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Isolation and Characterization of *Rhodococcus* spp. from Pistachio and Almond Rootstocks and Trees in Tunisia

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Abstract: The purpose of this study was to isolate and identify *Rhodococcus* spp. strains from almond and pistachio rootstocks and trees in Tunisia. Twenty-eight strains were identified through 16S rDNA and *vic*A genes amplification and sequencing. Pea bioassay was performed to determine the pathogenicity of the strains. Representative 16S rDNA and *vic*A sequences of eight strains from pistachio and seven strains from almond were closely related (>98% similarity) to *Rhodococcus* spp. accessions in GenBank. Phylogenetic analysis based on 16S rDNA sequences revealed that the yellow-colored strains clustered with phytopathogenic *Rhodococcus fascians*. The red and orange-colored strains were separated into a different group with *R. kroppenstedtii* and *R. corynebacteiroides* isolates. Eleven strains affected the pea seedlings' growth and exhibited different levels of virulence. The number of shoots was significantly higher in seedlings inoculated with four *Rhodococcus* strains, whereas the other three strains caused up to 80% of plant height reduction and reduced root secondary growth compared to non-inoculated pea seedlings. These strains, most of which are epiphytes from asymptomatic hosts, showed strong pathogenicity during pea bioassay and were established endophytically in pea tissues. Ten *att* and five *fas* genes were detected in four strains and may represent a novel model of plant pathogenic *Rhodococcus* virulence. The results of our survey showed that *Rhodococcus* is present but not prevalent in all visited orchards of almond and pistachio rootstocks and trees. Our surveys complemented the investments being made on ornamental species in Tunisia and unveiled the presence of undocumented plant-associated *Rhodococcus* spp. on economically important crops.

Keywords: *Rhodococcus*; epiphyte; inoculation; endophyte; pathogenicity; virulence genes

1. Introduction

Members of the genus *Rhodococcus* are Gram-positive bacteria, most of which are benign, and they have been frequently found in hydrocarbon-contaminated soils and from extreme environments [1–5]. Several plant-associated *Rhodococcus* spp. isolates have been found in the rhizosphere [6], phyllosphere [7], and endosphere of plants [8–10], most of which are plant growth-promoters during the epiphytic stage [11–14], and others acquiring virulence factors can cause disease [15–17].

However, despite the association of several *Rhodococcus* isolates with plants, *Rhodococcus fascians* remains the only well-described plant pathogenic species [15,18,19]. *R. fascians* causes leafy galls [20–23] through the secretion and modulation of cytokinin pathways strictly encoded by genes of the *fas* operon located on the linear plasmid pFiD188 [24–30]. *Fas*R, an AraC type regulatory gene [31], *fas*A gene coding for a P450 monooxygenase [25], and *fas*D, an isopentenyl transferase [32], are confirmed to be necessary for pathogenesis of the leafy gall *R. fascians* inducer [15–17,33]. The ability of *R. fascians* to cause disease has

been found to be dependent on another locus of the pFiD188, the *att* locus that is essential for full virulence through the production of an autoregulatory compound [32,34]. In this locus, the presence of virulent and avirulent strains of *R. fascians* has been tested by the detection of the *att*A, *att*B, and *att*R virulence genes [15–17,35].

New plant species have been identified as hosts of the plant pathogenic *R. fascians* and other newly reported members of the genus *Rhodococcus* [33,36–40]. Two *Rhodococcus* isolates designated as PBTS1 and PBTS2, one of which is a *R. fascians*, have resulted in a significant loss to the US pistachio industry [36,37]. *Rhodococcus* PBTS isolates have shown a synergistic relationship altering plant development of UCB-1 pistachio rootstocks [33,37] and recently of tobacco sp. [33]. However, the pathogenic strategies deployed by these isolates are yet unknown and appear to be different from the leafy gall inducers [18,33]. Similar to PBTS, two new plant diseases in Tunisia caused by unique isolates of *Rhodococcus* spp. have been documented on ornamental plants originating in tissue culture [38,39]. The discovery of these two new pathosystems further demonstrates the risk of clonal plants to formerly undocumented isolates of *Rhodococcus*.

Pistachio (*Pistacia vera* L.) and almond (*Prunus amygdalus* Batsch) nut crops are considered among the most profitable nut crops production in the world [41], and they continue to increase every year in acreage and yield throughout the world [42]. Pistachio and almond production in Tunisia is very low and shares only 1% of the world production [42]. Currently, more than 250,000 and 30,000 ha are planted with almond and pistachio trees in Tunisia, respectively [43]. The production of these crops is based on the use of locally adapted clones generally located in the arid and semi-arid central and southern areas [43–45]. The production of pistachio is based majorly on one local pistachio cultivar "Mateur" that is either grafted on *P. vera* or *P. atlantica* rootstocks [46], whereas the almond production is based on diversified local cultivars propagated onto rootstocks of a number of interspecific hybrids including Garnem (peach hybrid: 'Garfi' x 'Nemared'), bitter almond (*Prunus amygdalus* var. amara), and GF677 (Prunus hybrid: *P. amygdalus* x *P. persica*) [43,45]. Despite the large cultivated area and the favorable warm region in Tunisia, the low genetic diversity of cultivars and rootstocks and traditional cultural practices are leading to sensitivity of almond and pistachio trees to both biotic and abiotic stresses [43,46]. Moreover, pests and diseases are considered as serious problems for nut crop production in the temperate zones and the Mediterranean regions, resulting in yield reduction and poor quality of the fruit [47–50].

The epidemiological background on the transmission and survival of plant pathogenic *Rhodococcus* spp. on nut crops [51] combined with disease emergence in propagation facilities and orchards urged the investigation of the risk to nut tree production in Tunisia. Since the prevalence of *Rhodococcus* spp. on permanent crops in Tunisia is unknown, the goal of this study was to determine whether *Rhodococcus* spp. are common or perhaps even ubiquitous epiphytes or endophytes of almond and pistachio, especially those propagated with ornamental plant species.

2. Materials and Methods

2.1. Surveys, Sites of Prospection and Sampling

During 2018 and 2019, many regions of the main pistachio and almond-producing areas in Tunisia were surveyed for the presence of *Rhodococcus* spp. Surveys were conducted visiting 30 orchards and 19 commercial nurseries located in Gafsa, Kasserine, Sidi−Bouzid, Manouba, Tunis, Kef, and Bizerte governorates. Investigated species and varieties of almond included bitter almond (*Prunus dulcis*), Garnem (*Prunus persica* x *Prunus amygdalus*), GF−677 (*Prunus persica* x *Prunus amygdalus*), 'Mazetto' green almonds (*Prunus amygdalus*), and Lauranne (*Prunus amygdalus*). Investigated pistachio species included majorly the *Pistacia vera* cv. 'Mateur' widely used as a rootstock and scion, and the pistachio rootstock *Pistacia atlantica*. Other pistachio scion varieties including 'Kerman', 'Meknassy', 'Ohadi', 'Safeed', 'Red Aleppo', 'Thyna 1', 'Thyna 2', 'Nouri', 'Lybie rouge', 'Achouri', 'Amri', 'Razzai', and 'Jebari' grown in the research station in Sfax governorate were also examined.

Ten pistachio and ten almond rootstocks were excavated with roots intact from each nursery. From each rootstock tree, ten leaves, ten stem segments (5 × 5 mm each), and ten root segments (1 cm each) were sampled for epiphytic and endophytic isolations. Twenty trees were randomly selected per orchard, and twenty leaves per tree were sampled for the determination of epiphytic and endophytic populations of *Rhodococcus* spp. We collected a bulk sample from asymptomatic trees and individual samples from symptomatic trees.

2.2. Bacterial Isolation and Growth Conditions

To detect epiphytic populations of *Rhodococcus* spp., 10 leaves from each sample were incubated in 50 mL of 1X sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7, 4). Ten stem fragments (1 cm each) were cut from each rootstock and incubated in 20 mL of sterile PBS buffer. Ten root segments (5 cm each) were excised from the rootstock tree, briefly washed under running tap water, and then incubated in 20 mL of sterile PBS buffer. All plant tissue samples incubated in the PBS buffer were placed on a rotary agitator for 30 min at room temperature with gentle shaking. The resulting suspensions were serially diluted up to 10^{-7} and 200 µL of each dilution of 10^{-5} through 10^{-7} were plated onto D2 medium, which is a semi-selective medium for Gram-positive *Corynebacterium* [52] (Kado and Heskett 1970) amended with cycloheximide (2%) and pimaricin (4.0 mg L^{-1}) [38,39]. To detect endophytic populations of *Rhodococcus* spp., the same plant tissues used for epiphytic isolation were surface disinfected as described in [38,39] and macerated in sterile distilled water (SDW). The resulting macerated plant tissues were assessed for serial dilutions and plating as described above. Isolation plates were incubated in the dark at 27 °C for 4 to 15 days for the detection of epi− and endophytic populations, respectively.

2.3. Identification of Bacterial Isolates and Virulence Detection

Bacterial colonies with colors ranging from cadmium yellow and deep chrome yellow to deep orange were selected for further analysis. Bacterial cultures were re-streaked onto D2 medium for single pure colonies, incubated for 2 days at 27 °C, and used for genomic DNA extraction and molecular characterization. DNA extraction was performed following the protocol used by innuPREP Bacteria DNA extraction Kit (Analytic Jena AG, Germany). Then, genomic DNA was visualized on 1, 5% agarose gel stained with ethidium bromide and photographed on a UV-transilluminator. DNA concentration (ng µL^{-1}) was also determined using a spectrophotometer (Maestro Nano GEN MN−913) for better PCR optimization. All DNA samples were stored in TE buffer (10 mmoL L^{-1} Tris, pH 8, 1 mmoL L^{-1} EDTA) at −20 °C until analyzed.

Amplifications and sequencing of the 16S rDNA gene and the specific chromosomal *R. fascians vic*A gene [35,53,54] were used to identify the bacterial isolates as indicated in Table 1.

Table 1. Primers nucleotide and PCR mix, cycling time, and conditions used in this study.

Primer	Nucleotide Sequence (5′ → 3′)	Target	Amplicon Size (bp)	Reference	Cycling Conditions	Total Amount (25 μL/PCR)
27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	16S ribosomal DNA	1500	[53]	1 cycle of initial denaturation: at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 90 s; 1 cycle of final extension at 72 °C for 5 min	50 ng: DNA 1X: Taq buffer 0.2 mM: dNTPs 4 mM: MgCl$_2$ 24 pmol: each primer 1U: Taq DNA polymerase
vicA1497FvicA1990R	TCTGGATCTCGAAGTGCAAACCGT AGCGTACAAGGCCTTCCTGAAAGA	Putative malate synthase	179	[54]	1 cycle of initial denaturation: at 95 °C for 5 min; 45 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 90 s; 1 cycle of final extension at 72 °C for 5 min	50 ng: DNA 1X: Taq buffer 0.24 mM: dNTPs 2 mM: MgCl$_2$ 10 pmol: each primer 1U: Taq DNA polymerase
JPEL JPER	GGGAATTCCGACCGTATCCAGTGT CGGGATCCATATCGAACCGCCCTC	*fas*−1 isopentenyl-transferase	225	[55]	1 cycle of initial denaturation: at 94 °C for 3 min; 33 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 60 s, and extension at 72 °C for 40 s; 1 cycle of final extension at 72 °C for 5 min	10 ng: DNA 1X: Taq buffer 0.4 mM: dNTPs 4 mM: MgCl$_2$ 10 pmol: each primer 1U: Taq DNA polymerase
p450−F p450−R	TATCCTTGCTGCGGAGTTCT CAACCACCGCAATAATTCCT	*fas*A, P450 monooxygenase	538	[56]	1 cycle of initial denaturation: at 94 °C for 2 min; 33 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s; 1 cycle of final extension at 72 °C for 5 min	50 ng: DNA 1X: Taq buffer, 1.5 mM: MgCl$_2$, 0.2 mM dNTPs, 10 pmol: each primer, 1U: Taq DNA polymerase.
Fas−F *Fas*−R	CAACACTACTTTGCCCAGCA GGCCAACTCCTCTGGTGTTA	*fas*D, isopentenyltransferase	195			
fasR F fasR R	ATCAACGTCGACCTCGGAAT GCACGGGTTACAGTCATT	*fas*R gene, putative transcriptional regulator	688	[31]		
attA−585F attA−879R	GCCTGGAAGCGCATCAACATCAAT TTCTTCTGCGGCATGATCGAGCTA	*att*A, Arginino−succinate lyase	505	[35]	1 cycle of initial denaturation: at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s, and extension at 72 °C for 60 s; 1 cycle of final extension at 72 °C for 5 min	10 ng: DNA 1X: Taq buffer 0.2mM: dNTPs 2 mM: MgCl$_2$ 10pmol: each primer 1U: Taq DNA polymerase
attR−683F attR−887R	GGTGCAGCAGTATTCGTTGTCGTT TGCACATCTCGTCTTCTGCAGTCA	*att*R, LysR type transcriptional regulator	320			

The detection of virulent *Rhodococcus* strains was performed targeting plasmid-borne virulence genes of the *fas* and *att* loci using published primers (Table 1) [34,35,54–56]. PCR cycling conditions of each specific gene, including *fas*A, *fas*D, *att*A, and *att*R genes were optimized by varying the annealing temperature, dNTPs, and $MgCl_2$ concentrations and the amount of DNA template. Primer sequences and PCR amplification conditions are given in Table 1. All PCR amplifications were performed with the Thermo Cycler 2720 (Applied Biosystems, Foster City, CA, USA, Thermo Fisher Scientific, Waltham, MA, USA) according to the protocols presented in Table 1.

The detection of *fas* and *att* genes was also determined from the whole genome sequences of three *Rhodococcus* strains. The selection of these three *Rhodococcus* strains, identified as *R. fascians* GS6, *R. fascians* SB10, and *R. kroppenstedtii* K5 [40,57], was based on their high pathogenicity effect during pea bioassay (this study). Each virulence gene from pFiD188 plasmid (GenBank accession number JN093097) was used to identify homologs of genes on GS6, SB10, and K5 genomes. The translated sequences of *att*A, *att*R, *fas*R, *fas*A, and *fas*D were used in BLAST searches against the NCBI nr database to identify homologous sequences. Sequences from pFiD188 (pFi_001−pFi_184) and 4804 protein sequences of the chromosome of D188 (NCBI Reference Sequence: NZ_CP015235.1) were downloaded and used as queries in searches against GS6, SB10, and K5 genome assemblies. Protein database searches were performed using compositionally adjusted substitution matrices [58] and Gapped BLAST and PSI−BLAST programs [59].

2.4. Multilocus Analysis

Nearest-neighbor phylogenetic trees based on 16S rDNA and *vic*A gene sequences were constructed using MEGA software version 6.06 [60]. Chromatograms of DNA sequences were analyzed using Chromas trace software version 2.6.6 (Technelysium, Pty Ltd., Brisbane, Australia). Publicly available 16S rDNA and *vic*A sequences of plant-associated and environmental type strains of *Rhodococcus* were collected from the NCBI database. Sequence reads were assembled in MEGA6 and aligned using the ClustalW algorithm. Phylogenetic trees were constructed using Tamura–Nei models [61] for neighbor-joining maximum likelihood with 1000 bootstrap replicates [62] and a support threshold of 70%.

2.5. Pathogenicity on Pisum Sativum

2.5.1. Bacterial Strains

Pure cultures of *Rhodococcus* strains were grown on D2 medium for 2 days at 27 °C. Each *Rhodococcus* strain was inoculated in a 100-mL Erlenmeyer flask containing 40 mL of nutrient broth (NB) and incubated for 2 days at room temperature (25 to 28 °C) with shaking at 200 rpm [63]. After two overnights, the culture was transferred to 50-mL centrifuge tubes and centrifuged for 15 min at 15 °C and $3000\times g$ [63]. The supernatant was discarded, and the pellets were resuspended in SDW [63]. Then, the cell suspensions were pooled and diluted to an Optical Density (OD) at 600 nm of 0.18 (advanced microprocessor UV/VIS Spectrometer, single beam Li−295, Lasany, MA, USA) in SDW, corresponding to approximately 2.5×10^7 CFU mL^{-1} [35]. Bacterial suspension was kept at 4 °C for a subsequent use within 2 h.

2.5.2. Pea Bioassay

Following the protocol described by [35], *Pisum sativum* var 'Lincoln' seeds were surface disinfected briefly in 70% ethanol and rinsed in 10% sodium hypochlorite for 10 min and washed three to four times in SDW. Following disinfection, seeds were soaked in SDW for one hour and then placed on 1.5% water agar for 4 days at 20 °C until germination. Once the radicals emerged, seedlings were soaked in 20 mL of each *Rhodococcus* suspension for 2 h and then placed in tubes containing Hoagland's medium. Control pea seedlings were soaked in sterile Hoagland's solution. Ten seedlings were used per treatment, and the bioassay was repeated twice. Inoculated pea seedlings were placed in a growth chamber with a 12-h photoperiod at 18 °C for two weeks. The main stem length and the number of

lateral shoots at the cotyledon node of each pea seedling were measured at 14 days post inoculation (dpi). The degree of virulence was based the on the percentage of pea seedlings showing stunting and fasciations as well as the presence of the *fas* virulence genes.

2.5.3. Re-Isolation of the Pathogen

Epiphytic and endophytic *Rhodococcus* strains were re-isolated from inoculated and uninoculated pea seedlings at 14 dpi, and the presence of the *vic*A and *fas* genes among these populations was determined by PCR amplification. Three symptomatic and three asymptomatic pea seedlings from each treatment were used for epiphytic and endophytic pathogen re-isolation. The aerial part of the inoculated pea seedling, including galls and lateral shoots, was separated from the roots and suspended in 20 mL of sterile PBS buffer and incubated for 20 min with gentle shaking. Serial dilutions up to 10^{-10} were determined and 200 μL from each of 10^{-8} to 10^{-10} dilutions was streaked onto D2 medium. For endophytic isolation of *Rhodococcus* strains, the same tissue sample was briefly surface disinfected in 70% of ethanol and then in 10% of sodium hypochlorite for 30 s and rinsed four times in SDW [35]. The surface-disinfected aerial part of the pea seedling was macerated with a sterile mortar in sterile PBS buffer. The resulting suspension was serially diluted to 10^{-10}, and 200 μL from each of the 10^{-8} to 10^{-10} dilutions was streaked onto D2 medium. Bacterial colonies were streaked onto D2 medium for purification, genomic DNA extraction, and PCR amplifications.

2.6. Statistical Analysis

The influence of inoculation treatments on stem length and lateral shoots of pea seedlings was determined with an analysis of variance (ANOVA) and means were separated using a Student–Newman–Keuls test (SAS 9.1; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Prospection and Sampling

Among the 30 visited pistachio and almond orchards, two orchards growing pistachio trees in the governorates of Kef and Bizerte yielded *Rhodococcus* spp. isolates (Table 2). The pistachio trees in these orchards exhibited multiple pale shoots at the crown with small leaves (data not shown). Among the 19 visited commercial nurseries, one nursery producing fruit and nut rootstock trees located in Kasserine governorate had bitter almond rootstocks showing abnormal growth (data not shown). More than 80% of these rootstocks exhibited bushy phenotype, shoot proliferation, severe stunting, and compact growth due to reduced internodes length (data not shown). However, one endophytic *Rhodococcus* isolate was obtained from the unusual growth of bitter almond rootstocks. In the same aforementioned nursery, *P. vera* and almond 'GF' rootstock trees exhibited similar symptoms but did generate *Rhodococcus* spp. (data not shown). Asymptomatic almond rootstocks and pistachio trees grown in five different commercial nurseries and one orchard have been found to harbor *Rhodococcus* spp. isolates (Table 2).

Table 2. *Rhodococcus* spp. isolates from pistachio and almond plants grown in a commercial nursery and orchard sources in different regions of Tunisia.

Nursery/Orchard	Isolate Code	Location	Crop	Rootstock/Scion Cultivars	Symptomatic/Asymptomatic	Epi−/Endophytic	Isolate Color	16S rDNA Gene GenBank Accession Number	*vic*A Gene GenBank Accession Number
Nursery	JZ1	Gafsa	Almond	Bitter almond	Asymptomatic	Epiphyte	Yellow chrome	MN366362	*
	JZ2			GF		Epiphyte	Yellow chrome	MN366363	MN544259
	BA2	Manouba	Almond	Mazetto/GF	Asymptomatic	Epiphyte	Red–orange	MN366364	MN544260
	BA3					Epiphyte	Yellow chrome	MN366365	MN544261
	F1	Tunis	Almond	GF	Asymptomatic	Epiphyte	Yellow chrome	MN366366	MN544262
	GS6	Kasserine	Almond	Bitter almond	Symptomatic	Endophyte	Yellow chrome	MK455765	MN544263
	SB10	Sidi Bouzid	Almond	Garnem	Asymptomatic	Endophyte	Yellow chrome	MK455764	MN544264
	CS	Gafsa	Pistachio	*P. vera* var. Mateur	Asymptomatic	Epiphyte	Yellow chrome	MN366367	*
Orchard	K4	Kef	Pistachio	*P. vera* var. Mateur	Symptomatic	Epiphyte	Yellow chrome	MN366368	MN544265
	K5					Epiphyte	Red–orange	MK455766	MN544266
	Mt1	Bizerte	Pistachio	*P. vera* var. Mateur	Asymptomatic	Epiphyte	Yellow chrome	MN366369	MN544267
	Mt2			*P. atlantica*	Symptomatic	Epiphyte	Yellow chrome	MN366370	MN544268
	Mt5					Epiphyte	Red–orange	MN366371	MN544269
	Mt9			*P. vera*	Symptomatic	Epiphyte	Yellow chrome	MN366372	MN544270
	Mt11					Epiphyte	Red–orange	MN366373	*

*: Bad DNA sequence quality, not acceptable for GenBank accession submission.

3.2. Isolation and Identification of Bacterial Isolates

Epiphytic and endophytic *Rhodococcus* spp. isolates were obtained from foliage of almond and pistachio rootstocks and trees (Table 2). Yellow and deep-orange pigmented, rough, and smooth bacterial colonies were observed 7 days post-incubation at 27 °C (Table 2). However, putative yellow-colored *Rhodococcus* colonies were the most abundant colonies over the orange ones (data not shown). In many pistachio and almond commercial nurseries and field orchards, yellow and orange-colored *Rhodococcus* isolates were isolated from the same leaf tissue (Table 2). Overall, most of the *Rhodococcus* strains were phylloplane of pistachio and almond leaves. No epiphytic or endophytic *Rhodococcus* strains were obtained from pistachio and almond roots or stems. Only two endophytic *Rhodococcus* strains were obtained from leaves of bitter almond and Garnem rootstocks propagated in the commercial nursery of Kasserine (Table 2). A total of 28 bacterial isolates were cultured for genomic DNA extraction and PCR amplification. Only fifteen isolates generated positive *vic*A amplicons and were therefore used for subsequent 16S rDNA gene identification. Based on sequence analysis of the 16S rDNA and *vic*A genes, eight *Rhodococcus* strains were identified from pistachios and seven *Rhodococcus* strains were identified from almonds (Table 2). Representative 16S rDNA and *vic*A sequences of *Rhodococcus* strains were deposited into GenBank, and accessions numbers are presented in Table 2.

3.3. Multilocus Analysis of Bacterial Strains

Sequences of the 16S rDNA and *vic*A genes of the pistachio and almond *Rhodococcus* strains were highly similar (>98 %) to *Rhodococcus* spp. accessions in GenBank (Figure 1A). Based on 16S rDNA sequence analysis, the deep-orange-colored strains K5, Mt5, BA2, and Mt11 from pistachio and strain BA2 from almond were 100% similar to *Rhodococcus* sp. hm1 (MT012181.1), *Rhodococcus* sp. NQS13 (MN822627.1), *Rhodococcus* sp. OS−11 (EF612310.1), and *R. corynebacterioides* cqsA3 (MN826595.1) isolates (Figure 1A).

Based on a 16S rDNA gene-based tree, K5, BA2, Mt5, and Mt11 strains formed a distinct phylogenetic lineage with other isolates, including *Rhodococcus* sp. strain B4 (MH685557), *Rhodococcus* sp. strain N2 (MN150688), *Rhodococcus* sp. PBTS1 (CP015219.1), *R. kroppenstedtii* DSM 44908 (NR_118599.1), *R. enclensis* NIO−1009 (NR_134154.1), and *R. corynebacterioides* strains DSM 20151 (NR_119107.1), and C2 (MG280772.1) (Figure 1A).

Analysis of the 16S rDNA sequences of the yellow-colored strains from almond JZ1, JZ2, GS6, and SB10 were highly similar (99, 9 to 100%) to phytopathogenic *R. fascians* D188 (CP015235.1) and *Rhodococcus* sp. PBTS2 (CP015220.1) (Figure 1A). Some yellow-colored strains including BA3, F1, K4, Mt1, and Mt9 were highly similar (99.9 to 100%) to other strains of *R. fascians* (MK178494.1, MF620062.1, KY775507.1) and *Rhodococcus* spp. (MH725305.1, MT012184.1, MT012166.1) (Figure 1A). The remaining yellow-colored strains CS and Mt2 were highly similar to *R. sovatensis* a19 (MK726114) and *R. cerastii* (KY775508, KY775501), respectively (Figure 1A). The three almond strains JZ1, GS6, and SB10 formed a well distinct clade with phytopathogenic *R. fascians* D188 (CP015235.1) and *R. fascians* DSM 20669 (X79186.1) (Figure 1A).

Based on *vic*A sequence analysis, our almond and pistachio *Rhodococcus* strains were highly similar to *Rhodococcus* spp. isolates affecting pistachio trees in California (PBTS1 and PBTS2) and New Mexico (NM−J PBTS) and to *R. fascians* D188 (Figure 1B). However, in the *vic*A-based phylogenetic tree, two clades were generated; one supported our *Rhodococcus* almond and pistachio strains with *Rhodococcus* spp. associated with diseased ornamentals in Tunisia and pistachio in New Mexico (Figure 1B). The other clade supported environmental species and plant pathogenic of *Rhodococcus* including *R. fascians* D188, *Rhodococcus* PBTS1, and PBTS2 (Figure 1B). However, the yellow and deep-orange-colored strains were not classified separately in distinct clades as in the 16S rDNA phylogenetic tree (Figure 1A).
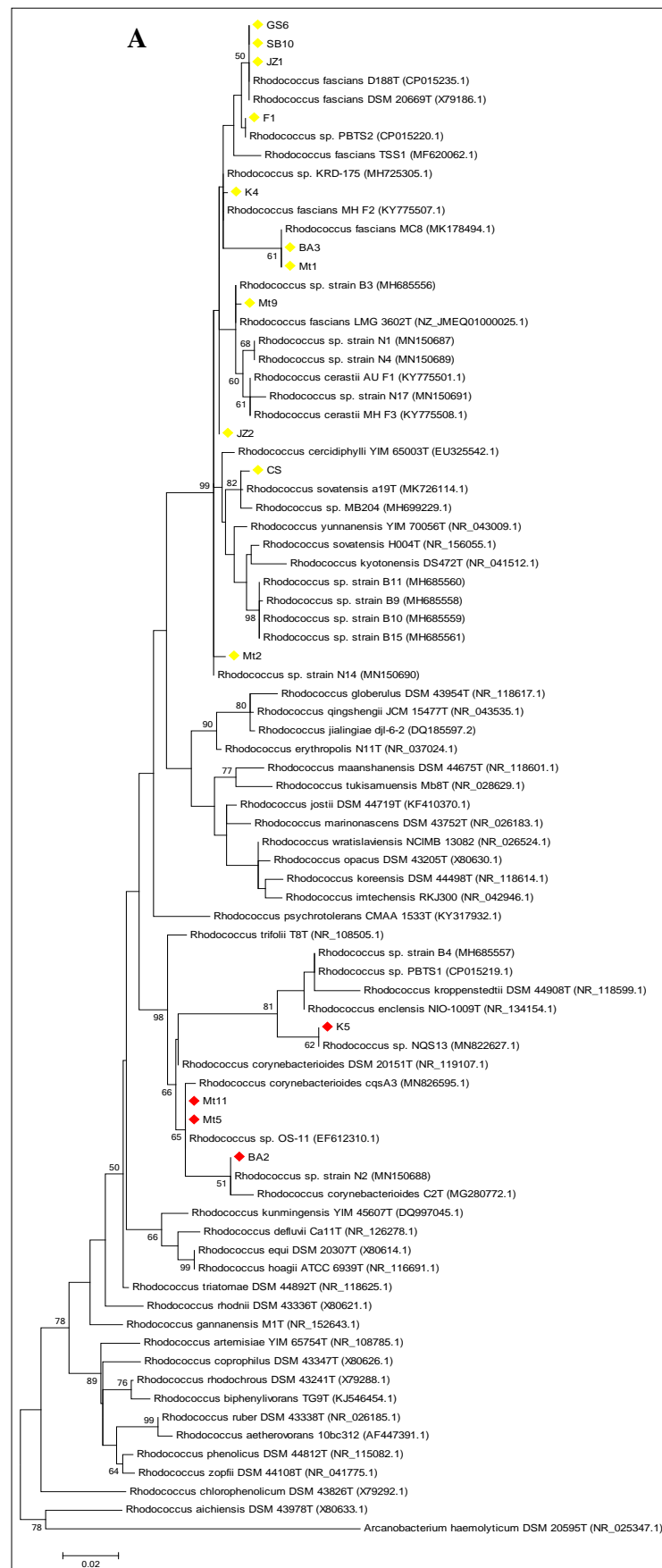
Figure 1. *Cont*.
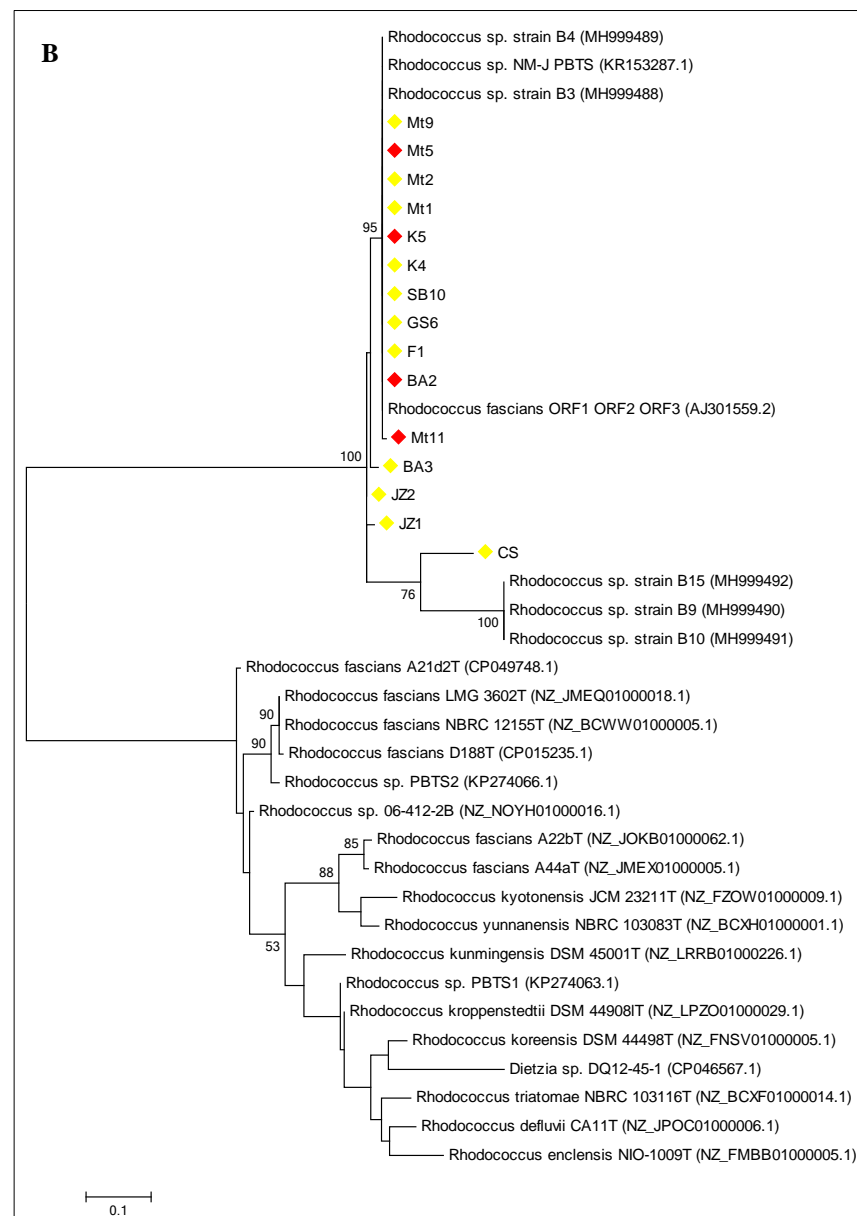
Figure 1. Phylogeny of partial 16S rRNA (**A**) and *vic*A (**B**) gene sequences of the fifteen *Rhodococcus* spp. isolates from pistachio and almond rootstocks and trees in Tunisia among different species of *Rhodococcus* spp. The tree was constructed using the neighbor-joining algorithm in MEGA. Nodes supported by greater than 50% of 1000 bootstrap replications are indicated. Our yellow strains are labeled by yellow dot markers and red–orange strains are labeled by red dot markers. Type strains were followed by the letter T.

3.4. Virulence Screening of Bacterial Strains

3.4.1. Virulence on Pisum Sativum

The pathogenicity of pistachio and almond *Rhodococcus* strains on inoculated pea seedlings was evaluated based on stem length and total number of shoots per seedling compared to the negative control. Symptoms developed on inoculated pea seedlings in this study were also compared to those caused by *Rhodococcus* spp. isolates from diseased ornamentals in Tunisia [38,39]. Fourteen days post-inoculation, most of the pistachio and almond *Rhodococcus* strains caused severe growth effects to pea seedlings showing the typical multiple shoot symptom of *R. fascians* infection (Figure 2, Table 3).

Figure 2. Phenotype of pea seedlings inoculated with pistachio and almond *Rhodococcus* strains 14 days post inoculation (dpi). (**a**) Negative control inoculated with Yeast Extract Broth. (**b**) and (**c**) Multiple shoots at cotyledon node of pea seedlings inoculated with *Rhodococcus* almond strains BA3 and BA2, respectively. (**d**) Multiple shoots and inhibited root growth of pea seedlings inoculated with *Rhodococcus* pistachio strain K5. (**e**) Leafy gall of inoculated pea seedlings with *Rhodococcus* almond strain GS6. (**f**) Multiple shoots of inoculated pea seedlings with *Rhodococcus* almond strain SB10. (**g**) Swollen stem and reduced shoot and root growth of inoculated pea seedlings with *Rhodococcus* pistachio strain Mt9. (**h**) Hypertrophied shoots of pea seedlings inoculated with *Rhodococcus* pistachio strain Mt11.

Symptoms on inoculated peas varied from multiple shoots developing at the cotyledon node (Figure 2b−h) to root growth inhibition (Figure 2d,g,h), swollen and hypertrophied shoots (Figure 2e−h), and stunted shoot and leaf growth (Figure 2b−h) when compared with negative control (Figure 2a). The same symptoms were observed in previous studies on pea seedlings inoculated with *Rhodococcus* spp. strains isolated from diseased ornamentals [38,39]. The number of shoots per seedling was significantly higher ($p \leq 0.05$) in seedlings inoculated with *Rhodococcus* strains SB10, K5, Mt5, and Mt11 compared to the negative control (Table 3). Inoculation with these strains resulted in the production of over three times the number of shoots per seedling ($p \leq 0.05$) of non-inoculated controls (Table 3). *Rhodococcus* strain SB10 from almond and *Rhodococcus* Mt11 strain from pistachio showed strong pathogenicity causing fasciations on 90 and 100% of inoculated

pea seedlings, respectively (Table 3). Moreover, another three almond *Rhodococcus* strains, BA3, F1, and GS6, showed strong pathogenicity and caused up to 60 and 80% of pea height reduction ($p \leq 0.05$) compared to non-inoculated peas (Table 3). Both epiphytic and endophytic *Rhodococcus* strains were recovered from inoculated pea seedlings but not from uninoculated peas. The *vic*A gene was successfully amplified by PCR from all of these strains, thus confirming their relatedness with abnormal growth of peas.

Table 3. Phenotypic characteristics of pea seedlings inoculated with *Rhodococcus* strains from almond and pistachio trees.

Strain	Fasciation Symptoms [x] (%)	Main Stem Length (mm) [y]	Total Number of Shoots [z]
Control	0	108.8 a ± 0.50	1 a ± 0.00
JZ1	0	61 bcdef ± 0.6	1.0 a ± 0.0
JZ2	0	73 abcdef ± 1.0	1.0 a ± 0.0
BA2	0	54 cdef ± 0.7	1.0 a ± 0.0
BA3	10	40 edfg ± 0.7	1.7 a ± 0.4
F1	20	17,1 g ± 0.14	1.3 a ± 0.21
GS6	40	32.2 fg ± 0.51	1.8 a ± 0.35
SB10	90	38 efg ± 0.48	3.3 b ± 0.33
CS	0	69 abcdef ± 1.4	1.0 a ± 0.0
K4	10	73.3 abcde ± 1.2	1.3 a ± 0.2
K5	60	58 bcdef ± 0.83	2.3 b ± 0.42
Mt1	20	85 abc ± 0.8	1.7 a ± 0.4
Mt2	0	77 abcde ± 1.4	1.0 a ± 0.2
Mt5	20	63 bcdef ± 1.1	2.3 b ± 0.8
Mt9	0	81 abcd ± 0.8	1.0 a ± 0.0
Mt11	100	59 bcdef ± 0.8	2.0 b ± 0.2

[x] Symptom development reflects the percentage of seedlings of cv. Lincoln (N = 10) showing fasciation symptoms at 14 days post-inoculation. [y,z] Means ± standard error represent the average of plant height and total number of shoots per seedling (N=10). Different letters indicate a significant difference between treatments according to Student–Newman–Keuls test at $p \leq 0.05$.

3.4.2. Fas and Att Virulence Genes Detection

PCR amplification of the plasmid-borne virulence genes *fas* and *att* was performed using *Rhodococcus* strains isolated from host of origin and those recovered from inoculated pea seedlings. PCR amplifications of *fas*-1, *fas*D, and *att*A genes in our *Rhodococcus* strains consistently produced multiple PCR products of various sizes (data not shown). However, amplicons of the expected sizes of *fas*-1 (225 bp), *fas*D (195 bp), and *att*A (505 bp) genes were detected in most of our strains using JPEL/JPER, *Fas*-F/*Fas*-R, and attA-585F/attA-879R primers, respectively. The primers, p450-F/p450-R designed for *fas*A gene amplification from pathogenic isolates D188 failed to yield a product from our strains. Additionally, the *att*R and *fas*R genes, predicted to encode LysR-type and AraC-type transcriptional regulators, respectively, and necessary for pathogenicity have not been detected in our strains using attR-683F/attR-887R and *fas* R F/*fas* R R primers.

After repeated attempts of PCR optimizations to detect clean PCR amplicons, the sequenced PCR fragment failed to give similar homologs to *fas*-1, *fas*D, and *att*A genes. However, there are seven and eight mismatches between the two primers JPEL and JPER designed to amplify the *fas*-1 gene and the *fas* sequence in pFiD188 (Supplementary Materials File F1). In order to investigate the characteristic virulence of our strains, we have sequenced the complete genome of *R. fascians* strains GS6 and SB10 from almond and *R. kroppenstedtii* strain K5 from pistachio (Dhaouadi et al., 2020b), which showed strong

pathogenicity effect on peas (this study). CDSs homologous to the *att* locus (attABCDE-FGHRX genes), *fas*R, *fas*B, *fas*C, *fas*E, and *fas*F genes of pFiD188 were present in the genome sequences of these strains (JAAFYX000000000, JAAFYW000000000, JAAFYU000000000). The Mtr1 and Mtr2 genes encoding a SAM (S-adenosylmethionine-dependent methyl-transferase) were also generated from these strains. No *fas*D (isopentenyl transferase) or *fas*A (putative p450 monooxygenase) genes, which were key virulence genes of the model strain D188 of *R. fascians*, were detected in our strains. The results of TBLASTN against the 4804 protein sequences of the chromosome of D188 and our *R. fascians* SB10 and GS6 assemblies showed that these two assemblies are quite closely related to D188 with ≈1/3 of the D188 proteins having >99% match to the genomes, which are very different from the linear plasmid results (data not shown).

4. Discussion

Previous studies on the occurrence of plant pathogenic *Rhodococcus* spp. isolates focused on ornamental plant species propagated in commercial nurseries (Dhaouadi et al. 2019, 2020a) with no data on the prevalence of *Rhodococcus* spp. on permanent crops in Tunisia. The present study has shown that *Rhodococcus* spp. isolates are present in different regions of the country growing pistachio and almond rootstocks and trees. While most of the isolates obtained from these crops were leaf epiphytes, only GS6 and SB10 strains were endophytes of leaf almond rootstocks. One commercial nursery in Kasserine governorate propagating fruit tree rootstocks had bushy almond rootstocks with stunted shoot growth with multiple shoots and considerable compact growth. The one endophytic strain obtained from these almond rootstocks, identified as *R. fascians* strain GS6 [57], caused severe growth effect on peas. However, the original cause of the symptoms observed on almond rootstocks is yet to be determined.

Since the identification based on 16S rDNA and *vic*A genes of our strains was in-sufficient in discriminating between species, a previous study [57] on concatenated gene sequence comparisons identified several of our strains as *R. fascians* and others as *R. kroppenstedtii*, while some remained unclassified and may represent novel species. The *vic*A gene has been reported to be implicated in *R. fascians* pathogenicity [35,64], but it was recently detected in red–orange *Rhodococcus* isolates associated with Pistachio Bushy Top Syndrome [36,37] and diseased ornamentals [38,39]. The *vic*A-based phylogenetic tree showed that our strains are separated from the plant pathogenic *R. fascians* D188 and the *Rhodococcus* PBTS isolates. However, many members of the Actinobacteria, including 407 *Rhodococcus* isolates, harbor this gene [16], and therefore, this chromosomal locus cannot be used as a phylogenetic trait in classifying plant pathogenic members of *Rhodococcus* neither in resolving members of this genus.

Eleven *Rhodococcus* strains obtained in this study showed a high degree of pathogenicity on pea seedlings. Strains related to *R. fascians* including SB10, BA3, F1, and GS6 strains were the most pathogenic, causing multiple shoots and reduced plant height and root growth of pea seedlings. Two of these strains, BA3 and F1, are epiphytic strains obtained from asymptomatic almond trees. Moreover, the red–orange *Rhodococcus* sp. Mt11, an epiphytic strain obtained from asymptomatic pistachio trees caused a systemic reaction in 100% of inoculated peas. Previous studies indicated that only endophytic virulent strains of *R. fascians* are capable of affecting the morphology of the plant, while epiphytic strains are nonpathogenic [16,28,29]. In our study, the epiphytic strains BA3, F1, and Mt11 have been established endophytically in peas and therefore are pathogenic. However, pea bioassay has been implemented for the evaluation of the pathogenicity and may not reflect what would occur on other host plants. For this matter, pathogenicity assays on pistachio and almond rootstocks are necessary.

The detection of virulence genes in our strains using published primers to target the plasmid-borne *fas* and *att* genes of *R. fascians* has been problematic, yet these strains influenced pea development. However, *fas*R and the 10 *att* genes have been found on contigs of the complete genome sequence of GS6, SB10, and K5 strains [40]. Surprisingly,

none of the other genes on the contigs of these strains are homologous to those on pFiD188 (S, Dhaouadi, personal communication). The ability of these strains and the other epiphytic strains to invade pea seedlings and produce symptoms may be conferred by chromosomal genes [14] or models of plant pathogenic *Rhodococcus* virulence are different from the conserved virulence plasmid pFiD188 of *R. fascians* D188 [15,17].

Epidemiological studies and the genomic characterization of *Rhodococcus* isolates from different nurseries suggested that these bacteria are transmitted due to independent introductions, reservoir populations, and point source outbreaks [16]. Recent findings illustrated the presence of plant pathogenic *Rhodococcus* spp. in two independent commercial nurseries in Tunisia causing diseases on *I. herbestii* and *E. japonicus* plants [38,39]. Consequently, the propagation of herbaceous plants and fruit trees in the same propagation facilities may represent a risk, since plant pathogenic strains of *Rhodococcus* infect primarily herbaceous plants [65]. Overall, sanitation from greenhouse to field situations is paramount to the prevention of a PBTS-like outbreak on pistachio and almond in Tunisia and the Mediterranean Basin.

5. Conclusions

The data in this paper could be used toward the development of a phylloplane ecology study that addresses the putative risk of these bacteria to economically important crops. We have documented for the first time in Tunisia a collection of a series of *Rhodococcus* isolates that includes eight strains from pistachio and seven strains from almond leaves. Ecological and pathological studies on members of this Genus are valuable for mitigation of future plant disease epidemics.

Author Contributions: Conceptualization, S.D., A.M.H. and A.R; methodology, S.D. and A.M.H.; software, S.D.; validation, S.D., A.M.H. and A.R.; formal analysis, S.D.; investigation, S.D. and A.M.H.; resources, A.R.; data curation, S.D. and A.M.H.; writing—original draft preparation, S.D. and A.M.H.; writing—review and editing, A.R.; visualization, A.R.; supervision, A.R.; project administration, A.R.; funding acquisition, A.R. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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