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Polycyclic Aromatic Hydrocarbons Effect on the phyllosphere bacterial community of *Gliricidia sepium* leaves

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***Abstract*—** *Plants and microorganisms can coexist in such a way that each of these two heterospecific organisms benefit from this association. In the environment of plants there are several habitats of bacteria among them the phyllosphere which is the aerial part of the plant. The phyllosphere can be influenced by several factors including hydrocarbons. Thus, polycyclic aromatic hydrocarbons (PAHs) have been used to assess their influence on the phyllosphere microorganisms of the leaves of Gliricidia sepium. The results showed that the atmospheric concentrations of PAHs are rather high in rural areas. The spatial patterns of atmospheric concentrations of PAHs showed higher concentrations of naphthalene in the two experimental group due to the high road traffic. In the different experimental groups, 93626 and 96954 OTUs were identified in the leaves collected on the road (SR) and out of the road (SH), respectively. In this present study, the leaves harvested on the road which are more exposed to PAHs present a strongly elevated relative abundance of Actinobacteria and Bacilli. It can therefore easily deduce that these bacteria could have developed a kind of resistance to these road PAHs. On the other hand, bacteria belonging to the Alphaproteobacteria class are significantly less represented in this rural area.*

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I. INTRODUCTION

Plant-microorganism interaction is a very interesting and well-studied subject in the world of science. Plants and microorganisms can cohabit in such a way that each of these two heterospecific organisms benefit from this association. It can be encountered in the environment of plants, several microorganisms such as bacteria, fungi, archaea and protozoa that can live inside, outside the plants or close to the plants roots. Therefore it can be distinguished the rhizosphere which is the zone of the soil close to the plants roots where the microorganisms are concentrated. This region is characterized by its microbial diversity, and in particular its bacterial richness and microscopic fungi (Asemoloye et al. 2017). This zone is the privileged place for exchanges between these microorganisms and plants. The endosphere however, is an internal tissue of any plant occupied by certain microbiomes, while the phyllosphere is the aerial part of plants, constituting an environment largely inhabited by bacteria (Fatima and Senthil-Kumar 2015; Fester et al. 2014). The phyllosphere can be subdivided into caulosphere (stems), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits) (Morris 2001), thus designating the community of microorganisms living in a symbiotic relationship with plants. The phyllosphere is a complex and relatively unknown world of microbes interacting with each other and with host plants. Studies of the rhizosphere are much more advanced than those of the phyllosphere. However, quite a large number of the phyllosphere reports are reported recently due to the massive production of data resulting from the use of omics and related technique. This has enhanced a significant advance in the understanding of microbial dynamics in the aerial organs of plants, mainly in the leaves. Although the nutrient content on the phyllosphere is poor, plants release an adequate concentration to support large microbial communities (Lindow and Brandl 2003), and microbial communities develop mechanisms to acquire other nutrients (Abdullah et al. 2020). The microorganisms benefit from the plant's carbon intake and play a protective role for this plant.

Recent scientific discoveries and numerous studies nowadays focus on different microorganisms for various scientific uses ranging from phylloremediation and biodegradation of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) (Wei et al. 2017), pest control (Tripathi et al. 2020), the invasion of pathogenic microorganisms on plants in general and leaves in particular (Wang et al. 2019), services for agriculture (Zhang et al. 2019b) and forestry, etc. Thus, after the soil, the phyllosphere ranks second as the habitat containing the greatest concentration of microorganisms on earth. Indeed, the leaf area of terrestrial plants is estimated at more than 6.4*108 Km² (Izuno et al. 2016). Given that the bacterial density on the leaf surface reaches 10^6-10^7 cells per cm² (Zhang et al. 2019b), the phyllosphere remains an indisputable habitat for different types of microorganisms.

Among the most prevalent and persistent contaminants, PAHs have attracted increasing attention following their carcinogenic effects on humans (Cabrerizo et al. 2011). PAHs are a ubiquitous group of organic pollutants, composed of two or more single or fused aromatic rings. They arise from both biological processes and by-products of incomplete combustion from natural combustion sources or caused by man-made sources (Kweon et al. 2014; Primost et al. 2018; Cristaldi et al. 2017). PAHs are therefore classified into 3 types: (1) Pyrogenic PAHs, formed by organic substances exposed to high temperature under conditions of low oxygen or no oxygen. (2) petrogenic PAHs formed during the maturation of crude oils and similar processes and (3) biological PAHs formed by biological processes (Keir et al. 2020). The biological approach, based on the capabilities of microorganisms with the necessary assets to degrade and/or detoxify/or biotransform organic contaminants, has proven to be the most recommended technology, due to the advantages without secondary pollution, their versatility and their environmentally friendly treatment (Morillo and Villaverde 2017). However, the adverse effects of PAHs are not only observed on humans but even microorganisms in the air and soil are not spared.

Our present study joins recent efforts to assess the impact of PAHs on leaf phyllosphere bacteria. The aim of our study is to (i) identify the major different PAHs released following road traffic in Moroni, (ii) analyze the phyllosphere bacterial population of the leaves of Gliricidia sepium and (iii) establish a correlation between the abundance of the bacteria phyllosphere with the PAHs identified in the study area. To do this, samples of the leaves of the Gliricida sepium plant were collected on the road and off the road to identify the different PAHs and the bacterial community found there.

. Cependant, les effets néfastes des HAP ne sont pas seulement observés sur les humains mais même les macros et microorganismes de l'air et du sol ne sont pas épargnés.

II. MATERIALS ET METHODS

1- Design and collection of samples

The leaves of *Gliricidia sepium* were collected on the Corniche road, Moroni, Comoros (longitude:

11°41'33'S, latitude: 43°15'08'E and altitude: 0m). The leaves sample were collected along the road (1 m from the road) and away from the road in the same area designated as SR and SH respectively. In each branch where the leaves were collected, we considered three levels which were: basal, noted Ni-1, middle (Ni-2) and apical noted Ni-3 where i can be 1, 2 or 3 depending on the case and N can be SH or SR. The leaves were collected with scissors sterilized with 70% ethanol on the spot. Sixty healthy and mature green leaves were collected at 1.5-2 m height. They were then sealed in 500 ml plastic tubes and brought to the laboratory. After collection, the leaf samples were divided into two groups; the first was used for bacterial experimentation and the second for the determination of PAHs. Two empty tubes without leaves were considered as control and marked CR1 and CR2.

2- Determination of the different PAHs on the leaves

To assess the concentration of different PAHs present on the leaves of *Gliricidia sepium*, the leaves of the plant were treated with dichloromethane as an extract and analyzed in high performance liquid chromatography (HPLC) as described in (Wang et al. 2016).

3- Extraction of bacteria from the leaves

At the Laboratory of Animal Biology, Faculty of Science and Technology, University of Comoros, leaves collected from the field were used to extract the bacterial phyllosphere content on the leaf surfaces of *G. sepium*. The leaves were transferred to 500 ml bottles containing sterile water (autoclaved), to suspend the bacteria from the leaf phyllosphere. The sample was alternately manually shaken for ten minutes four times. The leaves were then removed and the solution was used as an extract of phyllosphere bacteria and transferred to small tubes.

4- DNA extraction and amplification

Total genomic DNA from the different samples was extracted using an Ultra-Clean Microbial DNA Isolation Kit (Morio Laboratories, Carlsbad, CA, USA). Polymerase chain reaction (PCR) and amplification of 16S rRNA genes from the V3-V4 region of each sample was performed as described in (Huang et al. 2014), using the universal primers 338F (5' -ACTCCTACGGGAGGCAGCAG-3') and 806R (5'GGACTACHVGGGTWTCTAAT3'). The extracted DNA was sent to Sangon Biotec Institute (SBI) in Shanghai, China, for sequencing. DNA concentrations and purity were measured using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, USA.

5- Bioinformatics analysis

Deduplication and filter quantification of raw fastq files, sequence classification, annotation, and calculation of beta-diversity distance were performed using Quantitative Insights Into Microbial Ecology (QIIME Version 1.9.). The UPARSE software (version 7.0.1001) was then used to group the filtered sequences of the Operational Taxonomic Units (OTU) with a similarity threshold of 97%. At 97% confidence level, the taxonomy of each 16S RNA gene sequence was analyzed using the 16S rRNA Database and RDP Classifier (version 2.12). The distance matrix and similarity or difference in sample community composition was performed using UniFrac in QIIME Version 1.9.01.

6- Statistical analyzes

Physical and chemical data were subjected to statistical analysis of variance (ANOVA) in SPSS software (20). Differences between the means of multiple samples were made using the Duncan post-hoc with a confidence level of 95%. The Shannon index was calculated to describe the diversity and richness of the microbiota present. Various graphs were performed by using Origin pro software.

III. RESULTS

1- The different PAHs identified on the leaves of *G. sepium*

Table 1 below contains the concentrations of PAHs recording to the two experimental groups (SR and SH). 20 hydrocarbons were detected, and their concentrations vary depending on where the leaves were collected. Among the 20 PAHs identified, the concentration was significantly high on the road area (CR1, SR) compared to that recollected out of the road (CR2, SH). This confirms previous observations that road traffic is one of the sources of PAH emulsion.

Tableau 1 : the different PAHs identified on the leaves of G. sepium collected on the road and out of the road.

	Nap	Acy	Ace	Fln	Phe	Ant	Flt	Pyr	Bn21T	BghiF
CR1	0.033	0.02059	0.01784	0.05923	0.0766	0.0142	0.0346	0.0267	0.0194	0.05631
CR2	0.0025	0.0031	0.00623	0.0012	0.0021	0.0023	0.0026	0.0017	0.0031	0.0016
SR1	14.371	0.5956	0.2127	3.5225	3.0186	0.4871	4.2443	5.3182	0.1368	2.8261
SR2	14.7237	0.2868	0.2247	3.3637	2.8923	0.16	3.7286	4.9045	0.0517	1.9975
SR3	13.2	0.1904	0.2445	3.5419	2.6991	0.1851	3.5185	4.5002	0.0311	1.9979
SH1	3.2745	0.01977	0.0182	0.613	0.6989	0.00934	0.2854	0.2219	0.029	0.5452
SH2	3.2214	0.0138	0.01823	0.4842	0.6126	0.01444	0.1747	0.3366	0.0396	0.5314
SH3	3.3909	0.0699	0.02797	0.5777	0.4003	0.00712	0.9292	0.8653	0.032	0.4084
	BcP	Bn12T	Bn32T	BaA	CcdP	Tph	Chr	BbF	BkF	BjF
CR1	0.001853	0.009	0.005	0.008647	0.002842	0.003346	0.001121	0.002941	0.006745	0.007432
CR2	0.00543	0.003452	0.0012	0.00219	0.005632	0.00128	0.005321	0.002945	0.002934	0.001965
SR1	1.0599	0.841	0.388	5.2879	2.8413	1.3283	5.6339	5.2194	3.1308	3.4109
SR2	0.3606	0.157	0.0373	1.6244	1.4775	1.6282	4.3375	4.0197	3.5231	2.7816
SR3	0.3308	0.135	0.057	1.6094	1.4866	1.5935	4.1343	5.3962	3.1476	2.3702
SH1	0.02158	0.00101	0.056	0.9807	0.2522	0.2961	1.3682	1.6697	0.8627	0.9599
SH2	0.1839	0.00131	0.00373	0.9152	0.2783	0.3599	1.2042	1.2996	0.6365	0.7756
SH3	0.1396	0.0096	0.00269	0.6295	0.1824	0.2649	0.9051	1.3356	0.728	0.7686

Concentrations of 20 PAH congeners in the samples analyzed, expressed as ng g-1 leaf mass for the leaf samples (Nap: naphthalene; Acy: acenaphthylene; Ace: acenaphthene; Fln: fluorene; Phe: phenanthrene; Ant: anthracene; Flt: fluoranthene; Pyr: pyrene; Bn21T: benzo[b]naphtho[2, 1-d]thiophene; BghiF: benzo[ghi]fluoranthene; BcP: benzo[c]phenanthrene; Bn12T: benzo[b]naphtho[1, 2- d]thiophene; Bn32T: benzo[b]naphtho[3, 2-d]thiophene; BaA: benz[a]anthracene; CcdP: cyclopenta[cd]pyrene; Tph: triphenylene; Chr: chrysene; BbF: benzo[b] fluoranthene; BkF: benzo[k]fluoranthene; BjF: benzo[j]fluoranthene;

2- Bacterial community in the leaves of *G. sepium* species

After sequencing the 16S rRNA genes, the number of OTUs identified in the different leaves of the plant was significantly higher compared to those identified in the control (CR1 and CR2). In the different experimental groups, 93626 and 96954 OTUs were identified respectively in the leaves collected on the road (SR) and out of the road (SH) (Table 2). No significant difference was observed when comparing the results of the bacteria identified on the leaves collected on the road and those collected out of the road. The OTUs identified were different in the three different zones, taking into account the level of the collect (apical, basal and middle). The richness estimated by the Shannon and Chao indices showed no difference between the results obtained on the leaves collected on the road and those out of the road.

Tableau 2 : numbers of identified bacterial OUT, and the relative abundance of bacteria estimated in the different experimental groups as well as the diversity indices of Shannon and Chao.

Experimental group	Bar code	Seq_Num	Num- OTUs	Shannon index	Chao index
CR1	ATCGAC	5372	68563	2.06 ± 0.23	356.28 ± 36.33
CR2	GCCTAG	5387	59564	2.02 ± 0.24	346.14 ± 41.33
SR1	ATGACG	61408	95303	3.18 ± 0.23	476.18 ± 46.33
SR2	GACGAT	62803	90873	3.39 ± 0.67	411.14 ± 30.87
SR3	AGCATA	62494	94703	3.48 ± 0.22	445.11 ± 28.42
SH1	CGACAT	59882	98373	2.39 ± 0.24	478.17 ± 32.86

SH2	ACTATT	61075	97330	3.34 ± 0.26	467.16 ± 30.59
SH3	TGGCGG	61930	95160	3.53 ± 0.45	483.15 ± 34.61

Data shown are the mean of three replicates ± SD and were compared by Duncan's multiple range tests. Seq-Num is the number reads of the samples, Num OTU is the number of 16S rRNA OTUs sequences obtained by grouping and normalizing the samples.

3- Bacterial abundance and diversity on *G. sepium* leaves based on different taxa

3.1. Based on the phylum

The relative abundance of bacteria was assessed at the phylum level (Figure 1). According to the results, Proteobacterium and Baciliota are the two main phyla identified in the leaves of the plant *G. sepium* with respectively 24.27% and 72.06%. No difference was observed when considering the results obtained outside and on the road.

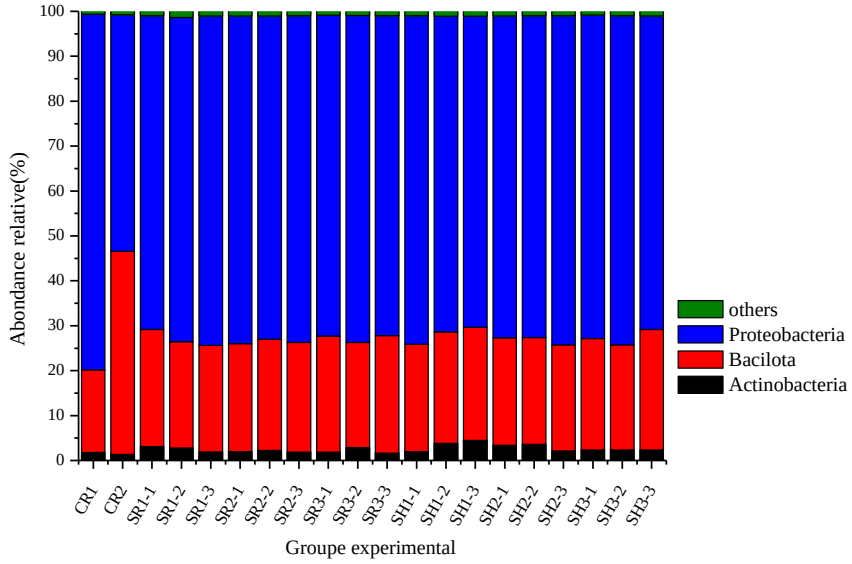

Fig.1: Relative bacterial abundance at the phylum level. The horizontal and vertical axis represent respectively the name of each sample and the abundance ratio in three repetitions. Each color corresponds to the name of the phylum and at the same time indicates the abundance of the different classes. SR= on-road, SH= out of-road, CR1= on-road control and CR2= out of-road control.

3.2 Based on the class level

Figure 2 represents the relative abundance of bacteria according to the different classes. It was found that the distribution of taxonomic classes differ by the relative abundance of bacteria in each class. In the leaves collected on the road (SR), Actinobacteria, Bacilli and Gammaproteobacteria were the most represented classes with 17%, 26% and 33% respectively. On the other hand, in the leaves collected outside the road (SH), Alpha, Beta and Gamaproteobacteria were the most abundant with respectively 24%, 29% and 38%. Compared to the two experimental groups, the relative abundance of Betaproteobacteria was significantly high in the two controls (CR1 and CR2)

Based on the genus

The relative abundance of bacteria was finally evaluated at the genus level (figure 3). In both experimental groups, several genera were identified. *Pantoea* was the most abundant genus with 18% followed by *Lactoccoccus* with 7% and *Pseudomonas* with 5%. These three genera show no significant difference between the different experimental groups.

3.3. Correlation between bacterial community in different samples

The scatterplot matrix presented in the figure 4 highlight the correlation between different phyla identified in the experimental group collected on the road and out of the road. The phylum Proteobacteria was strongly

correlated with, Actinobacteria and Baciliota (r = 0.83, $p < 0.05$). While the genera *Xanthobacter* was however correlated to *Pseudomonas*, *Martelella*, *Altererythrobacter* and *Sphingobium* (r = 0.86, $p < 0.05$). The phylum Baciliota was strongly correlated with Actinobacteria (r = 0.88, $p < 0.05$), while the genera *Altererythrobacter* was positively correlated to *Pseudomonas* and *Sphingobium* (r = 0.81, $p < 0.05$) and finally, the genera *Sphingobacter* was correlated to *Altererythrobacter* and *Kordiimonas* (r = 0.77, $p < 0.05$).

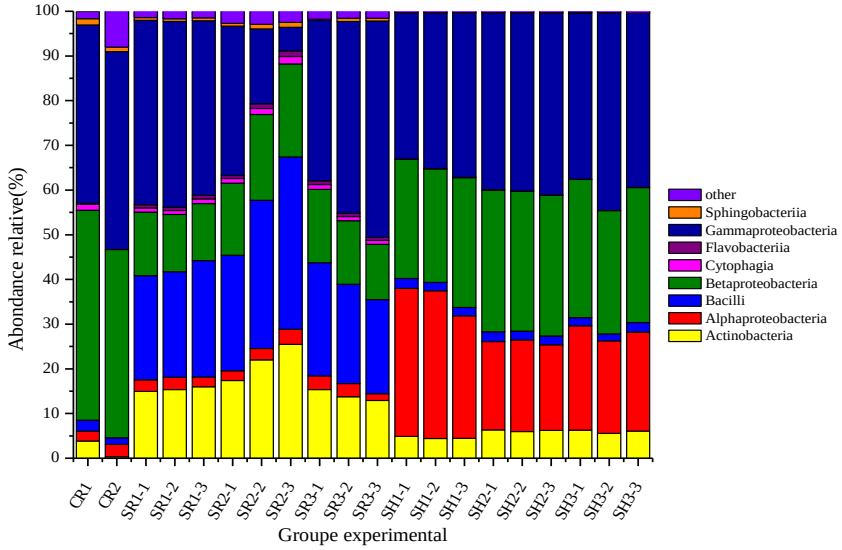

Fig.2: Relative bacterial abundance at the class level. The horizontal and vertical axis represent respectively the name of each sample and the abundance ratio in three repetitions. Each color corresponds to the name of the class and at the same time indicates the abundance of the different classes. SR= on-road, SH= out of-road, CR1= on-road control and CR2= out of-road control.

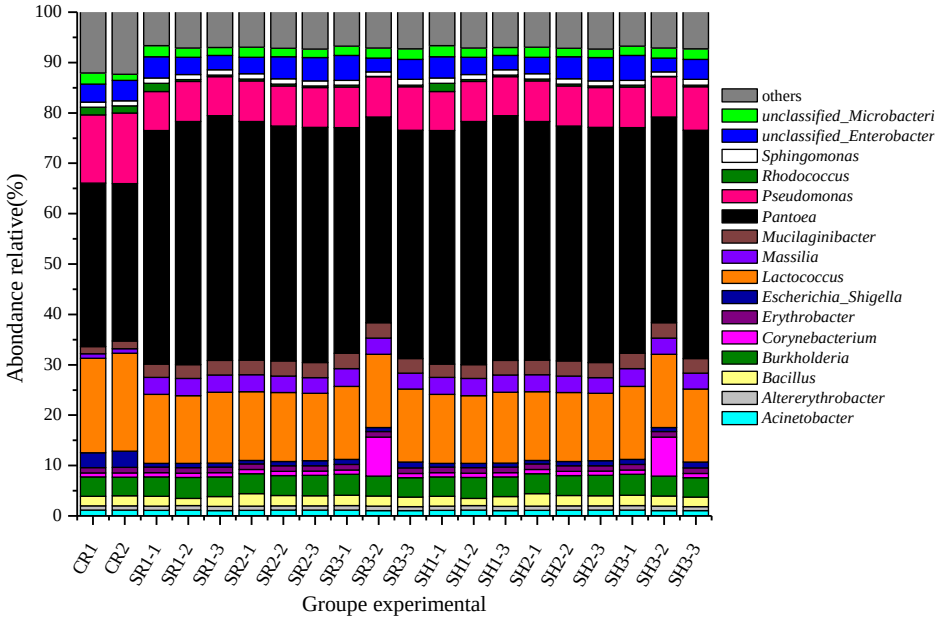

Fig.3 : Abondance relative bactérienne au niveau du genre. L'axe horizontal et vertical représente respectivement le nom de chaque échantillon et le rapport d'abondance en trois répétitions. Chaque couleur correspond au nom du genre et indique par la même occasion l'abondance des différentes classes. SR = sur la route, SH=hors de la route, CR1= contrôle sur la route et CR2= contrôle hors de la route.

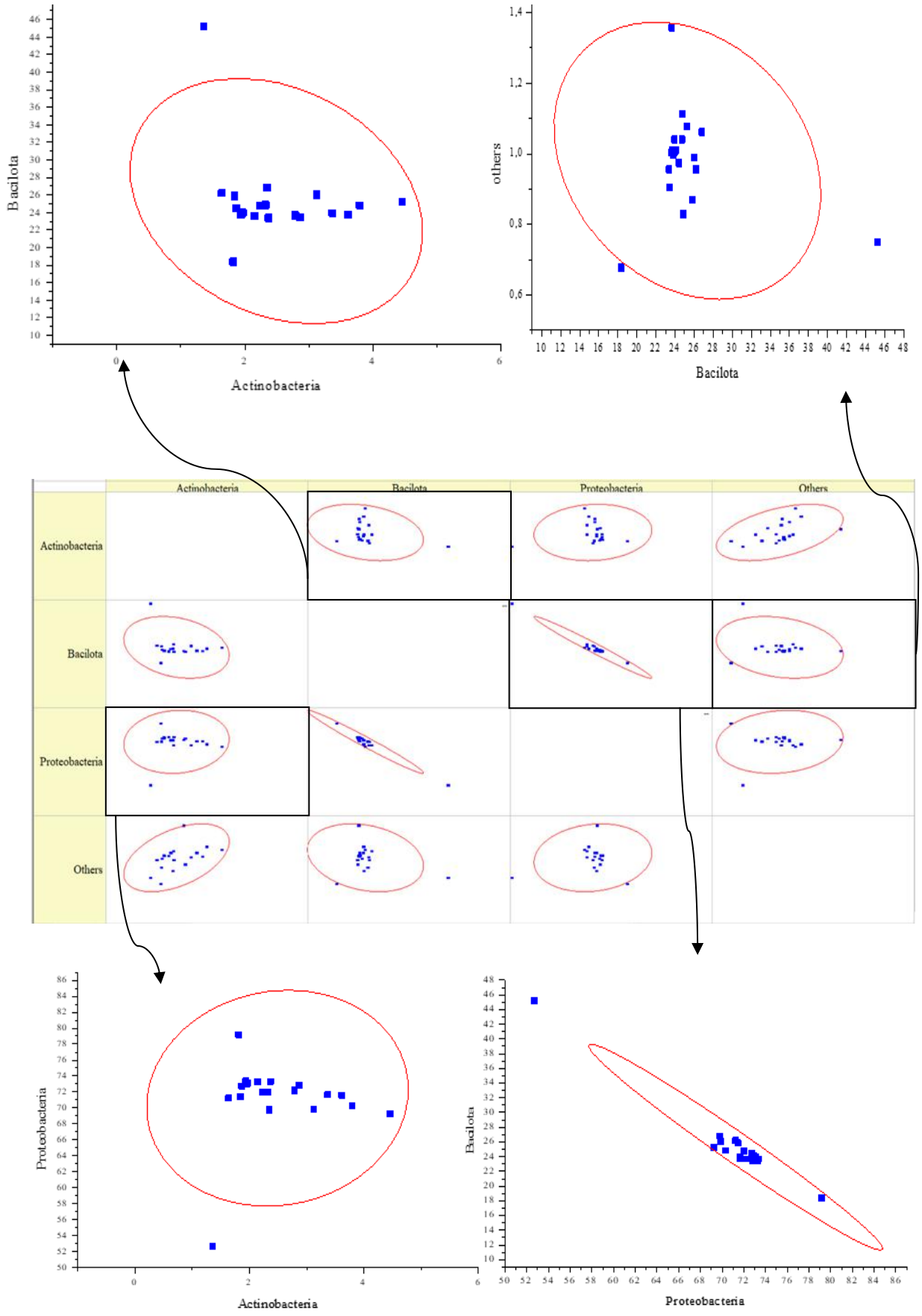

Fig.4: The scatterplot matrix presented highlight the correlation between different phyla identified in the experimental group for the leaves collected on the road and out of the road

IV. DISCUSSION

The critical role of plants in removing PAHs from the atmosphere has been known for over 20 years, when Simonich and Hites in 1994 estimated that over 40% of atmospheric PAHs were trapped by vegetation and released into the soil, while more recent works report lower values (Zhang et al. 2019a). The spatial patterns of atmospheric concentrations of PAHs that we observed in this present study were consistent with those reported in previous studies, which showed higher concentrations of PAHs are rather observed in rural areas where road traffic is high. The spatial trend of PAH concentration extracted from leaf samples in the present study was generally consistent with airborne concentrations. This finding is consistent with several previous reports of PAH deposition on plant leaves which showed leaf concentrations to be higher in urban areas compared to per urban or remote areas (Andrea et al. 2020). *Gliricidia* leaves are known to have a high wax content (Aranda et al. 2017). Yet previous scientific reports indicate that the concentration of PAHs on leaves increases with wax content (Wang et al. 2008). Therefore, in this present study, only one species of plant was used, the relation "wax content-PAH concentration" cannot be a strong argument to explain the different concentrations of PAH on the leaves collected on the road contrary to those collected out of the road.

Among the PAHs identified in this study, naphthalene was the most abundant compound in most leaf samples. Such an abundance of naphthalene on the leaves could be due to the high vapor pressure of the lower molecular weight PAHs, which facilitates both direct uptake by the atmosphere through the stomata and particulate phase exchange at the wax-rich surface of the plants leaves. The stomatal conductance of a leaf, in particular, can determine the capture efficiency of semi-volatile pollutants such as low molecular weight PAHs (Abdullah et al. 2020), while high molecular weight PAHs are usually deposited on the plant surface bound to particles in wet and dry deposition (Alagic et al. 2016).

Epiphytic bacteria, living in the aerial parts of the plant and on the surface of the leaves in particular, are directly exposed to many variable environmental factors, but especially to atmospheric pollutants (Lindow and Brandl 2003). For this reason, they were able to develop a kind of adaptive and metabolic capacities towards these atmospheric pollutants, which can play a potential role in the processes of air bioremediation. Despite their continuous exchange with airborne populations, phyllosphere bacteria are not random assemblages, but rather form true communities resulting from certain selection processes (Vorholt 2012; Rastogi et al. 2012). These communities undergo selection processes resulting in predictable microbial communities represented by a few dominant phyla and other less represented taxa. The few bacteria holding the power of resistance due to different genetic assets are essential in these environments where living conditions are constantly changing. In this present study, the leaves collected on the road which are more exposed to PAHs present a strongly elevated relative abundance of Actinobacteria and Bacilli. We can therefore easily deduce that these bacteria could have developed a kind of resistance to these road PAHs. On the other hand, bacteria belonging to the Alphaproteobacteria class are significantly less represented in this road area. This could be explained simply by the fact that PAHs are toxic to these bacteria.

V. CONCLUSION

The present study not only identified the major different PAHs released as a result of Moroni road traffic, and analyzed the phyllosphere bacterial population of *Gliricidia sepium* leaves, but also established a correlation between the abundance of the phyllosphere epiphyte bacterial population living in the leaf surface of *Gliricidia sepium* with the PAHs identified in the study area. It was therefore demonstrated that the spatial trends of atmospheric concentrations of PAHs were consistent with those reported in previous studies, showing that the higher concentrations of PAHs are rather observed in rural areas where road traffic is high and where their concentrations in the air are quite substantial. The variation of bacteria in the road area and that outside the road is simply a consequence of the development of resistance to PAHs by certain taxonomic groups which were able to impose themselves unlike other less resistant groups. However, although a great information has been gained from individual plant microbiome studies, we suggest that meta-analyses controlling for differences in methodology are needed to better understand leaf-microbe associations in plants. Acclimatization studies in crops subjected to PAH stress would be of great use to better apprehend and understand PAH-microbe interactions in the phyllosphere of the leaves.

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