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DNA barcoding analysis of the microbial community in Olive Orchards: *Olea europaea* L. Bacterial and fungal diversity in Tunisia

Houda Gharsallah[1], Ines Ksentini[1], Karama Hadj Taieb[1], Christina Schuster[2,3], Mohamed Ali Triki[1], Mohieddine Ksantini[1], Andreas Leclerque[2,3]

[1] Laboratory of Improvement and Protection of Olive Tree Genetic Resources, Olive Tree Institute, Sfax, Tunisia

[2] Institute for Microbiology and Biochemistry, Geisenheim University, Germany

[3] Department of Biology, Technische Universität Darmstadt, Germany

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Abstract

Olive production is the main agricultural activity in Tunisia. This study investigated the microbial diversity isolated from soil and the major olive tree insect pests. We aimed to explore the scarcely known microbiota in Tunisian olive orchards as the first step to elucidating the microbial interactions that meddle with biological control. Bacterial and fungal strains were randomly isolated from different biotopes situated in Sfax (Tunisia). The 16S rRNA and ITS gene sequencing were used to identify the microbial community. 88 isolates were shown as belonging to 41Operational Taxonomic Units (OTUs) identified toward the genus level and 39 OTUs identified at the species level. The different olive cultivars depicted distinct communities and exhibited dissimilar amounts of bacteria and fungi with distinct ecological functions that could be considered promising resources in biological control.

Keywords: Biological control, Microbial diversity, Bacteria, Fungi, *Olea europaea*

1. Introduction

Tunisian olive growing is one of the main strategic economic sectors, particularly in agriculture. Tunisia ranks fourth in the world in terms of olive trees number, with nearly 60 million trees, and second in terms of surface area, around 1.6 million hectares, i.e. nearly a third of the country's arable land FAOSTAT (2018). For production, FAO and EBRD (2015) report that Tunisia generally ranks among the top six world producers of olive oil. To consolidate the position of the olive sector in Tunisia and improve the competitiveness of Tunisian olive oil on an international scale, efforts should be focused on controlling production costs, and improving and enhancing quality.

In this context, the phytosanitary aspect of the olive tree is considered among the important factors acting both on the production costs and on the quality of the product FAO and EBRD (2015). Indeed, the olive tree is subject to several harmful

insects attacking its productive potential. In Tunisia, this crop is subject to the attack of mainly three pests which are the olive fly *Bactrocera oleae* (Diptera, Tephritidae), the olive moth Prays oleae (Lepidoptera, Yponomeutidae), and the olive psyllid olive *Euphyllura olivina* (Hemiptera, Psyllidae) COI (2007). These three pests are subject to chemical treatments every year.

However, in front of the environmental and health problems caused by insecticides, biological control is considered a vital component of sustainable agriculture that preserves natural resources and the environment through the use of microorganisms selected for their high efficiency and safety Warlop (2006). Within the framework of enhancing the biodiversity of the olive tree agroecosystem, the Tunisian olive institute developed a biological and integrated management program against the main pests of Olivier through research aimed at promoting certain autochthonous entomopathogenic bacteria, for the biological control of the aforementioned insects.

Nowadays, biological control agents are being developed worldwide, including organisms such as fungi and bacteria, reducing the populations of pests affecting olive crops.

The aim of the present study was the use of the 16S and ITS region in a metabarcoding approach to investigate the bacterial and fungal microbiota in Tunisian olive orchards. Representative sequences were phylogenetically analyzed with selected reference sequences to enable the most accurate possible identification of putative species.

2. Material and Methods

2.1. Microbial collection and isolation

Sampling was carried out during the year 2017 in 5 olive orchard siteslocated in the region of Sfax. Microbial isolation was performed from insect pests and the soil of the olive tree. This isolation was done on the LB medium (Luria-Bertani) for bacteria and for fungal strains the cultures are carried out in PDA medium (Potato Dextrose Agar). A total of 88 samples (52 Bacteria and 36 Fungi) were collected, according to a standardized protocol (Barer and Harwood, 1999).

2.2. Bacterial identification

DNA extraction from bacteria was performed using the kit DNeasy blood and tissue kit. The extracted DNA served as a template for amplifying the 16S region of ribosomal DNA. PCR amplification was performed using the two complementary universal primers fD1/rP2 (Table1).

Table 1. Sequences of primers used. fD1 and rP2 are complementary universal primers for amplifying the 16S region. ITS4 and ITS5 are standard primers for amplifying the ITS region.

Primer	Sequence
fD1	AGAGTTTGATCCTGGCTCAG
rP2	ACGGACTTACCTTGTTACGACTT
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG

The PCR thermal cycler System MyCyclerTM used for carrying out PCR reactions has been programmed in an initial cycle of denaturation at 95°C for 2 min, followed by 40 cycles at 95°C for 30 sec, hybridization at 56°C for 30 sec and polymerization at 68°C for 3 min and ending with an elongation cycle at 68°C for 5 min. The purification of the PCR products was done using the PCR Purification Kit. The purified PCR products were subsequently sequenced. The chromatograms obtained were analyzed using the editor of CHROMAS sequences. A manual enhancement has been made for missing sequences of some nucleic acids. Alignment Sequential was conducted using MEGA7 software. Nucleic Sequences of the isolates were analyzed for their similarity by the basic BLAST online alignment search tool (http://blast.ncbi.nlm.nih.gov) which makes it possible to compare the sequences with those available in the GenBank database (NCBI).

2.3. Molecular identification of isolated fungi

Fungal DNA extraction was done by the DNeasy Plant Pro and Plant Kits (Qiagen®) according to an optimized protocol. The extracted DNA was used as a template for the PCR amplification of the ITS region of the rRNA of themolds.A PCR-based molecular study was performed with specific primers ITS4/ITS5. Amplification was programmed in an initial cycle of denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 1 min, 1 min at 52°C (hybridization), and 68°C for 2 min (polymerization), and ending with an elongation cycle at 72°C for 5 min. PCR reactions were performed in a PCR thermocycler. (MyCycler Thermal Cycler PCR Machine- BioRad– Hochschule Geisenheim University). In this study, PCR amplification was performed in a total volume of 50 µl for 1 sample containing5 µl of reaction buffer, 43.2 µl of water, 1 µl of dNTP, 0.25 µl of each primer (100 µM), 0.25 µl of Taq polymerase (New EnglandBiolabs®) and 1 µl of DNA. Sequencing was performed by StarSEQGmbt (Mainz, Germany).At the end of the analyses, the contracted company proceeded to send the nucleotide sequences of isolates. The latter were then assimilated to their similarity by the online basic alignment search tool BLAST (Basic Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov) which allows unknown gene sequences to be compared to known sources in the gene bank database (NCBI). The latter holds sequences from thousands of fungi and can be a powerful tool that can identify unknown organisms.

3. Results and Discussion

3.1. Microbial community isolation and identification

This study describes the composition of bacterial and fungal communities within olive trees different cultivars, insects, and soil located in the Sfax region, the main olive producing region, located in the center of Tunisia. The 30 olive trees'soil and pests sampled harbored 88 microorganisms. Fungal and bacterial isolates were obtained in all samples tested. All isolates were successfully identified, through the search for homologous sequences using BLAST at the NCBI, based on 16S for bacteria and ITS for fungi sequences analysis. The microbial collection was identified at the genus and species level in 100 % of the isolates. The size of the generated PCR products ranged from 350 to 500 bp (ITS) and from 600 to 800 bp (16S).

3.2. Structural diversity of the microbial community

The 88 fungal and bacterial isolates were identified as belonging to 41 OTUs identified at the genus level and 39 OTUs identified at the species level. The bacterial isolates were identified as belonging to 23 OTUs identified at the genus level and 21 OTUs identified at the species level. 9 strains *Alcaligenes* spp. showed identity with 2 species; *Alcaligenes aquatilis* and *Alcaligenes faecalis*. Two *Alcaligenes* strains were identified to the genus level. *Bacillus* spp. showed identity with 5 species; *Bacillus atrophaeus*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus thuringiensis* and *Bacillus safensis.* Bacteria belonging to *Bacillus* spp. have been previously detected in the microbial communities of the endophytic different olive trees with a percentage of 33% (Muller et al., 2015; Ruano-Rosa et al., 2017; Gharsallah et al., 2018). *Brevundimonas* spp. showed identity with 2 species *Brevundimona sbullata* and *Brevundimonas diminuta. Lysinibacillus* spp. showed identity with *Lysinibacillus sphaericus, Myroides* spp. showed identity with *Myroides injenensis.* For *Paenalcaligenes* spp. strains were identified to the genus level. *Proteus* spp. showed identity with *Proteusmirabilis. Providencia* spp. showed identity with 2 species; *Providencia rettgeri* and *Providencia vermicola. Providencia* bacteria had previously been found associated with insects but not with the olive fly (Juneja *et al*. 2009; Guerfali *et al*., 2018; Ksentini *et al*., 2018). *Serratia* spp. showed identity with *Serratia marcescens. Staphylococcus* spp. showed identity with 5 species; *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus warneri,* and *Staphylococcus xylosus* (Table 2). These findings are in agreement with results reported from microbial communities associated with the root system of wild olives (Aranda *et al*., 2011; Ruano- Rosa *et al*., 2017).

Table 2. Bacterial and Fungal OTUs identified to the genus level present in soil and Insect pests

		Origine				Total	%
		Insect pests	%	Soil	%		
OTUs	*Alcaligenes* spp.	9	11.5	-	-	9	10.2
	Alternaria spp	2	2.6	-	-	2	2.2
	Aspergillus spp	5	6.4	8	80.0	13	14.8
	Bacillus spp	19	24.3	-	-	19	21.6
	Brevundimonas spp	3	3.8	-	-	3	3.4
	Cladosporium spp	7	9.0	-	-	7	7.9
	Fusarium spp	2	2.6	-	-	2	2.2
	Lecanicillium spp	1	1.3	-	-	1	1.1
	Lysinibacillus spp	2	2.6	-	-	2	2.2
	Myroides spp	1	1.3	-	-	1	1.1
	Paenalcaligenes spp	2	2.6	-	-	2	2.2
	Penicillium spp	9	11.5	2	20.0	11	12.5
	Proteus spp	1	1.3	-	-	1	2.2
	Providencia spp	7	9.0	-	-	7	7.9
	Serratia spp	2	2.6	-	-	2	2.2
	Staphylococcus spp	6	7.7	-	-	6	6.8
Total		**78**	**100.0**	**10**	**100.0**	**88**	**100.0**

The Fungal isolates were identified as belonging to 6 OTUs identified at the genus level and 21 OTUs identified at the species level. *Aspergillus* spp. showed identity with 8 species; *Aspergillus calidoustus*, *Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus pseudodeflectus, Aspergillus tamarii, Aspergillus terreus,* and *Aspergillus ustus.* The genus *Alternaria* showed identity with one species which is *Alternaria consortialis*. *Cladosporium* spp. showed identity with 3 species; *Cladosporium cladosporioides, Cladosporium halotolerans, Cladosporium sphaerospermum. Cladosporium* spp. are described as secondary weak pathogens in many different host plants and occur as cosmopolitan saprophytic fungi (Tashiro *et al*., 2013). Wang and collaborators reported that *Cladosporium cladosporioides*is known as an effective bio-control agent (Wang *et al.*, 2013). Fusarium spp. showed identity with *Fusarium solani*. *Lecanicillium* spp. showed identity with *Lecanicillium aphanocladii*. *Penicillium* spp. showed identity with 7 species; *Penicillium chrysogenum*, *Penicillium citrinum, Penicillium crustosum, Penicillium freii, Penicillium pinophilum, Penicillium polonicum, and Penicillium verruculosum* (Table 2). Most of the identified fungi are already referred to as leading the diversity in olive trees (Abdelfattah *et al*., 2015; Martins *et al*., 2016; Gomes *et al*., 2018; Gharsallah *et al*., 2020). Concerning showed differences, the fungal communities could be associated with several factors such as an underestimated fungal diversity due to low spatial-temporal sampling, the type of vegetative tissue, and environmental factors at sample sites.

The most frequent strains obtained in this study, belong to the bacterial genus *Bacillus* 19 (21.6 %) appeared only in insects, followed by the fungal genus *Aspergillus* 13 (14.8%) isolated from insects and soil. The number of OTUs identified from insects was 38, from which the two species belonged to *Alcaligene feacalis* and *Providencia vermicola* being the most representative 6 (7.7%), followed by two OTUs representing 5 (6.4%) of the isolates *Bacillus atrophaeus* and *Bacillus cereus*. The number of OTUs identified from soil was 6, from which the genus *Aspergillus* was the most representative 6 (60.0 %) with the two species *Aspergillus ustus and Aspergillus calidoustus.* These observations are in line with studies emphasizing the largest widespread of *Penicillium* and *Aspergillus* identified as the dominant genera in most ecosystems and were the most ubiquitous fungal species in nature (soils, plants, and agricultural communities) (Godinho *et al*., 2015; Yee *et al*., 2016; Kazerooni *et al*., 2017).

4. Conclusion

In conclusion, the present study provides a comprehensive picture of the distribution of the microbial strains isolated from different olive cultivars in Tunisia. These results presented here give an important contribution to this field. The olive microbial community was found to contain known benefic and phytopathogenic microorganisms that can significantly impact olive production. This microbial community may be further explored as antagonists of important olive pathogens, and possibly be developed as effective biocontrol agents.

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