Preliminary PCR-TTGE analyses of bacterial communities associated with pollen from anemophilous trees: potential impacts on plants and human health

Françoise Fons, Stefaniya Hantova, Yasmine Hamdouche, Sylvie Rapior, Corinne Teyssier

To cite this version:
Françoise Fons, Stefaniya Hantova, Yasmine Hamdouche, Sylvie Rapior, Corinne Teyssier. Preliminary PCR-TTGE analyses of bacterial communities associated with pollen from anemophilous trees: potential impacts on plants and human health. Journal of Microbiology, Biotechnology and Food Sciences, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, 2018, 7 (5), pp.478-483. 10.15414/jmbfs.2018.7.5.478-483 . hal-02195580

HAL Id: hal-02195580
https://hal.umontpellier.fr/hal-02195580
Submitted on 26 Jul 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
PRELIMINARY PCR-TTGE ANALYSES OF BACTERIAL COMMUNITIES ASSOCIATED WITH POLLEN FROM ANEMOPHILOUS TREES: POTENTIAL IMPACTS ON PLANTS AND HUMAN HEALTH

Françoise Fons¹, Stefaniya Hantova², Yasmine Hamdouche¹, Sylvie Rapior¹, and Corinne Teysnier²-³

Address(es): D. Corinne Teysnier,
¹Université de Montpellier, UFR des Sciences Pharmaceutiques, Laboratoire de Botanique, Phytochimie et Mycologie, UMR 5175 CEFE / Equipe Substances Naturelles et Médiations Chimiques, 15 avenue Charles Flahault, 34093 Montpellier cedex 5, France.
²Université de Montpellier, UFR des Sciences Pharmaceutiques, Laboratoire de Bactériologie, Virologie et Contrôle Microbiologique, 15 avenue Charles Flahault, 34093 Montpellier cedex 5, France.
³UMR 95 QualiSud / Cirad TA B-95 / 16, 73 rue J-F Breton, 34398 Montpellier cedex 5 (France), Phone: (33) 4 67 61 57 78.

*Corresponding author: corinne.teysnier@unumontpellier.fr

do: 10.15414/jmbfs.2018.7.5.478-483

ARTICLE INFO

ABSTRACT

Received 23. 6. 2017
Revised 30. 11. 2017
Accepted 10. 1. 2018
Published 1. 4. 2018

Pollen from wind-pollinated plants is the predominant cause of pollinosis which corresponds mainly to allergic rhinitis and conjunctivitis. Bacterial communities associated to leaves and roots surface were analysed for many years with both culture-dependent and independent methods. However, microbiota of pollen grains was rarely investigated and never with a molecular fingerprint technique. The aim of this study was to perform a preliminary analysis of the pollen microbiota by applying PCR-TTGE method. Pollen samples were collected from various anemophilous trees genera (Cephalotaxus, Cupressus, Pinus, Platanus and Quercus) in Montpellier (France) in 2013. Thorough pollen preparation was essential to a successful recovery of DNA from pollen. Regarding the results, pollen microbiota was tree genus-dependent. In addition, intra-tree genus variations were also observed. The Grammaproteobacteria class was the most represented in pollen samples whatever the tree genus. Among this class, some bacteria were recognized as phytopathogens (Pseudomonas, Erwinia) but also opportunistic human pathogens (Pseudomonas, some enterobacteria). PCR-TTGE is a suitable tool to analyse the microbiota associated with pollen responsible for phytopathogenicity or affecting the human respiratory tract.

Keywords: pollinosis; anemophilous trees; pollen microbiota; health; PCR-TTGE

INTRODUCTION

Pollen grains represent a category of Primary Biological Aerosol Particles (PBAP) in addition to bacteria, virus, fungal spore, algae, cyanobacteria (Després et al., 2012). During pollination, pollen grains come into contact with human respiratory or conjunctival mucosa and could be responsible for pollinosis which correspond mainly to allergic rhinitis and conjunctivitis. In France, pollinosis affect from 10 to 15% of the general population and are increasing in urban areas (Ravault et al., 2005). In some cases, small pollen grains can induce asthma attacks (Heydenreich et al., 2012). Allergic rhinitis triggered by the pollen grains of some seasonal plants is commonly named “hay fever”, and appears mostly during haying season. However, atopic patients with seasonal allergies present hay fever throughout the year. The pollen grains that provoke hay fever change between individuals and from region to region. The hardly visible pollen of wind-pollinated plants such as trees, grasses, and weeds are yet the predominant cause (D’Amato et al., 2007). In Mediterranean region, Piniaceae and mostly Cupressaceae pollen grains constitute most clinically relevant trees pollen (D’Amato et al., 2007; Yáñez et al., 2013). Moreover, pollen is regarded as a source of not only allergens but also immunomodulatory molecules, which present major roles in sensitization and/or the exacerbation of allergies (Kamijo et al., 2009). Recently, nanovesicles containing allergens (named pollensomes) released during in vitro germination of pollen grains were described to contribute to allergic reaction (Prado et al., 2015).

For many years, bacterial communities associated to plants were analysed particularly root-inhabiting (Bué et al., 2009; Bulgarelli et al., 2012), leaf-surface inhabiting (Yashiro et al., 2011; Ishaki et al., 2013) and less frequently flower-inhabiting (Alekket et al., 2014) bacterial microbiota. Both culture-dependent and independent methods were performed to explore bacterial microbiota. Since the diameter of pollen grains from anemophilous species varies from 17 to 58 μm (Després et al., 2012), pollen grains may represent a support for bacteria. Moreover, many exudated compounds of pollen grains are known to be attractive for bacterial colonizers (Alekket et al., 2014).

Bacterial communities associated to pollen called bacterial pollen microbiota were rarely investigated. Coldhald and Carlsson (1968) isolated the Gram-negative Pseudomonas maltophilia (currently Stenotrophomonas maltophilia) from pollen samples. The presence of microorganisms on surface of pollen was then confirmed by scanning electron microscopy (Coldhald and Nilsson, 1973). Later, Spiewak et al. (1996a) highlighted the presence of a mixed microflora consisted of Gram-positive and Gram-negative mesophilic bacteria, thermophilic actinomycetes and fungi on allergenic pollen grains. Moreover, Spiewak et al. (1996b) showed that Gram negative bacteria such as Pantocha agglomeras endotoxin associated to pollen should be considered as a potential factor aggravating pollinosis. More recently, Heydenreich et al. (2012) reported that grass pollen grains were colonized by Gram-negative bacteria as Acinetobacter lwofii and Gram-positive bacteria belonging to the genus Bacillus which presents adjuvant activity inducing inflammatory T cell responses. Few studies on plant protection have been carried out in order to detect bacteria responsible for plant disease as Pseudomonas syringae pv actinidiae on the kiwifruit pollen grains (Yamamoto et al., 2013). All these studies were investigated with culture-dependent method based on the isolation of bacteria on different agar media. Molecular methods were never used.

Currently, bacterial communities associated with humans (McCarty, 2002; Roudrié et al., 2009; Michon et al., 2012), animals (Richards et al., 2005; Navarrete et al., 2012), plants (Lambais et al., 2014), foods (Ogier et al., 2004; Hamdouche et al., 2015) or environment (Lyautay et al., 2005) are analysed by culture-independent approaches such as genetic fingerprinting methods (PCR-DGGE, PCR-TTGE). The 16S rRNA gene is the most frequently gene used for amplification. Separation of PCR products in TTGE is based on the decrease of the electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing DNA denaturants according to a temperature gradient. Molecules with various sequences will have a different melting behaviour and will stop migrating at distinct position in the gel (Ogier et al., 2002). The aim of this study was to perform a preliminary analysis of the bacterial communities associated with pollen isolated from plants responsible for...
pollinosis by applying PCR-TTGE method. A PCR anchored on the bacterial 16S rRNA gene followed by a TTGE analysis was carried out on DNA extracts from several pollen grain samples collected in Mediterranean region. On the one hand, methodological aspects were developed including a comparative study of different pollen preparation methods. On the other hand, bacterial diversity of a set of samples were analysed with a 16S rDNA based PCR-TTGE approach.

**MATERIAL AND METHODS**

**Pollen grains sampling**

Pollen samples were collected from various anemophilous trees (Tab 1) in Montpellier, France (GPS data = 43.6° N & 3.9° W) between February and April 2013, in accordance with the pollinic bulletins of the French aerobiology network RNSA (Réseau National de Surveillance Aérobiologique). Collected pollen samples belonged to the genera *Pinus* (x = 5), *Cupressus* (x = 4), *Cephalotaxus* (x = 1), *Quercus* (x = 1), and *Platanus* (x = 1). Data concerning the samples figured in Table 1. According to the type of male inflorescence, pollens were either directly collected or spikes were cut with sterile scalpel. In both cases, pollen grains were aseptically collected in sterile storage bags after shaking and sieving through sterile gauze. Careful consideration was given to collect the pollen grains in order to avoid mixed pollen samples from different plants. Pollen samples were stored at 4°C in dark. Quantification of bacteria in pollen presented in Table 1 was performed according to the protocol described below.

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Date of sampling</th>
<th>Plant genus</th>
<th>Geographic location in Montpellier city*</th>
<th>Enumeration number of bacteria / g pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>I</td>
<td>4.0 10^7</td>
</tr>
<tr>
<td>P2</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>II</td>
<td>1.4 10^7</td>
</tr>
<tr>
<td>P3</td>
<td>2013/04/10</td>
<td><em>Pinus</em></td>
<td>I</td>
<td>6.7 10^6</td>
</tr>
<tr>
<td>P4</td>
<td>2013/04/14</td>
<td><em>Pinus</em></td>
<td>III</td>
<td>3.9 10^6</td>
</tr>
<tr>
<td>P8</td>
<td>2013/04/22</td>
<td><em>Pinus</em></td>
<td>IV</td>
<td>1.9 10^6</td>
</tr>
<tr>
<td>C1</td>
<td>2013/03/21</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>1.4 10^7</td>
</tr>
<tr>
<td>C1b</td>
<td>2013/03/21</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>5.4 10^7</td>
</tr>
<tr>
<td>C2</td>
<td>2013/02/23</td>
<td><em>Cupressus</em></td>
<td>V</td>
<td>2.0 10^7</td>
</tr>
<tr>
<td>C3</td>
<td>2013/03/25</td>
<td><em>Cupressus</em></td>
<td>I</td>
<td>5.7 10^7</td>
</tr>
<tr>
<td>Cx</td>
<td>2013/04/03</td>
<td><em>Cephalotaxus</em></td>
<td>VI</td>
<td>5.0 10^7</td>
</tr>
<tr>
<td>Q</td>
<td>2013/04/18</td>
<td><em>Quercus</em></td>
<td>VI</td>
<td>7.0 10^7</td>
</tr>
<tr>
<td>Pla2</td>
<td>2013/04/22</td>
<td><em>Platanus</em></td>
<td>VII</td>
<td>5.7 10^7</td>
</tr>
</tbody>
</table>

**Legend:** *I* Faculty of Sciences campus, *II* Faculty of arts campus, *III* campus, *VI* Garden of plants, *VII* Albert 1st square.

**Preparation of pollen samples**

Pollen samples were prepared by five different protocols (A, B, C.adn, C.pca and C.tsh) before DNA extraction (Fig 1).

**DNA extraction**

DNA was extracted from the five previous protocols of pollen preparation, following the recommendations of the MasterPure™ DNA purification Kit (Epicentre). Quality of DNA extracts was evaluated using UV spectrophotometry (rate 260 nm / 280 nm) and diluted to obtain a final concentration of 50 μg/ml.

**PCR –Temporal Temperature Gradient Gel Electrophoresis (TTGE) analysis**

A 199 bp-fragment (from position 338 to position 536, Escherichia coli numbering) overlapping the V3 variable region of 16S rDNA (position 338 to position 534, *E. coli* numbering) (Sundquist et al., 2007) was amplified using primers HDA1F-GC and HDA2 (Oger et al., 2002). A 40-bp GC-clamp was added to the forward primer. PCR was performed in a final volume of 50 μl containing 200 pM each dNTP (Fermentas), 200 mm each primer (Sigma), 2.5 μl of Fast Start Taq DNA Polymerase (Roche) in the appropriate 1x reaction buffer with 1.8 mM MgCl₂ and 1 μl of the DNA extract. Amplification program was carried out as follows: an initial denaturation step at 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min and 72 °C for 7 min for the final extension. PCR products were checked using conventional electrophoresis in 1.5 % (w/v) agarose gel with 1 μl of Tris-buffered 1x TAE buffer. DNA was checked using UV transilluminator.

**Figure 1** Presentation of the different protocols used to prepare pollen samples before DNA extraction. For protocols C.pca and C.tsh, an additional culture on PCA medium and TSH medium were performed respectively before extraction with the MasterPure™ DNA purification Kit (Epicentre).

The primary objective was to highlight the best protocol to extract effectively all bacterial DNA associated to pollen grains. For protocol A, 30 mg of pollen sample was suspended directly in 150 μl of Tris-EDTA (TE) buffer with 1 μl of lysozyme (Sigma) and incubated at 37 °C for 18 h. For protocol B, 30 mg of pollen sample were centrifuged (12,000 g for 10 min) thrice in 150 μl of TE buffer. Then, the final pellet was suspended in 150 μl of TE buffer with 1 μl of lysozyme and incubated at 37 °C for 18 h. The protocol C was based on the method described by Vanneste et al. (2011) including few modifications as follows: 30 mg of dry pollen suspended in 1 ml of sterile distilled water were sonicated for 5 min. Suspension was shaken with a rotary shaker for 60 min at 120 rpm. After settling, the supernatant was filtered through sterile gauze and centrifuged at 10,000 g for 20 min at 6°C. The supernatant was discarded and the pellet was rehydrated with 1 ml of sterile distilled water. This solution is referred to as the Final Concentrate (FC). From this FC, three different variations to protocol C called C.adn, C.pca and C.tsh were performed in order to optimise the recovery of DNA. For Cadn, DNA was directly extracted from 500 μl of FC. For C.pca, 100 μl of the FC were spread onto Plate Count Agar (PCA) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.tsh, 100 μl of the FC were spread onto Tryptic Soy Agar supplemented with 5 % of horse blood (TSH) (Bio-Mérieux) and incubated at 30 °C for 48 h. For C.pca and C.tsh, DNA was extracted from cultures collected at the surface of PCA or TSH plates respectively. The last step of protocol C consists of re-suspension in 150 μl of TE buffer with 1 μl of lysozyme and incubation at 37 °C for 18 h.

**Bacterial enumeration**

For protocol C, three decimal dilutions were carried out in saline serum from Final Concentrate (FC). Then 100 μl of FC and each dilution were plated in duplicate on PCA agar and incubated at 30 °C for 18 h.

**Table 1** Data concerning the pollen sampling. Quantification of bacteria in pollen was performed according to the protocol described below.

Legend: *F* Faculty of Pharmacy
TTGE bands analysis and sequencing

Dominant bands were cut from TTGE gels, rinsed twice with molecular biology grade water and eluted overnight in 10 mM Tris buffer (pH 8.5) at 37 °C. Extracted DNA was re-amplified using primers HDA1 and HDA2 without GC-clamp as previously described (Michon et al., 2010). Then PCR products were sequencing on an applied automatic sequencer (Cogenics) by using the forward primer HDA1. DNA sequences were visualized and analysed with BioEdit program version 7.0.9 (Hall, 1999). The 16S rRNA gene sequences were screened using GenBank’s Blast program (Altschul et al., 1990).

Fingerprinting and statistical analysis

The TTGE gel images were analyzed using Image Quant TL software v. 2003 (Amersham Biosciences). Individual lanes of gel images were aligned which permits detection and record of the relative position of each DNA band. TTGE patterns were manually scored by presence and absence of co-migrating bands between lanes. Pairwise community similarities were quantified using the Dice similarity coefficient (S_D = 2 N_c / N_a + N_b) (Heyndrickx et al., 1996) where N_a represented the number of bands detected in sample A, N_b represented the number of bands in sample B, and N_c represented the number of bands common to both samples. A cluster analysis was carried out using the similarity matrix to group pollen samples according to their similarity index. A Principal Component Analysis (PCA) was also used as a multivariate technique for exploratory data analysis using Statistica (version 7) software (StatSoft, USA).

RESULTS

Bacterial communities were analysed on pollen samples from various wind-pollinated trees in order to evaluate the microbiota associated to pollen. The comparison of microbial ecology of pollen grains from various anemophilous trees was performed by PCR-TTGE analysis.

Optimization of preparation of pollen samples and bacterial numeration

Five protocols were evaluated to prepare the pollen samples. Figure 2 shows TTGE patterns obtained from pollen samples of five Pinus specimens, i.e., P1-4 and P8 (Tab 1) prepared according to four different protocols (A, C.adn, C.pca and C.tsh).

For P8 sample, one band was detected for protocols A and C.adn while 16 bands were detected for protocols C.pca and C.tsh. Figure 3 presents TTGE patterns obtained from pollen samples of Cupressus (C1, C1b, C2 and C3), Cephalotaxus (Cx), Quercus (Q) and Platanus (Pla2) prepared according to five protocols (A, B, C.adn, C.pca, C.tsh).

The cluster analysis of TTGE patterns obtained from pollen samples of the five Pinus specimens prepared according to four protocols showed two main dissimilar clusters: the first cluster grouped patterns obtained from protocols C.pca and C.tsh and the second cluster comprised patterns obtained from protocols A and C.adn (Fig 4).

For C1b, one band was detected for protocol C.adn while 12 and 16 bands were respectively obtained for protocols C.pca and C.tsh. Thus, both protocols C.pca and C.tsh highlight the highest number of detected bands whatever the sample. The cluster analysis of TTGE patterns obtained from pollen samples of the five Pinus species prepared according to four protocols showed 70% of the variability of samples according to four protocols with or without a step of culture before DNA extraction (Fig 5).
Two distinct groups could be discriminated: the first group contained samples obtained from protocols with step culture (C.pca and C.tsh), a second group included samples prepared without step culture (protocols A and Caud). Bacterial enumeration from PCA medium varied from 1.4 $10^4$ to 4.0 $10^4$ CFU / g pollen and from 2.0 $10^6$ to 5.7 $10^6$ CFU / g pollen for Pinus and Cupressus, samples, respectively (Table 1). Bacterial numeration ranged from 7.0 $10^4$ to 5.7 $10^4$ CFU / g pollen for Quercus, Cephalotaxus and Platania.

Bacterial communities associated to pollen samples

The objective of this study was to evaluate the bacterial communities associated to pollen grains from different tree genera, i.e., Pinus, Cupressus, Cephalotaxus, Quercus, and Platania using PCR-TTGE.

Concerning pollen samples from Pinus, a total of 53 DNA bands were observed in total of the samples (Fig 2). Among the 53 observed bands, 46 bands were cut and sequenced. DNA sequences obtained from cut bands varied from 147 to 172 bp compared to the 199 bp of the V3 region. First, we demonstrated that several sequences corresponded to chloroplast DNA. Presence of chloroplast DNA was not unexpected because of the high similarity between bacterial and chloroplast 16S rRNA gene sequence in the context of endosymbiotic origin of chloroplast (McFadden 2001). As a result, bacterial but also chloroplast 16S rRNA genes could be amplified with primers used in this study. Then, we detected a majority of bacteria belonging to the Enterobacteriaceae family such as Erwinia sp., Pantoaea sp., Enterobacter sp., Klebsiella sp., and Providencia sp but also to Pseudomonas, Bacillus, Flavobacterium, Trabulsiella sp., and the genus Bacillus. Within the genus Pinus, the five TTGE patterns corresponding to C.pca protocol were very different from one tree to another. Indeed, we observed that bacterial communities associated to pollen seemed to vary among the genus Pinus. Only bacterial strains belonging to Pseudomonas genus seemed to be present in four Pinus samples from five.

Concerning pollen samples from Cupressus, Cephalotaxus, Quercus, and Platania, a total of 99 DNA bands were observed in total of the samples which permitted to obtain 81 DNA sequences. From Cupressus samples, we found bacteria belonging to Enterobacteriaceae family such as Erwinia sp., Pantoaea sp., Enterobacter sp., Klebsiella sp., and Providencia sp but also to Pseudomonas, Bacillus, Flavobacterium, Trabulsiella sp., and the genus Bacillus. Within the genus Pinus, the five TTGE patterns corresponding to C.pca protocol were very different from one tree to another. Indeed, we observed that bacterial communities associated to pollen seemed to vary among the genus Pinus. Only bacterial strains belonging to Pseudomonas genus seemed to be present in four Pinus samples from five.

DISCUSSION

In the present study, we analysed bacterial communities associated to twelve pollen samples collected from five anemophilous tree genera at different locations in Montpellier (France). We used a molecular fingerprint technique called PCR-TTGE which was already applied to determine bacterial communities associated to pollen samples from various plant genera (Spiewak et al., 2012; Navarrete et al., 2012). Five pollen preparation protocols were evaluated. Preparation of pollen samples before DNA extraction was a critical step of the PCR-TTGE analyses since it appeared decisive to obtain the totality of bacterial communities associated to a sample. As concerns the number of detected DNA bands for each TTGE profile, mean values varied according to the pollen preparation protocol. Thus, the best results were obtained with the protocol adapted from Vanneste et al. (2011) followed by a culture dependent step on a non-selective medium (PCA medium) or enriched medium (TSM medium) before DNA extraction, amplification and TTGE analyses. Indeed, the previous mentioned preparation of pollen comprising sonication and a 1-h shaking allowed to optimally recovery bacteria from exine pollen wall. Then, the culture-dependent step permitted to concentrate bacteria. We obtained TTGE patterns with a largest number of DNA bands corresponding to main but also less abundant bacteria present on pollen samples. However, potential viable but nonculturable (VBNC) bacteria could not be detected. The culture-independent protocol highlighted only the main bacteria. The bacterial enumeration of pollen samples varied from 7.0 $10^4$ to 5.7 $10^6$ CFU/g pollen. These results were concordant with those obtained by Spiewak et al. (1996a) for different trees.

The bacterial communities associated to pollen samples varied among two plant genera (inter- genera variations) but also inside a same genus (intra-genus variations). Indeed, we observed very different TTGE profiles for the five pollen samples from Pinus and for the four pollen samples from Cupressus. Bacteria belonging to the class Gammaproteobacteria (Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae…) were the most represented in pollen samples before bacteria belonging to the phylum Firmicutes, whatever the plant genera. Pollen samples were thus composed with those observed by Spiewak et al. (1996a) which highlighted in particular mixed microflora consisting of Gram-positive and Gram-negative mesophilic bacteria on allergenic pollen grains.

Bacteria belonging to Gammaproteobacteria class represented 73% of the 16S rDNA sequences obtained and were detected in most of samples. These results are in accordance with those of Wetzet et al. (2010) which showed that 81.2% of bacterial species isolated from edible flowers belonged to the class Gammaproteobacteria. Our results were in accordance with the intragenomic and intraspecific heterogeneity of the 16S rDNA gene, it is very difficult to obtain identification at the species level and even at the genus level. It is a classical disadvantage of the PCR-TTGE method (Michon et al., 2012). Some Enterobacteriaceae species are currently present in plant. We found DNAs of Erwinia sp. and Pantoaea sp. in Pinus and Cupressus samples. Erwinia is a well-known bacterial genus associated to plants. Some Erwinia species could lead to damages of plant structure, wilting, dieback, yellowing or rot (Thomson et al., 1999). The main pathophytopathic Erwinia species are E. amylovora and E. carotovora responsible for the fire blight of Rosaceae especially apple and pear (Piqüé et al., 2015), and soft rot diseases respectively (Parent et al., 1996). Pantoea agglomerans (formerly Erwinia herbicola) could be opportunistic pathogen in cases of weakness of the plant (Dutkiewicz et al., 2016). Dickeya sp., Enterobacter sp. and Klebsiella sp. DNAs were also detected in our pollen samples. The genus Dickeya comprises several species particularly D. chrysanthemi (formerly Erwinia chrysanthemi) known to be phytopathogens (Toth et al., 2011). Enterobacter asburiae, Enterobacter cloaceae and Enterobacter cowanii were already recovered from plants (Wetzet et al., 2010; Humann et al., 2011). E. asburiae was considered as a Plant Growth Promoting Rhizobacteria (PGPR) particularly for Citrus reticulata (Thokhcom et al., 2014) whereas E. cloacae is showed to be phytopathogenic for numerous plants such as tomato (Parent et al., 2005) and also to be acquired from apple tree (Dutkiewicz et al., 2012) but as Klebsiella oxytoca were reported to cause wilt in many plants (Sarkar and Chaudhuri, 2015). K. oxytica, Enterobacter sp., Erwinia sp., Tatumella pyseos etc. were also dominant taxa in a Greek vineyard (Nisiotta et al., 2011). To conclude on the Enterobacteriaceae species present on pollen grains, we observed that they were usually present in other parts of the plants where they potentially phytopathogens.

The genus Pseudomonas which also belongs to the Gammaproteobacteria class presents various species associated to plants, e.g., P. syringae (Kennelly et al., 2007), P. rhizosphere (Peix et al., 2003) and P. panacis (Park et al., 2005). Thus Pseudomonas spp. could be considered as phytopathogen, biological agent or PGPR (Vanneste et al., 2011; Noori and Saud, 2012; Mansfield et al., 2012). The genus Xanthomonas with X. campestris and X. oryzae causes also significant damage in a range of crops too (Ryan et al., 2011). The Enterobacteriaceae is a well-known family of bacteria which constitute human intestinal microbiota but several species or serotypes can also be pathogenic to human. Most of them are recognized as opportunistic pathogen and causes respiratory symptoms. In particular, Pantoaea strains are responsible for respiratory infections in immunocompromised patients (Flores Popoca et al., 2012; Kursun et al., 2012; Walterson and Strawinides, 2015; Dutkiewicz et al., 2016). Enterobacter asburiae was also found in human samples particularly in sputa (Koth et al., 2012). Klebsiellae cause various infections in humans including community-acquired pneumonia and nosocomial infections. Moreover, Tatumella pyseos strains were notified to be responsible for tracheobronchial/pulmonary infections (Costa et al., 2008). For the Pseudomonadaceae family, Pseudomonas aeruginosa is likely to be responsible for nosocomial infections (Parent et al., 2005) and for the hospital-acquired. For example, P. aeruginosa is the second most common cause of nosocomial pneumonia. P. aeruginosa is also recovered from respiratory tract of cystic fibrosis patients (Winstanley et al., 2016). In addition, P. mosselii should be taken into account as a potential human pathogen which was especially isolated from tracheal aspirate of a patient suffering from pulmonary infections (Leneveu-Jenvri et al., 2013). Regarding the phylum Firmicutes which represents 14% of the 16S rDNA sequences obtained, we underscored the presence of Bacillus strains in particular Bacillus cereus which is well-known as volatile human pathogen (Botton, 2010), and Staphylococcus pasteurii responsible for the few cases of infections in immunocompromised patients (Morfín-Otero et al., 2012). Pollen samples from Cephalotaxus, Quercus and Platania were also Gammaproteobacteria such as Enterobacteriaceae and Pseudomonadaceae strains.

Indeed, we detected in pollen grains from anemophilous (deciduous and coniferous) trees various bacterial genera, i.e., Pseudomonas, Erwinia and Enterobacter well-known as responsible for a wild range of human diseases and plant infections.

CONCLUSION

In this present study, we analysed the first time bacterial communities associated to pollen grains of five anemophilous trees. Whatever the plant genus, the pollen with those observed by Spiewak et al. (1996a) which highlighted in particular mixed microflora consisting of Gram-positive and Gram-negative mesophilic bacteria on allergenic pollen grains.
should be considered as vectors for these human opportunistic bacteria. In addition to being allergenic, pollen could participate to the dissemination of bacteria.