Rhizosphere bacteriome of the medicinal plant *Sapindus saponaria* L. revealed by pyrosequencing

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ABSTRACT. *Sapindus saponaria* L. of Sapindaceae family is popularly known as soldier soap and is found in Central and South America. A study of such medicinal plants might reveal a more complex diversity of microorganisms as compared to non-medicinal plants, considering their metabolic potential and the chemical communication between their natural microbiota. Rhizosphere is a highly diverse microbial habitat with respect to both the diversity of species and the size of the community. Rhizosphere bacteriome associated with medicinal plant *S. saponaria* is still poorly known.

The objective of this study was to assess the rhizosphere microbiome of the medicinal plant *S. saponaria* using pyrosequencing, a culture-independent approach that is increasingly being used to estimate the number of bacterial species present in different environments. In their rhizosphere microbiome, 26 phyla were identified from 5089 sequences of 16S rRNA gene, with a predominance of Actinobacteria (33.54%), Acidobacteria (22.62%), and Proteobacteria (24.72%). The rarefaction curve showed a linear increase, with 2660 operational taxonomic units at 3% distance sequence dissimilarity, indicating that the rhizosphere microbiome associated with *S. saponaria* was highly diverse with groups of bacteria important for soil management, which could be further exploited for agricultural and biotechnological purposes.

Key words: Pyrosequencing; Rhizosphere; Diversity; Culture-independent approach; *Sapindus saponaria* L.

INTRODUCTION

In Brazil, biotechnological research has targeted the diversity of microorganisms because of the potential for discovery of new bioactive compounds or bioprocesses (Schulz and Boyle, 2005; de Oliveira Costa et al., 2012; García et al., 2012b; Rhoden et al., 2012; Miguel et al., 2013; Polonio et al., 2015). Studies on microbiota associated with plants are important to understand and predict the spatial distribution of microbial communities and the ecosystem responses to global climate change (Singh et al., 2010). Most plant-microbe community relationships studied deal with plants that are economically important or have medicinal properties.

Sapindus saponaria L., belonging to the Sapindaceae family, is popularly known as soldier soap. The *Sapindus* species extracts have antimicrobial, spermicidal, antiulcer, antifungal, and anti-inflammatory properties (Pelegrini et al., 2008). Studies by Garcia et al. (2012a) demonstrated the antimicrobial activity of secondary metabolites from endophytic fungi isolated from *S. saponaria*.

Rhizosphere is a highly diverse microbial habitat in terms of both the diversity of species and the size of the community. Even though bacteria are the most abundant soil microorganisms (Gans et al., 2005; Pelczar et al., 2011), only a fraction of them can be cultivated in the laboratory, and there are many unidentified bacterial species in the soil (Gans et al., 2005; Schloss and Handelsman, 2006).

Cultivation-independent methods, such as pyrosequencing, for the study of microbial diversity are becoming increasingly popular for the estimation of the number of bacterial species present in different environments. The high performance and low cost of pyrosequencing allow a comprehensive assessment of the diversity of bacteria in the soil (Roesch et al., 2007). In addition, the 16S rRNA gene is an important molecular tool in this process, assisting in the identification and study of bacterial communities.

The complexity and specificity of rhizosphere habitat is still poorly known. Therefore, the objective of this study was to analyze the microbiome of *S. saponaria* rhizosphere.

MATERIAL AND METHODS

S. saponaria

The *S. saponaria* tree studied in this study was located in the campus of Universitdade Estadual de Maringá, Maringá, State of Paraná, Brazil (23°24'22.7''S; 51°56'28.5''W). The samples used for analyses were collected in December 2013.

Physicochemical composition of *S. saponaria* rhizosphere

The physicochemical analysis of *S. saponaria* rhizosphere samples was conducted by Laboratório Rural de Maringá, Maringá, Brazil.

Total DNA extraction from *S. saponaria* rhizospheric soil

Considering the definition of rhizosphere (Morgan and Whipps, 2001) and the size of adult trees and their roots, the samples of *S. saponaria* rhizospheric soil were collected from four points at a distance of 50 cm from the tree trunk, near the roots. Soil samples were collected from an average depth of 10 cm with a Dutch auger and were sieved to remove lumps, roots, and leaves. The samples were then mixed and total DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer protocol.

Analysis of rhizosphere microbiome via pyrosequencing

The quantity and quality of the pooled DNA extracted from the soil was assessed on 1% agarose gel. Bacterial diversity was assessed using the 16S rRNA gene, which was amplified using primers 968F (Nielsen et al., 1999) and 1378R. PCR was performed on a final volume of 50 μL, containing 1X enzyme buffer, 5 mM $MgCl_2$, 10 mM dNTPs, 0.1 M each primer, and 2U Taq DNA polymerase. The amplification was performed in a thermal cycler, with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 40 s, and a final extension at 72°C for 10 min. The PCR products were assessed using 1% agarose gel electrophoresis.

The PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK), following the manufacturer protocol. The purity and quantity of the amplicons were checked on 1% agarose gel. The PCR products of each treatment were mixed proportionally and were sequenced by a 454 Sequencing System (Roche Applied Science, Branford, CT, USA), using the pyrosequencing method.

Analysis of pyrosequencing data

DNA sequences were selected based on their quality and size using the Pipeline Initial Processing tool available in the Ribosomal Database Project (RDP) Pipeline. Selection was based on the quality of bases, size of the fragments, identified sequences of the primers, and presence of unidentified bases. In order to obtain a set of sequences with satisfactory quality for analysis, only the sequences with scores greater than 20 and with bases having maximum probability of 1% error were used. Sequences with unidentified bases and sequences smaller

than 380 bp were excluded. There was a 20% tolerance range for similarity with primers (without the adapter sequences). Thus, the adapter sequences were removed from the primer-flanking region, eliminating the risk of truncated sequences reducing the quality and interfering with the classification of the sequences.

Sequences were classified and species accumulation (thinning) was analyzed using the software Mothur v.1.34.4, which performs a similarity analysis against the RDP database containing tested gene sequences of 16S rRNA that have been previously evaluated for quality. The search parameters were based on similarity, with higher similarity resulting in the identification of the lower taxonomic levels. Analysis of sequence similarity was performed according to the recommended system: domain, >0%; phylum, >75%; class, >85%; order, >91%; family, >92%; genus, >95%; and species, >97%.

RESULTS

Rhizosphere bacteriome

Totally, 5367 bacterial 16S rRNA gene sequences were identified in the rhizosphere of *S. saponaria*, through pyrosequencing. After screening via RDP Pipeline, we obtained 5089 sequences.

On comparison of these sequences against RDP database, 26 phyla were identified (Figure 1), with a predominance of Actinobacteria (33.54%), Acidobacteria (22.62%), and Proteobacteria (24.72%). Other phyla were identified at rates below 6% of the total number of sequences. Within the phylum Actinobacteria, the orders Actinomycetales (18.04%), Solirubrobacterales (12.1%), Acidimicrobiales (2.77%), and five other orders at rates less than 1% of the total number of sequences were observed. Among the Acidobacteria, 19.69% of the sequences were divided into 12 taxonomic groups classified only at the phylum level, whereas 2.93% were classified at the order level. Proteobacteria were distributed into 33 orders, of which Rhizobiales (6.07%), Myxococcales (4.68%), Syntrophobacterales (3.42%), Rhodospirillales (1.59%), Desulfuromonadales (1.40%), and Xanthomonadales (1.20%) were most prominent.

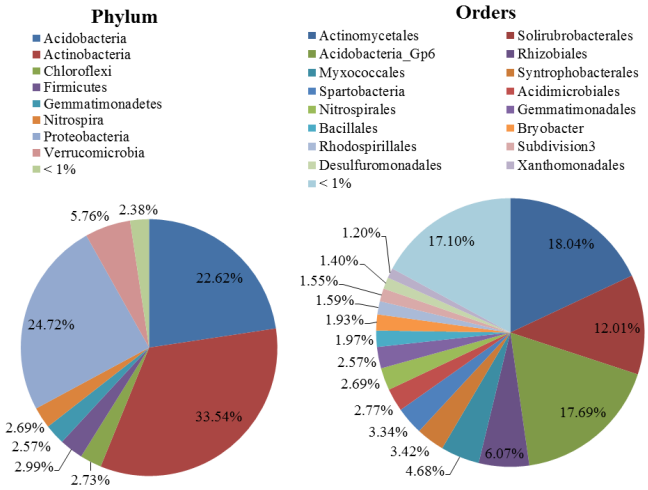

Figure 1. Taxonomic distribution of soil bacterial communities associated with the plant *Sapindus saponaria* L., at the phylum and order levels.

In the soil microbiome, we found nine sequences belonging to the class Gammaproteobacteria. Other phyllum, besides Proteobacteria, were present at a rate less than 1% in the soil microbiome.

Among the Firmicutes, we obtained 100 sequences (1.97%) belonging to the order Bacillales, whereas 0.22% Lactobacillales were present. Among Bacteroidetes, the order Flavobacteriales presented only four sequences.

Figure 2 shows a rarefaction curve depicting linear increase in bacterial diversity, measured by the richness of operational taxonomic units (OTUs) at a taxonomic level of 97% identity, based on the number of sequences obtained via pyrosequencing (Table 1). At this level of identity, OTUs would be formed by closely related species with similar phenotypic properties (Keswani and Whitman, 2001; Qi et al., 2012). Statistical analyses were performed and Shannon index, Simpson index, and Chao1 estimator were calculated to estimate the biodiversity of microbes in the sample (Table 1).

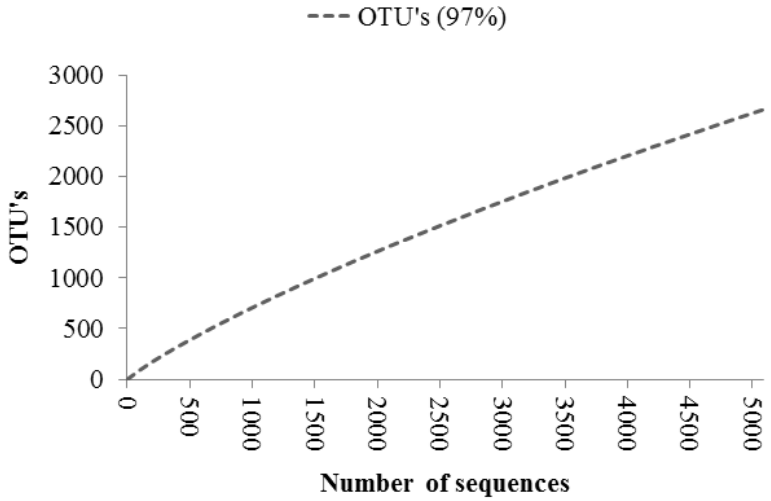

Figure 2. Rarefaction analysis of *Sapindus saponaria* rhizosphere. Rarefaction curve was constructed using the Mothur software and shows OTUs with differences not exceeding 3%.

Table 1. Biodiversity indexes for *Sapindus saponaria* L. rhizosphere microbiome.

Index	Order (91%)	Genus (95%)	Species (97%)
Simpson	0.0098	0.0049	0.0027
Shannon	6.013	6.8126	7.237
Chao1	4999.887	8587.804	10,790.46
OTUs	1563	2209	2660

Chemical composition of the *S. saponaria* rhizosphere

The analyses of macro- and micro-nutrient contents of the *S. saponaria* rhizosphere are demonstrated in Table 2.

Table 2. Physicochemical properties of rhizospheric soil associated with *Sapindus saponaria* L.

Parameter	Result
Soil acidity	
pH in calcium chloride	5.70
pH in water	6.45
pH in SMP buffer	6.73
Total acidity [H^+] ($cmol_c/dm^3$)	2.90
Physical composition of the soil	
Sand (%)	37.00
Clay (%)	56.00
Silt (%)	7.00
Macronutrients and micronutrients	
Organic matter (g/dm^3)	29.11
Carbon (g/dm^3)	16.89
Phosphorus (mgP/dm^3)	9.87
Potassium ($cmol_c/dm^3$)	0.47
Calcium + magnesium ($cmol_c/dm^3$)	5.67
Calcium ($cmol_c/dm^3$)	4.38
Magnesium ($cmol_c/dm^3$)	1.30
Hydrogen + aluminum ($cmol_c/dm^3$)	2.90
Aluminum	-
Sulfur (mg/dm^3)	3.58
Copper (mg/dm^3)	9.05
Zinc (mg/dm^3)	5.40
Iron (mg/dm^3)	30.50
Manganese (mg/dm^3)	78.50
Sodium (mg/dm^3)	2.15
Boron (mg/dm^3)	0.11
Nitrogen	-
Chlorine	-
Other components	
Crude protein	-

DISCUSSION

Rhizosphere is a microbial ecosystem occupied by specific microorganisms. Thus, the study of its microbial diversity is of utmost importance to assess the predominant genera and to understand the possible ecological relationships between plant hosts, endophytes, and the soil microbiome.

The complex interaction between these environments and their microorganisms is still unknown. Mendes et al. (2011) showed that microbial communities in the soil could influence plant immunity against root pathogens. They demonstrated that members of Proteobacteria have the ability to suppress the activity of *Rhizoctonia solani*, an important pathogen of various crops, such as sugar beet, potatoes, and rice, and that plants could benefit directly or indirectly from the ability of these bacteria to control plant pathogens.

We investigated the rhizosphere bacterial community associated with the medicinal plant *S. saponaria*. The soil examined harbored a diverse community of bacteria. We also analyzed the chemical composition of the soil to investigate whether environmental factors, such as macro- and micro-nutrients or pH, could explain bacterial richness in the rhizosphere. According to García-Salamanca et al. (2013), the taxonomical and functional structures of soil microbial communities are influenced by biotic and abiotic factors, including the physicochemical characteristics of the soil and presence of plants. The authors pointed out that plants exert selective pressure on the soil microbial population through modification

of the physicochemical characteristics of the surrounding soil and by the excretion of exudates consisting of amino acids, organic acids, proteins, and other chemicals that act as chemoattractants or chemorepellents. The values obtained in our samples were consistent with those from normal agricultural soil. The soil microbial biomass carbon and nitrogen, and nitrification, are also significantly positively correlated to the soil pH (Kemmitt et al., 2006).

The index values for the 2660 OTUs of *S. saponaria* rhizosphere, at 3% distance sequence dissimilarity, were much higher than the values obtained by García-Salamanca et al. (2013), who studied the bacterial diversity of maize rhizosphere. They obtained less than 60 OTUs from a relatively high pH, carbonate-rich soil typical of Southern Spain. The pH of *S. saponaria* rhizosphere was 6.45, which was less than that of maize rhizosphere from Spain (pH 8.5). However, other *S. saponaria* and maize rhizosphere characteristics, such as sand (37.00 and 37.02%, respectively) and clay texture (56.00 and 31.30%, respectively), were similar. The number of microorganisms in the soil depends on environmental factors, such as the amount and type of nutrients, moisture content, degree of aeration, temperature, and pH. Roesch et al. (2007) analyzed different types of soil from Brazil, USA, and Canada using pyrosequencing. They observed that the diversity of bacteria in the forest soil was higher than that in the agricultural soil. They highlighted pH as an important factor influencing this diversity. Similarly, Nacke et al. (2011) evaluated the bacterial community structure of different types of soil from forests and grasslands of Germany and concluded that the bacterial community composition and diversity, in the six types of soil environment, presented significant differences between the types of land use and forest. Furthermore, the structure of bacterial communities was largely dependent on the tree species and soil pH.

Statistical diversity indexes, such as Shannon index, Simpson index, and Chao estimator, were calculated. Chao1 estimator value for *S. saponaria* rhizosphere was 10,790.46. The Shannon and Simpson indexes were 7.237 and 0.0027, respectively, for the *Sapindus* rhizosphere (present work) and 3.42 and 0.059, respectively, for maize rhizosphere (García-Salamanca et al., 2013), indicating that the bacterial community in the *S. saponaria* rhizosphere was more complex than the maize rhizosphere. Similar to the observations of García-Salamanca et al. (2013), the most predominant 16S rRNA gene sequences in the *S. saponaria* rhizosphere were those of Actinobacteria, Acidobacteria, and Proteobacteria.

The bacterial community within a plant is prone to the influences of host genotype and changes in the host physiology (Hallmann and Berg, 2006). In the soil environment, most bacterial species are heterotrophic and sporulated bacilli are common. Species of *Bacillus*, *Clostridium*, *Arthrobacter*, *Pseudomonas*, *Rhizobium*, *Azotobacter*, and *Nitrobacter* are usually present in the soil. Actinomycetes bacteria, including species of *Nocardia*, *Streptomyces*, and *Micromonospora* are present in hot, dry soil. These bacteria can degrade many complex substances and are important for improving the soil fertility (Pelczar et al., 2011). Furthermore, they are known for their ability to produce antibiotics. In this study, the actinomycetes were the most frequent group observed in the *S. saponaria* rhizosphere.

Johnston-Monje et al. (2016) studied the bacterial populations in juvenile maize rhizospheres, originating from both seed and soil, by 16S rDNA fingerprinting and next-generation sequencing. They concluded that maize rhizospheres receive diverse bacteria from soil. Additionally, the rhizospheres are influenced by the genotype or treatment of the seed and are dominated by species of Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes. As many dominant 16S rDNA sequences were observed in the rhizospheres grown in both sterile and non-sterile substrate, Johnston-Monje et al. (2016) also concluded that most common

bacteria in juvenile maize rhizospheres are transmitted by the seeds.

According to Winston et al. (2014), understanding the relationship between microbes and *Cannabis*, a medicinally and economically important crop, can affect its agricultural practices, improving the ability of the plant to grow. They presented the first description of the endorhiza-, rhizosphere-, and bulk soil-associated microbiome of five distinct *Cannabis* cultivars using 16S rRNA gene sequencing. The bacterial community of the endorhiza is significantly cultivar-specific. The control of cultivar and soil type showed that microbial community structure differs significantly between plant cultivars, soil types, and among the endorhiza, rhizosphere, and soil. Comparing the non-rhizospheric and rhizospheric soil, the authors showed that there is a preference for bacterial communities in the rhizosphere to reduce the abundance of Acidobacteria, associated with an increase in Proteobacteria and Actinobacteria numbers. The Actinobacteria can produce various secondary metabolites with antibiotic properties (Silva-Lacerda et al., 2016), which can be important to balance the microbiome.

The highly diverse microbiome of *S. saponaria* could be even more diverse, considering the fact that fungi were not evaluated. The high complexity network communication between bacteria, fungi, and plants may influence the composition of the microbiome. In addition, considering that the rarefaction analysis showed linear increase in the bacterial diversity, a higher number of reads from pyrosequencing analysis of the *S. saponaria* rhizosphere microbiome could provide more information about the rare or less prominent bacteria.

CONCLUSION

Thus, for the first time, bacterial taxonomic composition of the *S. saponaria* rhizosphere was described using pyrosequencing. The soil associated with the medicinal plant *S. saponaria* supported a high diversity of bacteria, considering the rarefaction analysis, which indicated that soil microbial diversity was even greater than measured.

Conflicts of interest

The authors declare no conflict of interest.

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