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Analysis of the Cultivable Endophytic Bacterial Diversity in the Date Palm (*Phoenix dactylifera* L.) and Evaluation of Its Antagonistic Potential against Pathogenic Fusarium Species that Cause Date Palm Bayound Disease

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Abstract Biological control still remains an unexploited issue in southern countries such as Tunisia. Thus, the present study focused on the diversity of cultivable endophytic bacteria in the internal tissues (roots and leaves) of Tunisian date palm trees (*Phoenix dactylifera* L.). In order to assess their antagonistic potential towards date palm pathogens, particularly *Fusarium.* Indeed, the Genus Fusarium includes the causative agent of the Bayound disease, *Fusarium oxysporum*, a major treat for date production North Africa. Twenty two bacterial isolates presenting distinct colony morphology on TSA media were selected. The latter were characterized using Gram staining, biochemical tests, and molecular identification techniques based on 16S rRNA gene sequencing. Cultivable endophytic isolates were assigned into seven distinct groups. The species *Arthrobacter agilis* and *Bacillus subtilis* exhibited lasting antagonistic properties against a range of *Fusarium* species including the causing agent of the Bayoud disease, thus demonstrating their strong potential for future applications in the inoculation of date palm trees for biocontrol purposes. The isolates showed extracellular enzymatic activity including cellulase (76, 92%), protease (69, 23%) and amylase (38, 46%). This study thus demonstrates for the first time that the diversity of endophytic bacteria is abundant in date palm trees (*Phoenix dactylifera* L.) and could present varying biotechnological applications and particularly disease control.

Keywords: *palm date tree, endophytic bacteria, molecular identification, biocontrol, Fusarium spp., bayound deseases*

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1. Introduction

Date palm (*Phoenix dactylifera* L.) is a multipurpose tree typically cultivated in arid and semi-arid regions of the world. It is extensively cultivated for its edible fruit which is marketed worldwide. Current estimates indicate that North Africa is the world's tenth largest producer and first exporter of dates in value. In Tunisia, for instance, the date palm groves cover a total area of over 30,000 hectares and enclose over 4 million trees, 83.25% of which are grown in the regions of Djerid and Nefzaoua

(South of Tunisia). Tunisian annual production of palm dates is estimated to reach an average of 100,000 tons, with dominance of the Deglet Nour variety (about 60% of the total production).

Great efforts have been made to protect and expand the cultivation of date palms including *in vitro* culture of plantlets [1]. These efforts have, however, often been curtailed by plant diseases, some of which are still not well characterized. These include those from fungal origins, such as *Mycosphaerella tassiana*, often reported to cause brown spots on midribs, leaflets and thorns, and *Diplodia phoeniceum*, responsible for attacks of leaves of off-shoots, inducing deep, yellowish brown and necrotic lesions [2]. One of the most destructive fungal diseases of date palm remains a vascular wilt disease, commonly known as "Bayoud", which is caused by a soil-borne fungus, *Fusarium oxysporum* f. sp. *albedinis*. The Bayoud disease first appeared 1870 in Morocco, where it destroyed over 12 million trees [3,4]. It has more recently been reported to move to Algeria, where 3 million trees have been destroyed so far. This fatal disease has not yet spread to Tunisia but, due to the nonstop movement of people and goods to and from the country, the possibility of disease transfer remains a risk for the date palm cultivars in the country. In the case of date palm, a little is known about microorganisms that can protect plants against *Fusarium* and especially the causative agent of Bayoud [5,6]. Even less is known with regard to the induction of plant defense reactions [7]. Recently, it has been have shown that the pretreatment of date palm seedlings with a hypo-aggressive isolate of *F. oxysporum* was providing a protection against further attacks by an aggressive isolate of the same species [7]. The situation would become even more critical with diseases of unknown etiology. Several recent reports have, for instance, documented the emergence of a new lethal date palm disease, termed the brittle leaf disease, in a number of Tunisian groves, for which no etiological agent has so far been identified [2].

The inadequacies associated with the conventional methods employed for the management and control of date palm pathogens, including attempts to select plant pathogen resistant variants [8,9,10] underlines the need for alternative sustainable control strategies. Several plant growth-promoting bacteria (PGPB) have been shown to play a major role in controlling directly plant pathogens [11,12,13,14] and promoting the growth of various temperate [15,16] and tropical host plants [17] or promote host resistance [18]. However, such approaches have hardly yet been attempted with Saharian plants, and particularly, the date palm tree [5,6,7,19]. Indeed, despite the huge flow of data on endophytic bacteria in the literature, little work has so far been conducted on the characterization of the palm tree endophytes. Previous antagonistic assays performed against palm pathogens were realized with collection of antagonistic strains [19].

This work thus aims at (i) performing a first overview of the diversity of the cultivable endophytic bacterial microflora from the internal tissues of both roots and leaves of healthy Tunisian palm trees *Phoenix dactylifera* L., (ii) at characterizing potential PGPB biological activities of this flora. We specially focused on the functional characterization of bacteria with antagonistic potential towards date palm *Fusarium* species, among which is found the causing agent of the Bayoud disease, *F. oxysporum*. (iii) Finally, we aimed at assessing their GRAS status.

2. Material and Methods

2.1. Sampling of Date Palm Plants

The samples employed in the present study were collected from 'Deglet Nour' date palm trees grown at a local grove, the Jouali oasis, situated in the south west of Tunisia, on the algero-Tunisian border near Tozeur. This palm field is located in the major date production area in Tunisia and covers a surface of 4719 km^2. The oases at Tozeur typically consist of sandy-loam textured and carbonated soils. The oasis, from which the samples of the present work were collected, has a water pH of 8.1, total $CaCO_3$ content of 119 g/kg, organic matter, of 12.08 g/kg, Nitrogen, of 0.56 g/kg, Carbon, of 7.03 g/kg, K_2O, of 0.18 g/kg, CaO, of 49.1 g/kg, MgO, of 0.51 g/kg, Na_2O, of 0.53 g/kg, and P_2O_5, of 0.31 g/kg (Namsi et al. 2007).

2.2. Optic and Electronic Microscopy Observations for Sample Selection

Optic microscopy observations of transversal cuts of both leaves and roots were conducted to detect potential occurrences of symbiotic hyphae or bacteria and check for the absence of any histological degeneracy symptoms in the sample root and leaf tissues. Accordingly, root and leaf fragments were cut in both transversal and longitudinal sections using a Vibratum (Micron. HM 650 V, France). The fragments were colored with Carmine-green by immersion for 10 min, immersed (or not) in a hypochlorite 12°C solution for 15 min, and then rinsed four successive times in demineralized water. The slides were then left for 10 seconds in acetic acid pH 3.2, diluted with water (v/v), colored by hanging in carmine-green solution for 18 min, and finally rinsed three times in water. The observations were then performed using an optic microscope at maximal original magnification.

Electronic microscopy observations were based on the method developed by Jones [38]. For electron microscopy detection of putative bacterial or fungal mycelium in internal palm tissues, both leaf and root homogenates were studied by negative staining with 2% phosphotungstic acid (PTA), pH 7.0, using carbon/collodium-coated 200-mesh copper grids and observed in a JEOL 1200XII TEM (Transmission Electron Microscope) operating at 80 kV. Special attention was given to samples presenting rod-shaped structures that could indicate bacterial colonization of internal tissues. Degeneracy symptoms were also carefully tracked with the aim of selecting GRAS endophytes.

2.3. Isolation of Endophytic Bacteria

A total of 22 bacterial colonies of different characteristics and belonging to a range of species were isolated from the roots and leaves of date palm plants collected from the Jouali Oasis (Tozeur, Tunisia), and identified, suggesting that the diversity of the palm endophytic flora is indeed significant. In addition, isolates were here screened for their potential antagonistic

activities against a range of *Fusarium* species, including the causal agent of the Bayoud disease, with promising results for disease control.

The sampled healthy plant trees were approximately 50 years old with an average height in the range of 7 to 8 meters. In order to construct a systematic overview of date palm endophytes, the study involved the collection of both leaf and root samples from healthy trees. The latter included healthy mature leaves whose size ranged between 3.5 and 4.5 m collected from the central crown of the palm tree (the most active crown). They also included healthy roots with an average diameter of 0.3 ± 0.08 cm collected from the 0-20 cm surface soil layers.

Endophytic bacteria were isolated from the internal tissues of roots and leaves of three healthy palm date trees. Healthy leaves and roots were washed in running tap water for 30 min. From each washed leaf and root, 16 adjacent 1 mm x 2 mm segments were cut. The samples were surface- sterilized by sequential washes in 2% sodium hypochlorite for 10 min and 70 % ethanol for 1 min, and then rinsed five times with sterile distilled water and allowed to surface-dry under sterile conditions. This surface-sterilization method has previously been shown to effectively eliminate yeasts, fast-growing *Zygomycetes*, and other epiphyllous organisms from endophyte cultures [39,40], for a review of surface-sterilization methods).

The leaf and root segments were then placed on Petri dishes containing different type of medium like Miller Hinton Agar, Malt Extract Agar, and Tryptic Soy Agar (TSA), a medium commonly used in endophyte studies [41]. The plates were incubated at 28°C for 20 days. The bacterial population in the tissue samples was expressed as colony forming-units (CFU/g) of tissue. Morphologically distinct bacterial colonies were re-isolated by sub-culturing on appropriate media and stored at -80°C in 20% sterile glycerol for further studies.

2.4. Fungal Pathogenic Strains

Fusarium solani, *Fusarium oxysporum* and *Fusarium oxysporum f. sp. Albedinis* that were isolated respectively from potato, olive and date palm were used in this study. These *Fusarium* spp. were grown at 28°C on PDA for one week. They were also stored at -20°C in 20% glycerol solution for long time preservation.

2.5. *In vitro* Antagonism Test

Fusarium sp species previously isolated from three tissues of plant (palm date, Potato and olive) were used in this study for antagonistic tests. Twenty two bacterial isolates were tested for their ability to produce antifungal substances against *Fusarium* spp. *(Fusarium oxysporum* from Potato, *Fusarium solani* from olive and a Bayoud palm isolate of *Fusarium oxysporum f. sp. Albedinis*) using a dual-culture *in vitro* assay on PDA plates. A 5 mm agar disc of *Fusarium* sp. mycelia was used to inoculate one side of a Petri dish containing PDA medium. The dishes were then incubated at 25°C for 24 h. In parallel, each palm bacterial endophytic isolate was sub-cultured on Muller Hinton for 48 h at 30°C. A loop full of bacterial culture was then streaked 3 cm away from the disc of *Fusarium* isolates on the same dish. Paired cultures were incubated at 27-29°C. Dishes inoculated only with test *Fusarium* were used as controls. After 7 days, growth diameter of the fungus (distance between the point of inoculation of the fungal disk and actively growing edges of the fungus) was measured in both control and inoculated Petri dishes. The experiment was repeated three times for both control and treated samples (for each bacterial culture assessed). The percent growth inhibition (PGI) was calculated according to Erdogan and Benlioglu [42] method using the formula:

$$\text{PGI}\ (\%) = KR - R1 / KR\ x\ 100,$$

Where *KR* represents the distance (measured in mm) from the point of fungal inoculation to the colony growing edges on the control dishes, and similarly, *R1* represents the distance, on the dishes which received bacterial cultures, of fungal growth, in the direction of the antagonist, from the point of fungal inoculation to the fungal colony margin [43].

2.6. Biochemical Assessment of the Bacterial Endophytic Microflora in Relation with Antifungal Properties

Morphological characterization and microscopic features of the selected isolates were studied and classical biochemical characterization of the endophytic bacterial isolates was performed, including catalase and oxidase test. However, we focused particularly on activities that may act on the peptidoglycans which constitute major components of the fungal cell wall. Thus, protease, cellulase and amylase tests were performed following the methods described by Gerhardt et al. [44].

2.7. Isolation of Genomic DNA from Isolates of Endophytic Bacteria

Genomic DNA was extracted from 22 isolates of endophytic bacteria using the Kit Wizard SV Genomic DNA purification System (Promega, France) following the manufacturer's instructions. DNA quality and concentrations were assessed with NanoDrop (Spectrophotometer ND-1000, France) in accordance with the manufacturer's instructions.

2.8. PCR Amplification and Sequencing of 16S rRNA Genes of Cultivable Endophytic Bacteria

Bacterial 16S rDNA fragments were amplified from DNA extracted from endophytic bacteria by PCR amplification using universal bacterial 16S rRNA gene-specific oligonucleotide primers A (5'-AGAGTTTGATCMTGGCTCAG-3'), position 8-27 (*Escherichia coli* numbering Brosius et al. [45]) and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') position 1470-1492 (*Escherichia coli* numbering, Lane et al. [46]). The amplification reactions were performed using a thermal cycler (M.J. Research, Biometra, Stratagene) in volumes of 25 µl by mixing template DNA with polymerase reaction buffer, 25 mM $MgCl_2$, 10 mM dNTPs, the above mentioned primers (20 µM), and 5 U/µl Taq polymerase (Promega). The thermocycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 amplification cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, and a final polymerization step at 72 °C for 7 min. The PCR products

were electrophorized in a 1% (w/v) agarose gel and visualized by ethidium bromide staining.

2.9. Molecular Analysis of DNA and PCR Products

Products of the expected size were purified using a Zymoclean Gel DNA purification Kit (Zymo Research Crop, France), in accordance with the manufacturer's instructions, to remove primers in excess. They were then sequenced using the forward and reverse primers described above, respectively. The sequencer used was a 3130x1 Genetic Analyzer (Life Technologies, Technologies Corporation Tokyo Japan), and the sequences were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA). Internal primers B 5'-CTCCTACGGGAGCAGCAGT-3' (position 339-358 *E. coli* numbering system, Bottger [47]) were used when required to complete the 16S rDNA gene sequence.

2.10. Sequence Assembly and Analysis

At least two sequencing reactions were performed for each direction, and the sequences were assembled using SeqMan (Dnastar, UK). The assembled sequences were then submitted to a preliminary Blast analysis in the NCBI (National Center for Biotechnology Information) database and further nucleotide sequence similarity searches in the ribosomal database project (http://rdp.cme.msu.edu). Their identity was recursively established by comparison with 16S rDNA gene sequences of type strains from closest matching species and their related sequences available in the RDP database presenting correct identifications (obviously incorrectly annotated sequences were manually removed from the selection). Multiple sequence alignment was then carried out using Clustal W [48] at the European bioinformatic Institute website (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses were performed using programs from the PHYLIP package [49], and phylogenetic tree was constructed by the neighbor joining (NJ) algorithm [50] using Kimura 2-parameter distance. The robustness of the inferred tree was evaluated by bootstrapping (100 replications).

2.11. Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported here were deposited in the Gen Bank under the accession numbers PDR 13: JN934383, PDL W: JN934384, PDL Q: JN934385, PDL F4sain: JN934386, PDR T: JN934387, PDL H: JN934388, PDL Fb3sain: JN934389, PDR P: JN934390, PDR V: JN934391, PDR Rb2: JN934392, PDR Rb5: JN934393, PDL F2st1: JN934394, and PDR 15: JN934395.

2.12. Assessment of Generally Recognized As Safe (GRAS) Status of Selected Isolates

Pre-cultures of selected isolates were used to re-inoculate young plantlets of date palm trees that were produced beforehand in our laboratory (Drira N, personal communication). A record of the health status of the plants was kept for three months. A literature data mining was also performed in order to confirm the lack of previously reported general pathogenic activities for the species of interest to which the assessed isolates belonged.

3. Results

3.1. Optics and Electronic Microscopy Observations

Under the conditions described in the present work, the observation of the transversal leaves or roots using optic microscopic, showed typical features of monocotyledoneous palms, no degeneracy symptoms and no occurrences of bacteria, fungi, or structures typical of plant-bacterial or plant-fungal associations. However, when a transmission electronic microscopy was used at an original magnification of 12000 X, classic rod-shaped structures characteristic of bacteria were observed especially in the cytoplasm of subsurface chloroplastic cells from leaves samples, suggesting a bacterial colonization of palm leaves. Samples from such trees were selected for bacterial enrichments.

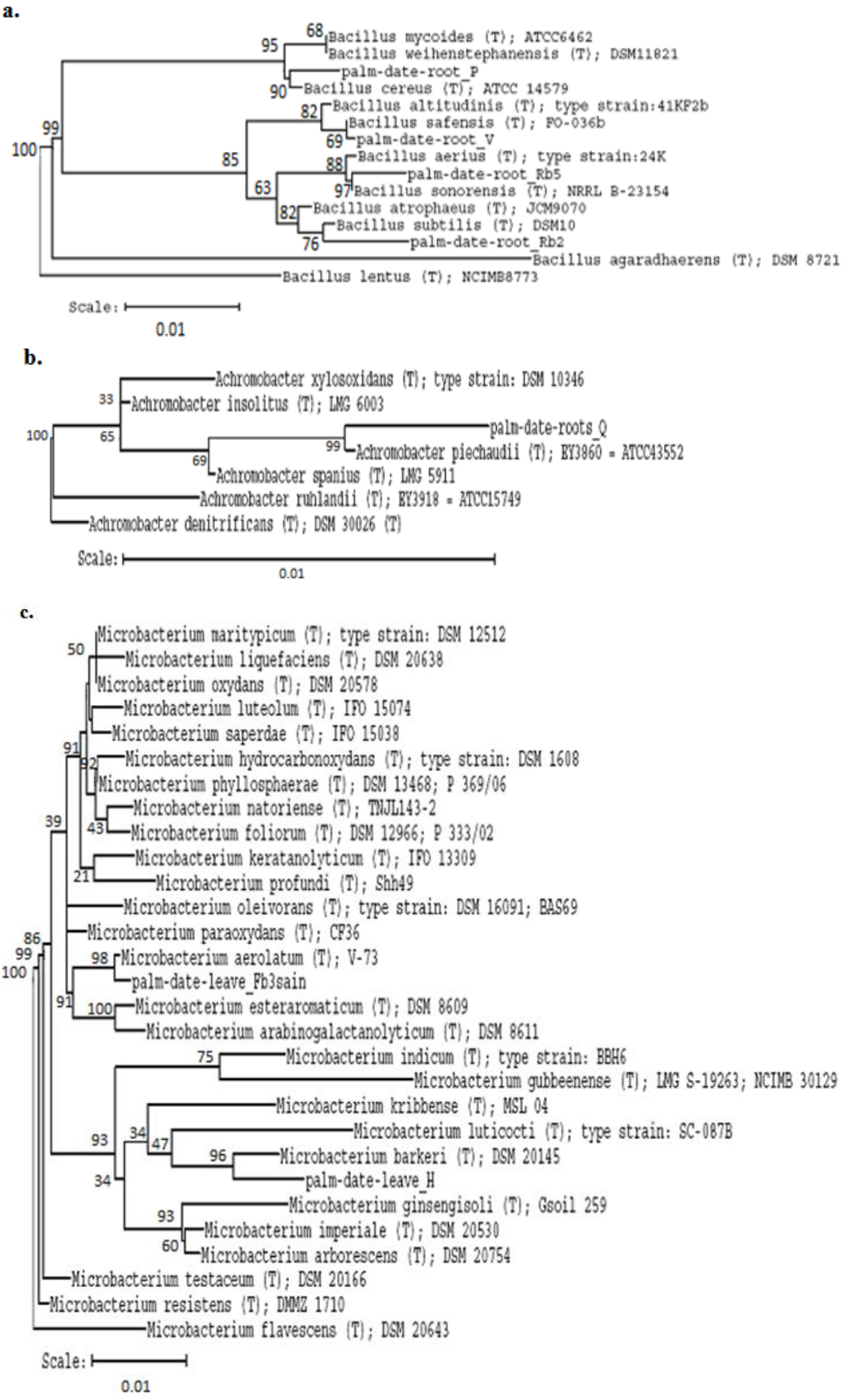
3.2. Colony Diversity of Endophytic Bacteria

Interestingly, a wide range of colony morphotypes were observed on TSA agar, with a total of 22 bacterial colonies presenting distinct morphologies being re-isolated from the macerates of leaves and roots on this culture medium. Conversely, no colonies were noted to develop on Muller Hinton Agar or Malt Extract Agar. The 22 colonies observed on TSA agar consisted of 14 strains that were isolated from root samples and 8 other strains that were isolated from the leaves. They were encoded according to the organ they originated from, PDR (for palm-date-root) and PDL (for palm-date-leave), respectively. Assessed for basic tests, most of the isolates appeared to be Gram-positive bacilli. However, one Gram-negative cocci (PDR13) and three Gram-negative bacilli (PDLQ, PDRT, and PDR15) were also noted. All the Gram-negative and Gram-positive bacilli were oxidase positive, but PDLF3S and PDLH were oxidase negative. On the other hand, the PDR13 coccus was oxidase negative. All isolates were catalase positive.

3.3. Molecular Analysis of DNA

The 16S rRNA gene of the 22 isolates was identified. Among the Gram-positive ones, both *Firmicutes* (*Bacillus*) and *Actinobacteria* (*Microbacterium, Arthrobacter*) were observed. The Gram-negative ones included *gamma-Proteobacteria* (*Serratia*, *Pseudomona*s and *Acinetobacter*) and *beta-Proteobacteria* (*Achromobacter*). More precisely, our findings allowed the clustering of the mentioned date Palm endophytic bacteria into 7 distinct groups. Group I consisted of members of the *Bacillaceae* family. The isolates of this group were related to *Bacillus safensis, Bacillus sonorensis, Bacillus subtilis, and Bacillus cereus* (Figure 1a). Group II contained a single representative of the *Alcaligenaceae* family related to *Achromobacter piechaudii* (Figure 1b). Group III included 4 strains related to *Microbacteriaceae*, which were all isolated from leaves (Figure 1c). Group IV encompassed only two isolates that related to *Micrococcaceae* (Figure 1d). Group V was composed of members of the *Enterobacteriaceae* family. The isolates of this group were related to *Serratia* (Figure 1e). Again, most of the enteric bacteria in this group were isolated from date palm leaves. Group VI contained only one isolate, related to *Acinetobacter*

calcoaceticus (Figure 1f). Group VII consisted of 2 strains related to *Pseudomonadaceae (Pseudomonas stutzeri* and *Pseudomonas sp)*, which were isolated from leaves and roots (Figure 1g). Overall, while the roots were noted to harbor a number of different genera with prevalence of *Bacillus*, bacterial strains isolated from leaves belonged to 4 genera with a large predominance for *Microbacterium*.



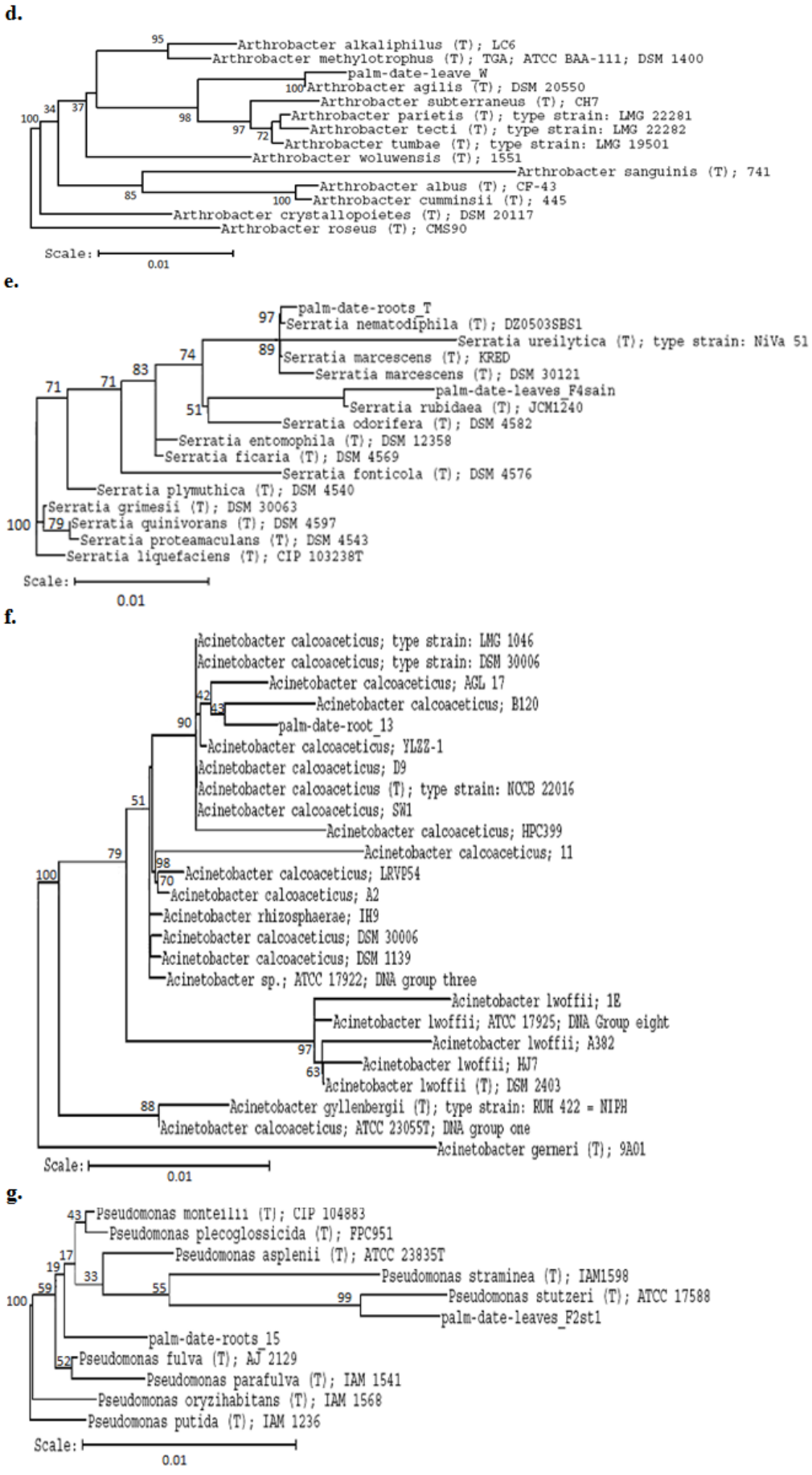


Figure 1. Phylogenetic tree of strains isolated from date palm and related species based on 16S rDNA sequences by the maximum parsimony analysis. The bootstrap percentages of more than 50% are shown above each branch. **a.** The *Bacilliaceae* with *Bacillus lentus* as the outgroup, **b.** The *Alcaligenaceae* with *Achromobacter denitrificans* as the outgroup, **c.** The *Microbacteriacea* with *Microbacterium flavescens* as the outgroup, **d.** The *Micrococcaceae* with *Arthrobacter roseus* as the outgroup, **e.** The *Enterobacteriaceae* with *Serratia liquefaciens* as the outgroup, **f.** The *Moraxellaceae* with *Acinetobacter gerneri* as the outgroup and **g.** The *Pseudomonadaceae* with *Pseudomonas putida* as the outgroup

3.4. *In vitro* Assessment of Enzymatic Activities

In the present study, all the endophytic isolates assessed showed extracellular multi enzyme activity. Indeed, a large majority of isolates were cellulase (*Acinetobacter, Arthrobacter, Achromobacter, Serratia, Bacillus, Pseudomonas*) and protease (*Acinetobacter, Arthrobacter, Achromobacter, Serratia, Bacillus*) producers, with respectively, 76.92% and 69.23%, while 38.46% of the isolates only, identified as *Microbacterium, Bacillus* and *Pseudomonas* species showed amylase production.

3.5. *In vitro* Antifungal Activity

All assessed bacterial strains had a delaying effect on the growth of the tested *Fusarium*. However, only 2 strains showed lasting (over 15 days), significant, antagonistic properties against pathogenic *Fusarium* species isolated from different plants. Our work included especially the strain causing the Bayoud disease *Fusarium oxysporum f. sp. Albedinis*. Indeed, in dual culture tests, *B. subtilis* and *A. agilis* strains only were shown to present lasting growth inhibition activity against assessed *Fusarium* (Figure 2 a, b, c). Additionnally, antagonistic activity of *A. agilis* was observed against all the 3 *Fusarium* tested, with Percent Growth Inhibition (PGI) reaching 36.48%, 37.14% and 38.81% for *F. oxysporum*, *F. solani* and *F. oxysporum f. sp. Albedinis*, respectively (Figure 3 a, b). This suggested that *A. agilis* could possibly present antagonistic activities against a wide range of *Fusarium.*

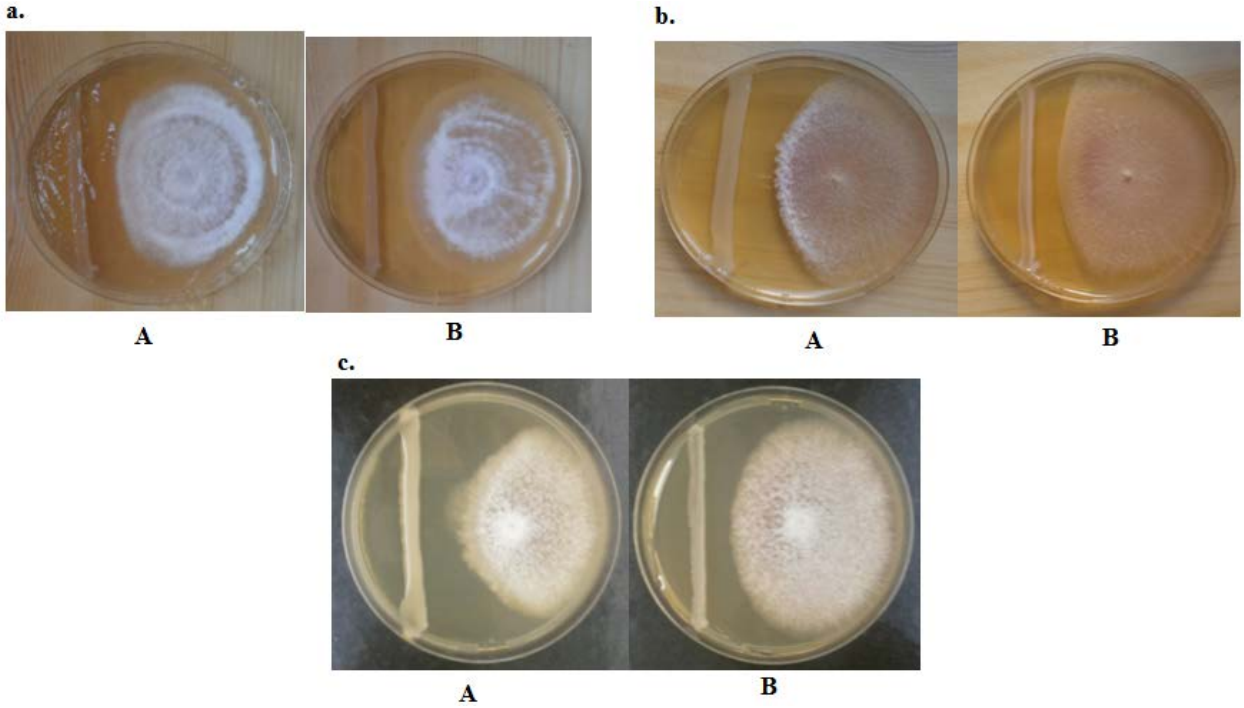


Figure 2. Antagonistic effect of bacterial palm endophytes (A) *A. agilis* and (B) *B. sublilis* on *Fusaria* as evaluated by dual-culture on PDA medium after 7 days. (a) *Fusarium solani* from potato tree, (b) *Fusarium oxysporum* from olive tree and (c) *Fusarium oxysporum* from palm date tree

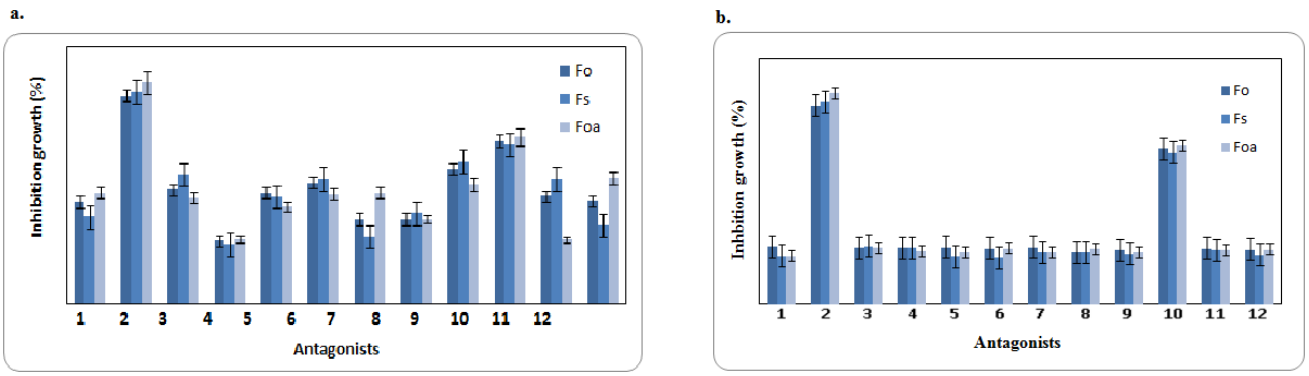


Figure 3. Growth inhibition (%) of different *Fusarium* spp (Fo: *Fusarium oxysporum*, Fs: *Fusarium solani* and Foa: *Fusarium oxysporum f. sp. albidinis)* induced by palm endophytic bacterial isolates (1: Acinetobacter calcoaceticus**;** 2: *Arthrobacter agilis***;** 3: *Achromobacter piechaudii***;** 4: *Serratia nematodiphila*; 5: *Pseudomonas sp;* 6: *Microbacterium aerolatum***;** 7: *Microbacterium barkeri***;** 8: Serratia *rubidaea***;** 9: *Bacillus cereus***;** 10: *Bacillus subtilis***;** 11: *Bacillus safensis* and 12: *Bacillus sonorensis* using the dual culture methods. Percentages were determinate (a) after 7 days and (b) after 15 days incubation

3.6. GRAS Status Assessments

Three months after the inoculation of young plantlets of *Phoenix dactylifera* with the candidate strains presenting antagonistic properties against *Fusarium* sp. *in vitro*, no negative effect on the palm date tree was observed. No significant differences in terms of growth, leave number but also leave shape (results not shown) were detected

between the inoculated plantlets and non-inoculated controls. Similarly, plantlet height and stem diameter could not significantly be distinguished between inoculated and control plantlet, although, a slight but not significant reduction could be observed for plantlet inoculated with the *B. subtilis* strain. Furthermore, literature data mining showed that *A. agilis* was previously reported as a saprophytic species, with water, soil, and human skin as common habitats. No instances of pathogenic activities were previously reported in the literature for this species, suggesting that it could presumably be considered as GRAS. In addition, PGPR activities where even recently reported for this species [51] which acts in favor of its GRAS positioning.

Table 1. Diversity and relative distribution of cultivable endophytic isolates from the leaves and roots palm date tree of Tunisia

Taxonomic	Order	The dosest type strain	Number of isolates	Leaf		Root		Total		Over lap
Identification				N[a]	%[b]	N[a]	%[b]	N[a]	%[b]	(bp)
Actinobacter	Actinomycetales	Arthrobacter agilis(JN934384)	2	02	26:00	-	-	02	09:00	1413
		Micrbacterium barkeri (JN934388)	3	03	37.50	-	-	03	13.60	1403
		Microbacterium aerolatum(JN9389)	1	01	12.50	-	-	01	04.50	1405
Fimicutes	Bacillales	Bacillus cereus(JN934390)	3	-	-	03	21.50	03	13.60	1431
		Bacillus safensis(JN934391)	1	-	-	01	07.41	01	04.50	1437
		Bacillus subtilis(JN934385)	1	-	-	01	07.14	01	04.50	1437
		Bacillus sanorensis(JN934398)	1	-	-	01	07.14	01	04.50	1428
Betaproteobacteria	Burkholderiales	Achromabacter piechaudii(JN934385)	1	-	-	01	07.14	01	04.50	1422
Gammaproteobacteria	Pseudomonadales	Pseudomonas stutzeri(JN934394)	1	01	12.50	-	-	01	04.50	1419
		Pseudomonas sp(JN934395)	1	-	-	01	07	01	04.50	1426
		Acinetobacter calcoaceticus (JN934383)	1	-	-	01	07	01	04.50	1428
	Enterrobacteriales	Serratia rubidaes(JN934386)	1	01	12.50	-	-	01	04.50	1432
		Serratia nematodiphila (JN934387)	5	-	-	05	36	05	22.0	1425

[a]: Number of entophytic bacteria isolates
[b]: Percentage of entophytic bacteria isolates.

4. Discussion

Endophytic bacteria have previously been reported in a wide range of plant species, including rice, banana, wheat, sugarcane, carrot, maize, soybean, potato, citrus plant, and, more recently oil palms [29,30,52,53]. However, to the authors' knowledge, no previous studies focused on endophytic populations in date palm trees. The present study thus reports, for the first time, the cultivable endophytic bacterial population of the roots and leaves of date palm plants (*Phoenix dactylifera L.)*.

Indeed, the morphological and biochemical characterization of the bacterial isolates indicated that a diversity of both Gram-negative and Gram-positive bacteria was recovered from palm date tissues (*Phoenix dactylifera L.*). This was further confirmed by a molecular approach that showed that the root and leaf tissues were inhabited by a wide variety of microorganisms from diverse phylogenetic affiliations. Most of those endophytic bacteria were already reported as endophytes in other plants [54,55,56]. However, this flora was quite different from the one previously reported for another palm species, oil palms, especially by Sapak [52]. In fact, this earlier study reported a flora composed mainly of actinomycetes as well as *Pseudomonas aeruginosa*, *Burkhorderia cepacia*, and *Serratia marcesens*. Conversely, in this study, members of the Genus *Bacillus* have been found to be the dominant group in the root tissues of date palm trees, with four species (*B. subtilis, B. safensis, B. cereus and B. sonorensis)* being identified. This finding is in agreement with previously reported results showing that *Bacillaceae* (27.27%) are frequent plant tissue colonizers commonly found in several plant species, including cotton [54], corn [57], oak [58], cucumber [59], citrus [56], *Eucalyptus* spp. seeds [60], and clover [61]. The second dominant group of endophytes in palms tissues was identified as *Enterobateriaceae* (27.27%), which is also consistent with previous results reported in the literature for rice [62], cotton [63], and coffee [64] among several other plant species [65,66,67]. In our case, *Proteobacteria* included *Enterobacteriaceae* (45.5%) and other isolates that belonged to *Achromobacter*, *Acinetobacter*, *Serratia and Pseudomonas* species, which is consistent with other studies [68,69,70]. In particular, the group of *Pseudomonadaceae* has frequently been reported in endophytic association with legume plants, such as alfalfa [33], clover [61], and pea [55]. The member of the group of *Moraxellaceae*, accounting, in our study, for (4.5%) of the flora, was identified as *Acinetobacter*. These species have also been isolated from the xylem of lemon roots (*Citrus jambhiri*) [71]. Furthermore, *Alcaligenaceae* (4.5%) represented another group that was identified from the date palm tissues and was included under *beta-Proteobacteria*. The latter were previously reported in endophytic association with rice (*Oryza sativa L.*) roots [72] and *Citrus jambhiri* roots [71]. Finally, the last group identified for date palm endophytes was represented by *Actinobacteria*, among which *Microbacterium* (18%) and *Arthrobacter* (9%) were observed. These species have been already reported in endophytic association with different plants and maize kernels [73,74,75]. Several *Microbacterium* species have also been reported in the work of Conn and Franco [74] on the endophytic populations in the roots of wheat (*Triticum aestivum* L.).

The preliminary characterization of endophytic bacteria showed that indigenous bacterial populations isolated from the roots and leaves were different. In fact, several genera, such as *Microbacterium* and *Arthrobacter*, were recorded in the leaf tissues only. Conversely, *Acinetobacter*, *Achromobacter* and *Bacillus* were recorded in the root but never in the leaf tissues. However, *Serratia* and *Pseudomonas* were recorded from both tissues. The plant-associated habitat is a dynamic environment in which many factors

may indeed affect the structure and species composition of the microbial communities that colonize roots and leaves [76], the leaves being a source of nutrients while the roots constitute a nutrient sink [77]. Additional factors affecting plant associated flora include types of plant tissues [77] and plant habitats, as well as other environmental factors [78]. Other palm varieties could of course harbor specific flora. In our case, we concentrated our efforts on the characterization of the Deglet Nour variety, which is, at least in Tunisia, the dominant date producer.

The natural environmental suppressiveness towards *F. oxysporum* is known for long. One of the first reports can be found in a study performed on tomato plants by Lemanceau et al. [79] who compared a suppressive soil from the Chateaurenard area (France) and a conducive soil from Carquefou (France) with regards to *F. oxysporum*, and attributed that suppressiveness potential of Carquefou soil was due to a bacterium, *Pseudomonas fluorescens*, which presented a clear antagonistic effect against *F. oxysporum*. In the case of date palm, several studies have reported the non-receptivity of given soils (i.e. in the region of Marrakech city in Morocco) to the development of Bayoud [9,80,81]. This non-receptivity has been related, later on, to direct competition between the causal agent *F. oxysporum f. sp. Albedinis* and other non-pathogenic *Fusarium* species that were predominant in these soils. It was also shown that this non receptivity could act against several bacteria and actinomycete [6]. Additional studies indicated that various individual PGPB strains could be applied as treatment as suppressor of plant fungal pathogens [82,83].

In particular, endophytic microorganisms have been explored for extra cellular enzyme activity and such exploration was known to have biotechnological and industrial application [84]. In this study, the presence of proteolytic activity confirmed that some species of *Bacillus*, *Serratia*, *Acinetobacter*, *Achromobacter* and *Arthrobacter* tended to synthesize proteolytic enzymes. All isolates have cellulotyic activities except *Microbacterium* and *Pseudomonas*. This enzyme may play a role in the mechanisms by which endophytic bacteria penetrate into and persist in the host plant [28,85] but may also contribute to their antagonistic potential. The amylolytic activity was here observed for isolates of *Microbacterium barkeri*, *Microbacterium aerolatum*, *Bacillus subtilis*, *Bacillus sonorensis* and *Pseudomonas stutzeri*. These species were already reported to produce extracellular amylase [86,87].

In this study, all bacterial isolates were selected from root and leaf of healthy palm date in fields suggesting their GRAS status. Some bacterial isolates showed high inhibition activity on *Fusarium* sp, whereas others showed only mild or no activity at all. Indeed, most isolates tested exhibited no or weak antagonistic activity against the *Fusarium* on PDA plates assay. Moreover, in the case of the present work, given date palm endophytes were related to species that are classified as Generally Recognized As Safe, which makes them potential strong candidates for future application for biocontrol purposes.

For biotechnological reasons, further attention was given to *A. agilis* and *B. subtilis* which (i) presented significant potential to delay the growth of *Fusarium* sp. on PDA medium and (ii) produced proteases that may take part to the wall degradation of fungal pathogens [88], (iii) were not previously reported as pathogenic species for humans, animals, or plants in the literature, and finally, (iv) showed no negative effects on date palm trees. Under *in vitro* test conditions, *A. agilis* appeared as the most effective strain. Its inhibition effect on *Fusarim oxysporum* might be the result of the release, by this strain, of antifungal compounds into the culture media [89]. Alternatively, it was suggested that fungitoxic compounds and/or hydrolytic enzymes produced by antagonists, could diffuse in a short distance into the agar that disorganize the pathogen hyphae before any physical contact [19]. Further, study should focus on the mechanisms of *Arthrobacter-Fusarium* interactions. However, this study is the first to report the antagonistic properties of an *A. agilis* strain against plant pathogenic fungi. However, it is not the first to observe the antagonist potential within the *Arthrobacter* genus, in general, since the antagonistic properties of other *Arthrobacter* species against plant pathogens were previously reported in barley and winter rape [90]. In addition, the antagonistic potential of *A. arilaitensis* and *A. bergerii* were already reported against the food pathogen *Listeria monocytogenes* [91], potentially based on competition for iron [92]. Moreover, no reports are currently available on the pathogenic or opportunistic behavior of *A. agilis.* As far as the GRAS status of *Arthrobacter* sp is concerned, environmental *Arthrobacter* sp were described since the 1950's as being involved in the degradation of numerous pesticides, such as the 2, 4-D for *A. globiformis* [93] and atrazine for *A. aurescens* [94], as well as heavy metal metabolism, such as *Arthrobacter sp*. [95]. More generally, *Arthrobacter* is a ubiquitous genus for which the soil constitutes the main environment. Several species of this genus were isolated from various biotopes (skin, cheeses, diverse animals, insects, plants, etc.), among which the species that are considered as pathogens *A. cumminsii*, *A. luteolus*, *A. oxydans*, *A. nasiphocae*, *A. gandavensis*, and *A. woluwensis*. These species were previously isolated by sampling humans (urinary infections, infection of the skin, the blood, etc.). However, no track of pathogenic potential has ever been reported for *A. agilis*, which is typically described in the literature as a commensally organism. The success of a biological control agent in turning-on plant defense mechanisms against pathogens depends of their ability to establish metabolically active populations in the host that could mediate host protection and/or compete directly or indirectly with the pathogens for nutrient resources [12,18,22,96]. This could be the case for *A. agilis* that appears as an active palm tree colonizer.

Interestingly, the findings of the present work indicate that *A. agilis* exhibits a number of promising activities that makes this strain an attractive candidate for potential biocontrol applications against *Fusarium*, which, though with different degrees of severity and emergency, constitute one of the major actual or looming threats to the date palm plantations in the Mediterranean as well as other regions throughout the world. The validation of this point requires however comparative studies in infected areas and the *in vivo* confirmation of the activity of *A. agilis*, a work that cannot be performed in Tunisia for obvious epidemiologic reasons. Indeed, the Bayoud disease which appeared over 150 years ago in Marocco has not yet affected Tunisian palm groves. This fact results from strict regulations which have of course to be maintained.

However, our study offers potential for additional active anticipation against the disease progression.

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