.
3. Comparisons of carbon source utilization and the diversity indices showed differences in the microbial community of composition.
4. The samples from the plantation established in Otrębusy revealed that the most dominant phylum of bacteria was Bacteroidetes and Proteobacteria.
5. A metagenomics analysis showed that most bacteria colonizing *Paulownia* leaves were classified to PGPB. In general, the microbial communities of the PB samples were characterized by the lowest Shannon–Weaver (H) diversity index in both metagenomics as well as the metabolic analysis.

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