ANALYSIS OF *AGROBACTERIUM* POPULATIONS ISOLATED FROM TUNISIAN SOILS: GENETIC STRUCTURE, AVIRULENT-VIRULENT RATIOS AND CHARACTERIZATION OF TUMORIGENIC STRAINS

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SUMMARY

The aim of this work is to assess the presence of agrobacterial populations in a variety of Tunisian soils to provide information regarding the diversity of strains occurring in agricultural soils. Twenty five soil samples were collected from seven different regions, which had or didn't have a history of crown gall. *Agrobacterium* spp. members were dominant in Tunisian soils, isolated from 23 samples and assigned to the genomospecies G4, G7 and G9 by specific primers. The majority of samples yielded strains belonging to more than one genomospecies and showed a predominance of G4 and/or G7 members. Partial *recA* gene sequencing revealed new alleles and a high allelic diversity at both local and country scales. BOX-PCR fingerprinting of non-tumorigenic strains from dominant alleles did not show a clear correlation with geographic origin/soil plantation type, or a clear clonal spread of *Agrobacterium* strains in Tunisian soils. Ti plasmid-containing strains were only recovered from soils of fields with evidence of crown gall disease, and were exclusively allocated to the genomospecies G4. Tumorigenic strains isolated from soils with galled grapevines were distinct from tumorigenic strains isolated from soils with galled stone fruit trees, based on *recA* sequences, Ti plasmid type, sensitivity to the strain K84 and L-tartrate utilization.

Keywords: *rec*A, BOX-PCR, Ti plasmid, strain K84, L-tartrate utilization.

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INTRODUCTION

The soil-borne tumorigenic agrobacteria cause a disease known as crown gall on at least 643 different plant species (De Cleene and De Ley, 1976). Crown gall causes significant negative economic impact on a wide variety of woody perennials, particularly in the Mediterranean basin and in several regions where tree crops are grown under Mediterranean-like climatic conditions.

Nomenclature and classification of agrobacteria species are still controversial and there is a large disagreement concerning the classification of *Agrobacterium* (*Rhizobium*) species and the genus itself (Young *et al*., 2001; Farrand *et al*., 2003; Young *et al*., 2006; Mousavi *et al*., 2015). Nevertheless, recent phylogenetic and genomic analyses propose that *Agrobacterium* must be retained as a valid genus limited to the single clade gathering *A. rubi*, *A. larrymoorei* and related species such as *A. skierniewicense* along to the so-called biovar 1 agrobacteria that are dispatched in several genomic species (or genomospecies) (Ramirez-Bahena *et al*., 2014; Kuzmanović *et al*., 2015; Mousavi *et al*., 2015). Except for *A. pusense* (G2), *A. radiobacter* (G4), "*A. fabrum*" (G8), *A. nepotum* (G14) and A. *arsenijevicii*, biovar 1 genomospecies have not yet received a Latin binomial and were thus just numbered from G1 to G9 plus G13 and G14 (Costechareyre *et al*., 2010; Lindström and Young, 2011). For their part, the former *Agrobacterium rhizogenes* (earlier called biovar 2) is now definitively designated *Rhizobium rhizogenes* (Lindström and Young, 2011), and the former *Agrobacterium vitis* (earlier called biovar 3) was proposed to be transferred to *Allorhizobium vitis* by Mousavi *et al*. (2014, 2015). In the present study, *Agrobacterium* spp. will be used to design the entire biovar 1 agrobacteria genomospecies according to the newest genus delineation proposed by Mousavi *et al*. (2014, 2015), which excludes indeed both *R. rhizogenes* and *Al. vitis*, and the term species or sp. will be used as an alternative to genomospecies.

Agrobacterium spp. members are common soil and rhizosphere inhabitants that, in their avirulent form, may

Fig. 1. Map of Tunisia showing the location of regions selected for the soil sampling: Regueb (10 samples), Gonna (5 samples), Oued Meliz (4 samples), Knaies (3 samples), BeniKhedach (1 sample), Morneg (1 sample) and Tunis (1 sample).

exhibit beneficial attributes to the plant (Hao *et al*., 2012). However, once they acquire a tumor-inducing (Ti) plasmid, agrobacteria become tumorigenic and can induce crown gall disease. Unfortunately, some soils facilitate the persistence of tumorigenic *Agrobacterium* for decades; this may render these soils unsuitable for the culture of susceptible crops (Krimi *et al*., 2002).

In Tunisia, a serious outbreak of crown gall in nurseries occurred in the early 1980's after the importation of contaminated stone fruit plants. To stop the propagation of the disease, the Tunisian Agriculture Ministry authorized the use of crops from nurseries only if they exhibited less than 1% of visible galls (Anonymous, 1981), while all the contaminated plants were destroyed and the production in the contaminated nurseries was halted (Boubaker, 1999; Rhouma *et al*., 2005). Until 2010, this sanitization practice was efficient and symptoms were limited to the almond trees in nurseries and particularly to some regions in the North and center of Tunisia. At that time, a serious problem was reported in vineyards and some peach orchards, in the nearby regions (Abdellatif *et al*., 2013), suggesting the possibility of contaminations through naturally infested soils. Interestingly, the presence of crown gall disease has never been reported from the southern region of Tunisia. In fact, Tunisian studies of *Agrobacterium* have been limited to tumorigenic isolates and to the indigenous population found in galls (Rhouma *et al*., 2001, 2006; Costechareyre *et al*., 2010). To our knowledge, there has not been any exhaustive search for the presence of *Agrobacterium* across a wide geographic range in Tunisian soils. In addition, little is known about the presence and genetic diversity of either tumorigenic or non-tumorigenic agrobacteria, and what impact this may have on the epidemiology of crown gall disease. Since Ti plasmids are conjugative, Ti plasmid-free agrobacteria can readily acquire the Ti plasmid rendering non-tumorigenic strains tumorigenic. As a result, both conjugant and trans-conjugant agrobacteria may contribute to the spread of the disease and the perennial soil contamination. To provide an initial database of *Agrobacterium* spp. populations in Tunisian agricultural soils, the work reported here addresses two topics: i) the presence of tumorigenic and non-tumorigenic agrobacteria in soils across Tunisian regions that had or didn't have a history of crown gall disease and ii) the genetic diversity of *Agrobacterium* spp. and the characteristics of tumorigenic strains on both a field and country-wide scale.

MATERIALS AND METHODS

Soil sampling, bacterial isolation and reference strains. Bulk soil samples (approx. 50 g per site) were collected from a soil depth of 10-20 cm, then placed in sterile plastic bags, transported to the laboratory on ice and stored at 4°C. Agrobacteria were isolated from these soils within one week. Soils were collected from 25 locations across Tunisia (Fig. 1). When the crown gall symptoms were present, the bulk soil underneath the infected plants was sampled.

One gram of thoroughly mixed soil from each sample was added to 1 ml of distilled water and shaken at 200 rpm for 30 min at room temperature (20-22°C). Ten-fold serial dilutions were made in distilled water before one hundred microliters of the 10^{-3} dilution was plated into MG agar plates and incubated at 28°C (Ophel and Kerr, 1990). Single colonies were selected and purified on LPGA media (Yeast extract 5 g/l, peptone 5 g/l, glucose 10 g/l, agar 20 g/l, pH 7.2) and cultured at 28°C.

Bacterial reference strains, which were used for comparative analyses are available at CFBP (Collection Française de Bactéries associées aux Plantes, INRA, Angers, France) and are listed in Table 1.

Biochemical and physiological tests. Putative *Agrobacterium* spp. isolates were identified by the presence of urease, esculinase and enzymatic ability to aerobically convert lactose to 3-ketolactose (Bernaerts and De Ley, 1963; Shams *et al*., 2012). Tartrate utilization was tested on AB minimal medium supplemented with 0.5% (w/v) sodium-potassium-tartrate, 25 mg/l bromothymol blue and 1.2% (w/v) agar (Szegedi *et al*., 2005). The sensitivity of different isolates to the bio-control agent *Rhizobium rhizogenes* K84

was tested using the double layer method described by Rhouma *et al.* (2008). Zones of inhibition were recorded after 24-48 h.

Pathogenicity tests. Carrot (*Daucus carota*) taproots were sterilized with 12% sodium hypochlorite (3 min) followed by a 70% ethanol rinse (7 min) and then cut transversely into 5 mm diameter disks. Two disks per isolate were placed on damp sterile filter paper in a Petri dish and inoculated with 200 µl of an *Agrobacterium* spp. suspension (10^8 CFU ml^{-1}). Petri dishes were incubated in the dark at 28°C for 7-10 days. Stems of tomato plants (*Solanum lycopersicum* cv. Rio Grande) were stabbed with a sterile needle and inoculated with 200 µl of 10^8 CFU ml^{-1} bacterial suspension. Plants were grown under greenhouse conditions (14-16 h light and 20 ± 2°C) and checked for tumor formation 4 weeks after the inoculation. The strain C58 of *Agrobacterium* sp. G8 ("*A. fabrum*") was used as a positive control, while sterile distilled water was used as a negative control.

Primers and PCR conditions. PCR was performed on freshly made suspensions of studied strains in NaOH (20 mM) to lyse the cells. The standard PCR mixture contained 1× Taq polymerase buffer, 200 µM of dNTPs, 1.5 mM $MgCl_2$, 0.8 µM of primers, 2.5 U ml^{-1} of Taq polymerase (Invitrogen, Carlsbad, USA) and 2 µl of freshly lysed cells. The cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, primer set specific annealing temperature for 45 s, 72°C for 1 min; and a final extension step at 72°C for 5 min. All reactions were conducted in a Biometra thermocycleur Whatman (Biometra, Goettingen, Germany). Primers used for *Rhizobiaceae* and *Agrobacterium* spp. genomic species identification were described previously by Shams *et al.* (2013). VCF3-VCR3 primers were used to detect virulence genes (virC1-C2) of Ti and Ri plasmids (Suzaki *et al.*, 2004) while F14 (Picard *et al.*, 1992) and F749 (Mougel *et al.*, 2001) primers were used to detect virulence genes (virB-virG) of octopine- and nopaline-type Ti plamids, as described by Shams *et al.* (2012).

BOX-PCR was performed on 50 ng of DNA extracted from each isolate using the Masterpure DNA kit (Epicentre Madison, WI, USA) to generate strain-specific fingerprint patterns. The primer BOX-A1R CTACGGCAAGGCGACGCTGACG was used as described by Rademaker *et al.* (2000). Computer-assisted analysis of the genomic fingerprints was performed using the commercially available GelCompar version 4.1 (Vauterin and Vauterin, 1992), as described by Rademaker and de Bruijn (1997) and Rademaker *et al.* (1999).

Gene sequencing and phylogenetic analysis. Partial *recA* (779 bp) gene amplification was carried out with the *Rhizobiaceae*-specific primers F7386 and F7387 (Shams *et al.*, 2013) and purified fragments were sequenced by the Genoscreen Company (Lille, France). The nucleotide sequences were deposited at Genbank under accession numbers KT153061 to KT153075. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for sequence similarities in the NCBI DNA database. Sequences were aligned and BioNJ trees were inferred with the SeaView4 software with 1000 bootstrap samplings to assess the confidence limits of branchings. Sequence polymorphism was analyzed with DnaSP v.5.10.01 (Librado and Rozas, 2009) and Ka/Ks ratio was calculated by the polymorphism and divergence analyses command.

Table 1. List and description of reference strains used in this study.

Strain	Species	Country	*recA*-allele[a]
TT111	*A.* sp. G1	USA	*recA*-G1-1
LMG146	*A.* sp. G2	Belgium	*recA*-G2-1
CIP107443	*A.* sp. G3	France	*recA*-G3-1
B6	*A.* sp. G4	USA	*recA*-G4-1
CFBP7273	*A.* sp. G4	Tunisia	*recA*-G4-1
CFBP7128	*A.* sp. G4	Tunisia	*recA*-G4-4
CFBP7126	*A.* sp. G4	Tunisia	*recA*-G4-5
CIP107445	*A* sp. G5	France	*recA*-G5-1
NCPPB925	*A.* sp. G6	South Africa	*recA*-G6-1
Zutra3/1	*A.* sp. G7	Israel	*recA*-G7-3
CFBP7125	*A.* sp. G7	Tunisia	*recA*-G7-5
CFBP7124	*A.* sp. G7	Tunisia	*recA*-G7-7
CFBP7129	*A.* sp. G7	Tunisia	*recA*-G7-8
C58	*A.* sp. G8	USA	*recA*-G8-4
Hayward 0362	*A.* sp. G9	Australia	*recA*-G9-1
PGF0122	*A.* sp. G13	France	*recA*-G13-1
C4.3.1	*A.* sp. G14	Poland	*recA*-G14-1 6 6
AF 3.10	*A. larrymoorei*	USA	-
CFBP5523	*Al. vitis*	Australia	-
K84	*R. rhizogenes*	Australia	-

[a]: *recA* allele according to Shams *et al.* (2013). *A.: Agrobacterium; Al.: Allorhizobium; R.: Rhizobium; A. sp.* G2*: A. pusense; A. sp.* G4*: A. radiobacter; A. sp.* G8*: "A. fabrum"; A. sp.* G14*: A. nepotum.*

RESULTS

Agrobacteria identification and genomospecies affiliation. One hundred twenty six isolates obtained from 23/25 soil samples (Table 2) were identified as belonging to the *Rhizobiaceae* family by using the F7386-F7387 primer set. One hundred twenty-two isolates showed positive reactions for urease, esculinase and 3-ketolactose tests and thus were identified as *Agrobacterium* spp. members (biovar 1). The genomospecies diversity of the *Agrobacterium* spp. population in soils was assessed using a set of species-specific primers described by Shams *et al.* (2013). The 122 *Agrobacterium* isolates were found to belong to genomospecies G4 (62/122), G7 (56/122) or G9 (4/122). Members of G4, G7 or G9 were obtained from soils collected underneath grapevines or stone fruit trees as well (Table 2).

Table 2. Distribution of genomospecies and *recA* STs of *Agrobacterium* strains recovered from Tunisian soils.

Soil sample	Locality	Plantation type	Crown gall symptoms	*Agrobacterium spp.*[b]	No. of *A. sp.* G4	*recA*-G4-8*	recA-G4-7*	recA-G4-7	recA-G4-10	recA-G4-2*	recA-G4-3*	recA-G4-3	recA-G4-5	No. of *A. sp.* G7	*recA*-G7-ST01	recA-G7-5	recA-G7-ST03	recA-G7-ST04	recA-G7-ST05	No. of *A. Sp.* G9	recA-G9-2	recA-G9-ST07	Tumorigenic strains[b]	L-tartrate utilizing strains[b]
						ST08	ST09	ST10	ST11	ST12	ST13	ST14	ST15		ST01	ST02	ST03	ST04	ST05		ST06	ST09		
						1[a]	1[a]	0[a]	0[a]	1[a]	1[a]	0[a]	0[a]		8[a]	0[a]	9[a]	7[a]	6[a]		0[a]	5[a]		
S1	Gonna	grapevine	yes	11	2	**1**						1		9	3	1	1	2	**2**	0				3
S2	Gonna	grapevine	yes	6	6	1+(**1**)		(**1**)	1	1		1		0						0			2	2
S3	Gonna	grapevine	yes	11	6			3		**2**		1		5	1	1	1	2		0				2
S4	Gonna	grapevine	yes	8	3	(**1**)				1		1		4	1	1			2	1		1	1	1
S5	Gonna	grapevine	yes	10	2	**1**						1		8	2	**1**		3	2	0				2
S6	Regueb	grapevine	yes	7	2							**2**		5		2			3	0				2
S7	Regueb	grapevine	yes	7	3			1		1		1		4	**1**	1	1		**1**	0				2
S8	Regueb	grapevine	no	2	1								1	0						1	1			0
S9	Regueb	grapevine	yes	15	6			1+(**2**)		1+**1**	1			9	2	2	3	1	1	0			2	3
S10	Regueb	grapevine	yes	7	5			(**1**)	1	2		1		2		2				0			1	1
S11	Regueb	peach	yes	1	0									1		1				0				0
S12	Regueb	peach	yes	3	2					1			(1)	0						1		1	1	0
S13	Regueb	peach	yes	3	1								(1)	2	1				1	0			1	0
S14	Regueb	peach	yes	2	0									1		1				1		1		0
S15	Regueb	peach	No	2	1					1				1		1				0				0
S16	Knaies	not cult.	No	4	3			1			1	1		1	1					0				0
S17	Knaies	grapevine	yes	6	4	1		(**2**)		1				2	1	1				0			2	2
S18	Knaies	not cult.	No	6	6		2		2	1		1		0						0				0
S19	Oued Mliz	almond	yes	3	2					1			(1)	1		1				0			1	0
S20	Oued Mliz	almond	yes	3	2						1	1		1		1				0				0
S21	Oued Mliz	almond	yes	1	1					(1)				0						0			1	0
S22	Oued Mliz	almond	yes	0	na									na						na				0
S23	Morneg	not cult.	No	3	3					2			1	0						0				0
S24	Beni Khed.	not cult.	No	1	1					1				0						0				0
S25	Tunis	olive tree	No	0	na									na						na				0
TOTAL				122	62	6	2	12	4	18	3	12	5	56	13	17	6	8	12	4	1	3	12	20
pTi+						2	0	6	0	1	0	0	3		0	0	0	0	0		0	0		
Tar+						4	0	6	0	3	0	2	0		1	1	0	0	3		0	0		

[a]: number of polymorphic sites with the closest reference allele described by Shams *et al.* (2013). *indicates allele with one mismatch to reference allele. [b]: numbers indicate number of strains. Number in brackets indicates number of Ti plasmid-harboring strains. Number in bold indicates number of tartrate-utilizing strains. Beni Khed.: Beni Khedach. not cult.: agronomic but not cultivated soil. na: not applicable. pTi+: number of Ti plasmid-harboring strains per ST. Tar+: number of tartrate-utilizing strains per ST.

Genetic diversity, population structure and geographical distribution of *Agrobacterium* spp. A partial sequencings of the *recA* gene was done to investigate the infra-specific diversity of local agrobacterial populations. The *recA* phylogenetic tree confirmed that *Agrobacterium* spp. strains (122/126) were divided into three main clusters corresponding to genomospecies G4, G7 and G9, and at the infra-specific level into 8, 5 and 2 different *recA* sequence types (STs) – with at least one nucleotide difference – into G4, G7 and G9, respectively (Fig. 2). The four remaining isolates were identified as *Rhizobium* spp. by partial sequencing of the *recA* gene (data not shown).

Based on sequence similarity to all previously deposited sequences in the NCBI gene database (BlastN algorithm), 74 of the local strains matched seven *Agrobacterium* spp. *recA* alleles previously described by Shams *et al.* (2013) (*recA*-G4-2*, *recA*-G4-3, *recA*-G4-5, *recA*-G4-7, *recA*-G4-10, *recA*-G7-5 and *recA*-G9-2), while the 48 remaining *Agrobacterium* spp. strains had one to nine mismatches and 99% similarity to one of the known *recA* alleles. To

avoid a wealth of allele description and because a single mismatch is something that happens in clonal populations, allele sequences that differed from a known one by a single mismatch were not considered as new and a star-coding (*) was applied to distinguish them from reference allele sequences (Table 2). The majority of samples yielded strains of several *recA* STs. There was no correlation between the allelic identity and crops or the geographic origin (Table 2). For instance, strains exhibiting ST12 (identical to the *recA*-G4-2* allele) were found in all regions and under grapevines, stone fruit trees or in not cultivated soils as well.

In spite of the high diversity of the *recA* gene sequence documented in this study, it turned out that all G4 alleles resulted in one identical protein sequence, while G7 alleles resulted in two different proteins differentiated by one amino acid change, A/S_{209} (already reported for known strains of the genomic species G7). Moreover, the *recA*-G9-ST07 had a unique protein sequence and was differentiated from the rest of the known G9 strains by one amino acid (V/I_{41}). To test whether the *recA* locus was under a selective pressure, the degree of selection was estimated by calculating the K_a/K_s ratio representing the ratio of non-synonymous substitutions to synonymous substitutions. For the *recA* gene of our isolates, this ratio was significantly below 1, which means that this locus is far from being under a strong positive selective pressure (Table 3).

BOX-PCR was performed in an attempt to look for differences within STs. BOX-PCR fingerprinting and subsequent cluster analysis of twenty local strains belonging to six main STs and seven collection strains of *Agrobacterium* sp. G4 and G7 (Fig. 3) confirmed that G4 and G7 strains are form two remarkably different groups joined at a low degree of similarity (48%). The maximum level of similarity (86%) was observed between isolates AT8 and AT9, which were recovered from the same soil sample, had the same ST and indeed likely belonged to the same clonal population. No correlation between genetic profile and geographic origin could be detected. The profiles of *Agrobacterium* local strains with the same STs were barely joined at a high degree of similarity, suggesting that they are markedly different strains.

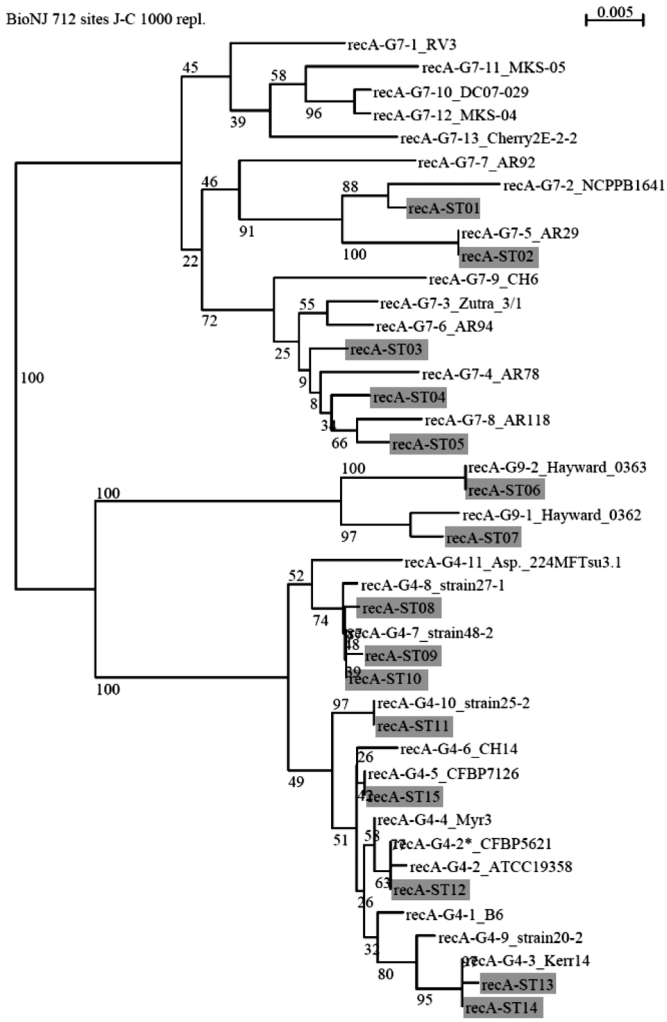

Fig. 2. *recA* phylogenetic relatedness of Tunisian soil strains of *Agrobacterium* spp. compared to the known *recA* allelic diversity of *Agrobacterium* genomospecies G4, G7 and G9. Neighbor-joining tree with 1000 bootstrap resamplings was performed with partial *recA* gene sequences (712 positions). ST01: 13 isolates; ST02: 10; ST03: 3; ST04: 3; ST05: 15; ST06: 1; ST07: 3; ST08: 6; ST09: 2; ST10: 12; ST11: 4; ST12: 24; ST13: 3; ST14: 18; ST15: 5.

Plasmid diversity and distribution amongst *Agrobacterium* spp. and *recA* alleles. Twelve of the 126 strains were shown to presumptively harbor a Ti or Ri plasmid as revealed by using the VCF3-VCR3 primers. They were all able to induce galls on carrot discs and tomato plant stems, confirming thus the presence of a Ti plasmid. Remarkably, tumorigenic strains were obtained from half the soils collected underneath crown galled material (5/10 and 4/7 underneath crown galled grapevine or underneath crown galled stone fruit trees, respectively), while no tumorigenic isolates were obtained from the six samples of soils free of diseased material (Table 2). The four tumorigenic strains obtained from soils underneath stone fruit trees contained nopaline-type Ti plasmids as determined by using F14-F749 primers. As expected with nopaline-type Ti plasmids, those strains were found to be sensitive to the *R. rhizogenes* K84 strain. The eight tumorigenic isolates obtained from soils underneath crown galled grapevines did not yield a PCR product with F14-F749 primers and were resistant to the action of the strain K84. Ti plasmids obtained from soils underneath crown galled plants were thus different according to diseased plants; with nopaline-type Ti plasmid and "non-nopaline/octopine-type" Ti plasmids for stone fruit trees and grapevines, respectively.

Ti plasmids were detected exclusively in *Agrobacterium* spp. members and thus, the four *Rhizobium* spp. isolates were eliminated from further analysis. All tumorigenic strains belonged to the genomospecies G4 and were distributed into four different *recA* STs: *recA*-ST08, *recA*-ST10, *recA*-ST12 and *recA*-ST15; corresponding to

Table 3. Analysis of the *recA* gene fragment in local *Agrobacterium* spp. strains.

Genomic species	Size of the fragment (bp)	No. of alleles	No. of polymorphic sites	K_a/K_s ratio
G4	712	8	18	0.000
G7	712	5	31	0.008
G9	712	2	15	0.017
G4+G7+G9	712	13	86	0.011

recA-G4-8*, *recA*-G4-7, *recA*-G4-2* and *recA*-G4-5, respectively. Tumorigenic strains recovered from soils collected directly underneath crown galled grapevines belong to *recA* STs: *recA*-ST08 (2/6) and *recA*-ST10 (6/12). Tumorigenic strains isolated from soils underneath crown galled stone fruit trees belong to *recA*-ST12 (1/12) and *recA*-ST15 (3/5) (Table 2). These observations clearly show that pathogenic strains collected underneath crown galled material are clearly different at both the Ti plasmid and the chromosomal levels.

Tartrate utilization. The ability to use L-tartrate was investigated because this trait has been described as an adaptation of agrobacteria to live in the L-tartrate rich sap of grapevine (Kado, 1998; Salomone *et al*., 1998). Remarkably, the 20 strains able to use L-tartrate as the sole carbon source were only found amongst the 88 strains recovered from soils collected underneath galled grapevine plants and never amongst the 34 strains recovered from outside grapevine yards. This trait was found in strains belonging to 5 and 2 STs of G4 and G7, respectively, and in both tumorigenic (8/8) and Ti plasmid-free strains (12/80) from soils of grapevine plants (Table 2).

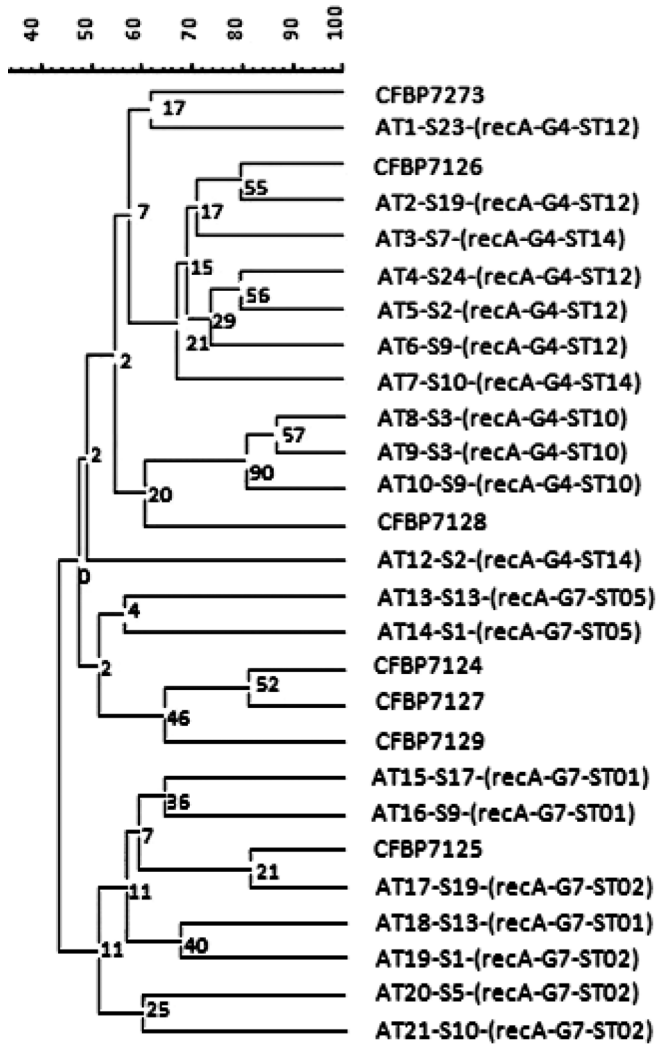

Fig. 3. Dendrogram based on BOX-PCR fingerprints showing phylogenetic relationships between local strains of *Agrobacterium* (AT) of principal *recA* STs: G4 (ST10, ST12 and ST14) and G7 (ST01, ST02, ST04 and ST05) recovered from different soils in Tunisia (S1 to S24), compared to reference strains (CFBP).

DISCUSSION

This work is directed toward analyzing indigenous populations of agrobacteria in Tunisian soils at the local and the landscape scales as a first survey for the presence of the *Agrobacterium* spp. in agricultural fields. For this purpose, twenty five different locations spanning from the North to the South of the country were considered. Eighteen soil samples were collected underneath plants exhibiting crown gall (10 samples from soils underneath galled grapevines and 8 samples from soils underneath galled stone fruit trees). Seven samples were collected from soils underneath healthy plantations in regions with no history of crown gall.

Agrobacterium members were recovered from 23 of the 25 sampled sites. This observation confirms the fact that *Agrobacterium* spp. is ubiquitous and widely spread in soils (D'Hondt *et al*., 2004; Süss *et al*., 2006). However, *Agrobacterium* members were absent in two sampled sites (S22 and S25). A second anomaly was our inability to detect any *Agrobacterium* species from around a galled almond tree in the sampled site S22. In fact, agrobacterial populations in the soil underneath galled grapevines were greater than in the soil underneath galled stone fruit trees and non-cultivated soils. This may be explained by the more recent appearance of crown gall on stone fruit trees as compared to grapevines that have exhibited galls for more than two years. The reason for this result could be also in the medium used. Although all agrobacteria and rhizobia can be isolated on MG medium (Ophel and Kerr, 1990), this medium is not selective and many other soil bacteria could easily grow and thus overgrow agrobacteria. One of the most effective types of media for agrobacteria isolation is MG medium supplemented with tellurite (Mougel *et al*., 2001; Campillo *et al*., 2012).

Our results confirm the dominance of *Agrobacterium* spp. (biovar 1) in Tunisia as previously reported (Zouba and Hammami, 1988; Benjama *et al*., 2001), but we add to these studies that several genomospecies were involved. The dominance of *Agrobacterium* spp. may be a trait

specific to Tunisian soils since many studies from other countries found that *R. rhizogenes* was more abundant than *Agrobacterium* spp., e.g. South Africa (Duplessis *et al*., 1984), Italy (Peluso *et al*., 2003) and Poland (Pulawska *et al*., 2012). The dominance of *Agrobacterium* spp. in Tunisia can be explained by the climatic and edaphic conditions of the country, as hypothesized previously by Rhouma *et al.* (2006). Thus, for example, according to Moore *et al*. (2001), populations of biovar 1 agrobacteria tolerate higher temperatures more than *R. rhizogenes*, and the average year round temperatures in Tunisia are significantly higher than those in South Africa, Italy or Poland.

The *Agrobacterium* populations that we detected were composed of only three genomic species: G4, G7 and G9 and most samples yielded more than one genomic species. The diversity of genomic species is surprisingly limited given the landscape scale of this study. In Tunisian nurseries, the populations of *Agrobacterium* spp. were more diverse and showed the presence of G4, G6, G7 and G8 genomic species (Rhouma *et al*., 2006; Costechareyre *et al*., 2010). This result may be explained by the fact that these previous studies were carried out on strains isolated from galled nursery plants, many of which were imported from a variety of European countries. Saïdi and coworkers (2011) also reported the presence of *Agrobacterium* sp. G2, G4, G9 and two atypical strains subsequently identified as G14 (*A. nepotum*) (Pulawska *et al*., 2012; Ramirez-Bahena *et al*., 2014) in the rhizosphere of grain legumes from other Tunisian regions. Such divergence in the identities of genomospecies detected in different Tunisian environments may be explained by the nature of the biotope (i.e., galls, rhizosphere or soil) and host plant species. In a similar study, 63 strains of *Agrobacterium* spp. obtained from 72 Slovenian soil samples all fell into genomic species G1 and G4. The authors suggested that the prevalence of G1 and G4 in their samplings could be due to the great ability of these species to grow quickly on selective media (Lamovšek *et al*., 2014). If this is the case, the absence of G1 members in our sampling must be underlined because it appears as a marked trait that differentiates Tunisian from European soils since G1 was also found in France (Vogel *et al*., 2003).

Even though we only detected three genomospecies in our study, partial sequencing of the *recA* gene revealed 15 different sequence types (STs). At a single sample site, more than one genomospecies and multiple *recA* STs were almost always discovered. The diversity of *Agrobacterium* has even been observed in 1 cm^3 of agriculture soil (Côte-Saint-André, France), where 42 different ribotypes were distributed into three genomospecies: G1, G4 and G8 (Vogel *et al*., 2003). Finally, in addition to the *Agrobacterium recA* diversity described by Shams *et al.* (2013), our study revealed five new alleles (4 *recA*-G7 and 1 *recA*-G9), which were found exclusively in Tunisian soils to date. Thus, this study is contributing to the improvement of the international *recA* database. Moreover, among the new alleles reported here, two alleles (one *recA*-G7 and one *recA*-G9) coded for new proteins with one amino acid changed. The high *recA* allelic diversity of Tunisian strains showed no positive selection pressure for this locus. It is most likely that *recA* locus was not a subject of recombination, which provides additional evidence that it could be suitable for preliminary differentiation of agrobacteria (Costechareyre *et al*., 2010; Shams *el al*., 2013).

This study has revealed that the genomic species G4 (and particularly the allele *recA*-G4-2*) is a ubiquitous member of the *Agrobacterium* spp. population in Tunisian soils. However, in France (Vogel *et al*., 2003) and Slovenia (Lamovšek *et al*., 2014), the most frequently isolated genomic species from non-contaminated soil was G1. The widespread presence of G4 and G7 genomic species, (regardless of the presence or absence of crown gall-affected plants in the sampling sites) opens the question of how far agrobacteria can spread across the landscape. Thus, repetitive sequence-derived PCR using BOX-A1R primer was used to generate genomic fingerprints of twenty local strains chosen from main *recA*-G4-STs and *recA*-G7-STs. Using BOX-PCR, reproducible fingerprints can be obtained, as mentioned in previous studies with important human and plant pathogens bacteria such as *Salmonella* (Oliveira *et al*., 2007), *Pseudomonas* (Louws *et al*., 1994, Marques *et al*., 2000), *Xanthomonas* (Louws *et al*., 1994) and *Erwinia* (Barionovi *et al*., 2006; McManus and Jones, 1995; Shrestha *et al*., 2007; Teixeira *et al*., 2009). The variation in genome sizes, as well as in the location of BOX elements among different isolates of a particular genomic species, leads to the generation of multiple strain-specific fingerprint patterns, which allows BOX-PCR typing to better discriminate among different strains than *recA* gene sequencing. Although BOX-PCR was useful to identify bacterial pollution sources in genotyping of environmental *Escherichia coli* isolates (Somarelli *et al*., 2007; Mohapatra and Mazumder, 2008) and *Pseudomonas aeruginosa* (Wolska *et al*., 2011), there was no evidence to suggest that genetic profiles of non-tumorigenic *Agrobacterium* spp. Tunisian strains were correlated with geographic location or the plantation type of the soil. The absence of a correlation between the genetic diversity of *Agrobacterium* spp. and the geographical origin or the host plant was also reported by Pulawska and Kaluzna (2012), using *gyrB* sequencing, RFLP and RAPD methods. In fact, populations of non-tumorigenic as well as tumorigenic agrobacteria are very diverse, both on the phenotypic and genetic level, compared to other plant pathogenic bacteria like e.g. *Erwinia amylovora*, as mentioned by Pulawska (2010).

In spite of the genetic relationship between strains from soils of different locations as given by *recA* sequence data, the BOX-PCR data presented here showed a high genetic diversity within non-tumorigenic *Agrobacterium* local strains and this likely do not support the notion that a clonal dispersion of non-tumorigenic agrobacteria occurred in Tunisia.

Tumorigenic *Agrobacterium* strains were detected in nine out of the eighteen soil samples collected underneath galled plants and only in this case. Moreover, our results clearly show that Ti plasmid-free agrobacteria were dominant – if not always alone – in soils even in fields with a high crown gall incidence confirming the report done by Krimi *et al.* (2002). All tumorigenic strains recovered from multiple sites were of the genomic species G4 and a narrow range of *recA*-STs. This suggests that pathogenic strains disperse more clonally than by Ti plasmid transfer to other *recA*-STs members (X. Nesme, unpublished data). Further genetic and biochemical analysis showed that tumorigenic strains from soils with galled grapevines were distinct from tumorigenic strains isolated from soils with galled stone fruit trees. First, tumorigenic strains from soils underneath galled grapevines harbored a Ti plasmid different than the nopaline Ti plasmid of the tumorigenic strains recovered under galled stone fruit trees. It was reported that the nopaline type of Ti plasmid was the most common within tumorigenic *Agrobacterium* strains in Tunisia from galls of stone fruit trees (Rhouma *et al*., 2006). In the case of pathogenic *Agrobacterium* spp. occasionally occurred on grapevine, it was mentioned that some isolates contain an octopine/cucumopine type pTi that is a characteristic of *Al. vitis* (Szegedi *et al*., 2005). Second, in addition to the specificity between the opine type of the pTi and the plantation of the soil (grapevines/ stone fruits), tumorigenic G4 strains recovered from soils with grapevines showed a specific capacity to use the L-tartrate as a sole carbon source, and thus are better adapted to grapevines. This property, although contributing to the grapevine/*Agrobacterium* interaction (Kado, 1998; Salomone *et al*., 1998) is not essential for the presence of *Agrobacterium* spp. in grapevine, as mentioned by Szegedi *et al.* (2005). In fact, *Agrobacterium* strains from grapevine probably possess this trait as a consequence of plasmid acquisition (Ridé *et al*., 2000; Palacio-Bielsa *et al*., 2009). Third, tumorigenic strains from soils with galled grapevines were resistant to the action of the *R. rhizogenes* K84 contrary to what has been observed with tumorigenic strains from soils underneath galled stone fruits. Even though the correlation between host plant and the characteristics of *Agrobacterium* strains from their galls has been previously reported (Lopez *et al*., 1988; Sobiczewski, 1996; Peluso *et al*., 2003; Rhouma *et al*., 2006), the current research is the first work to report that tumorigenic agrobacteria from soils underneath grapevines were distinct from those of soils underneath stone fruit trees, based on *recA* sequence, Ti plasmid type, L-tartrate utilization and sensitivity to the strain K84. Moreover, Ti plasmid-containing strains were only recovered from soils of fields with a high evidence of crown gall and this can be an additional insight into the soil contamination by Ti plasmids.

This study is the first assessment of agrobacterial populations' diversity in a variety of Tunisian soils as a prerequisite for a better knowledge of the crown gall disease in Tunisia. Culture-based method analyses remain important in epidemiological investigations when they are conducted at the population level of both tumorigenic and non-tumorigenic *Agrobacterium* populations and not at the single strain level. Here, we joined specific ecological traits to the corresponding genetic diversity of *Agrobacterium* in an effort to find an epidemiological clue for the spreading of pathogenic agrobacteria in soils across Tunisian regions. Finally, our observations need to be supported by laboratory experiments in order to reveal environmental factors that affect the survival of *Agrobacterium* in soil and manage the genetic structure of populations. This may lead to a better understanding of specific ecological adaptations involved in the speciation process of bacteria and eventually to better control strategies of perennial soil contaminations by tumorigenic agrobacteria.

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