Molecular Detection and Pathogenic Capability of *Agrobacterium tumefaciens* Isolates from Olive

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ABSTRACT

Crown gall, caused by *Agrobacterium tumefaciens*, first reported on olive in Libya. A partial 16SrRNA gene sequence confirmed the identity of the *A. tumefaciens* bacterial isolates. The Ti-plasmid (induce tumors formation) is the real reason of their pathogenicity. Meanwhile, the prerequisite for the control of *A. tumefaciens* is to have a rapid and reliable detection method of pathogenic isolates in order to select healthy plants therefore, we detected two plasmidic genes, virD2 gene of the virulence (*vir*) region (amplified by virD2A/C and virD2A/E primers), and T-DNA (on *tms* gene). The PCR amplified bands analysis had a correspondence to the pathogenicity assay (carrot tumorgenicity) and the biochemical determents' of the isolates genus.

Key words: *Agrobacterium tumefaciens*; PCR; Ti-plasmid; virD2; pathogenicity

INTRODUCTION

Agrobacterium tumefaciens is the causal agent of crown gall disease; the common neoplastic disease of dicot plants, including many woody shrubs and various herbaceous plants (DeCleene *et al*., 1976; Young *et al*., 2003; Rhouma *et al.,* 2006; Younis *et al*., 2016). Knowledge of the ecology of *Agrobacterium* spp.-in soil and on plants- is limited, but such information is required to find better methods of managing crown gall disease. Tumor inducing assay relayed on isolation of pathogen on semi-selective media and performing the pathogenicity test (Moore *et al* 2001). Although time consuming and laborious, pathogenicity assay is the only reliable method for determining tumorgenicity of bacteria associated with grapevine crown gall. *Agrobacterium* strains may inhabit nursery soils for years before causing disease (Haas et al., 1995). Therefore, the pathogen can reside undetected in soils of locations where nurseries will be established. Thus, it is important to develop sensitive and reliable tools to detect *A. tumefaciens* in soils and plant material and to distinguish disease-producing strains from nonpathogenic strains (Kuzmanović *et al*., 2016). Since a virulence assay for *Agrobacterium* typically takes 3-4 weeks, several other methods have been developed, including the introduction of the polymerase chain reaction (PCR) in plant pathology (Burr *et al.,* 1990) which, opened up new possibilities for rapid detection and identification of *Agrobacterium* in agriculturally important plants. First studies were started in the early 90s (Dong *et al*., 1992; Schulz *et al*., 1993). Pathogenic tumor-inducing isolates of *Agrobacterium* had a common feature which could permit their identity through DNA analysis: they contain the tumor-inducing (Ti) plasmid (Wang *et al*., 1990 ; Gelvin, 2017). Virulence depends upon two regions of the Ti plasmid: the transferred DNA (T-DNA) and the virulence (vir) genes (Zhang *et al*., 2017 ; Duan *et al*., 2018). Primers were designed to detect T-DNA and the *vir* region (Ponsonnet and Nesme, 1994; Haas *et al*., 1995; Sawada *et al*., 1995; Pulawska and Sobiczewski, 2005; Bini *et al*., 2008). The universal primers virD2A/C and virD2A/E were used to amplify the highly conserved DNA sequences in the virD2 gene of the *vir* region on the Ti plasmid. These results are supporting to identify of these isolates as *A. tumefaciens* (Eastwell *et al*., 1995; Szegedi and Bottka, 2002; Tolba and Soliman, 2014). Here, the goal of this study was to use a sensitive, specific and more rapid PCR-based method for detecting pathogenic *A. tumefaciens* in plant. Such a method is essential for early detection for nursery production of stone fruit trees and other plants susceptible to Agrobacterium crown gall disease.

MATERIALS AND METHODS

Bacterial isolation and culture media

Isolation trials were carried out from infected olive seedlings showing roots tumors, crown galls with well developed symptoms, collected from Benghazi region, Libya during 2017.The bacteria were isolated from symptomatic materials and streaked on glycerol nutrient agar according to described method of Moore *et al.* (2001) **.** After 48 h of incubation at 28°C the colonies were observed, isolated and kept purified on 2% glycerol nutrient agar slants for later use. In addition we used the strain C58 (kindly provided from Plant Pathology Department, Faculty of Agriculture, Alexandria University) as a reference in this study.

Phenotypic identification

The morphology of the bacterial isolates was examined by light microscopy. The physiological and

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biochemical characteristics were carried out according to protocols described by Jones and Geider (2001).

Detection of tumor-inducing isolates in carrot slices

Tumorgenicity of the selected isolates was tested on the carrot slices (*Daucus carota*) according to Burr and Katz (1983).

DNA extraction

The DNA of all bacterial isolates was extracted as a method described by Ausubel *et al*., (1995).

PCR analyses by 16Sr RNA, *virD2* and *tms2* genes

A partial portion of 16S rRNA gene was amplified for all bacterial isolates using universal primers P0 and P6, for detecting the *virD2* gene (virD2A/C and virD2A/E) and for amplifying *iaaH* (*tms2*) gene (tms2Fl/tms2B) (Table 2). PCR amplifications accomplished with 2µL of template DNA, 12.5µL of master mix (iNtron, Korea), 0.5µL of 10 pmol of each primer as what gene detects and molecular grade water to be added up to 25µL. The amplification was performed as one cycle at 95°C for 5 min followed by 34 cycles each with 45 s at 95°C for denaturation, 1 min at 50°C annealing for all genes, and 1 min at 72°C for elongation and for final extension 7 min at 72°C. The PCR products were suggested to run in 1% agarose gel in Tris Acetate EDTA (TAE) buffer, stained with Ethidium bromide and visualized on transilluminator.

Sequencing of 16S rRNA gene and alignment

The purified fragments of partial 16S rRNA gene were sequenced by Macrogen inc., Soul, Korea. To identify the bacterial isolates, the achieved sequences were blasted in GenBank database at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi. nlm.nih.gov), nucleotide sequences were submitted to the GenBank submission portal and assigned to have accession numbers. Phylogenetic relationships were evaluated using the 16S rDNA sequences of isolates identified from olive seedlings, and other sequences from GenBank reference using the maximum parsimony method of MEGA 6. The bootstrap consensus tree was inferred from 1000 replicates.

RESULTS AND DISCUSSION

Isolation and initial characterization

Four bacterial isolates were isolated from infected olive roots tumors as stated in materials and methods compared with the reference isolate C58. Distinct colonies that had typical morphological and biochemical characteristics of *Agrobacterium tumefaciens* as recorded by Schroth *et al* (1965); Moore *et al*. (2001); Adenemo and Onilude (2014) and Ali *et al*. (2016) were purified and listed in (Table 1).

Pathogenicity of *Agrobacterium* isolates

On the basis of carrot tumorgenicity test carried out on carrot slices, all the tested *Agrobacterium* isolates were characterized as tumor-inducing (Fig.1). In other speech, as Tolba and Soliman, (2014) declared that the tumorgenicity should be the clear evidence on the belonging of the isolate to the genus *Agrobacterium*. Therefore, pathogenicity test was conducted at the outset of the study in order to selecting the tumorigenic isolates.

Table 1. Morphological traits, physiological, biochemical reactions, isolate code and accession numbers of bacterial isolates obtained from infected samples, compared with the reference strain C58 of *Agrobacterium tumefaciens*

Bacterial isolates	Characteristic																										
	Shape(rods)	Gram staining	Motility	Anaerobic growth	3-Ketolactose production	Growth at 37°C	Catalase test	Mucoid growth	Oxidase reaction	Pigment on kingsB	Indole production	R. substance from sucrose	Urease production	Growth in 5% NaCl	Citrate utilization	Malonate utilization	Alkali from tartaric acid	Glucose	α-methyl glucoside	Maltose	sucrose	lactose	Dulcitol	Manitol	trehalose	Isolate code	Accession number
A. tumefaciens	+	-	+	+	-	-	+	+	+	n	-	-	-	-	+	-	+	-	-	-	a	a	a	a	a	AT2	MH921293
A. tumefaciens	+	-	+	+	-	-	+	+	+	n	-	-	-	-	+	-	+	-	-	-	a	a	a	a	a	AT3	MH921294
A. tumefaciens	+	-	+	+	-	-	+	+	+	n	-	-	-	-	+	-	+	-	-	-	a	a	a	a	a	AT4	MH921295
A. tumefaciens	+	-	+	+	-	-	+	+	+	n	-	-	-	-	+	-	+	-	-	-	a	a	a	a	a	AT5	MH921296
A. tumefaciens	+	-	+	+	-	-	+	+	+	n	-	-	-	-	+	-	+	-	-	-	a	a	a	a	a	C58	

+ = More than 80% of isolates gave positive reaction - = Less than 20% of isolates gave negative reaction, a=acid, n=not tested

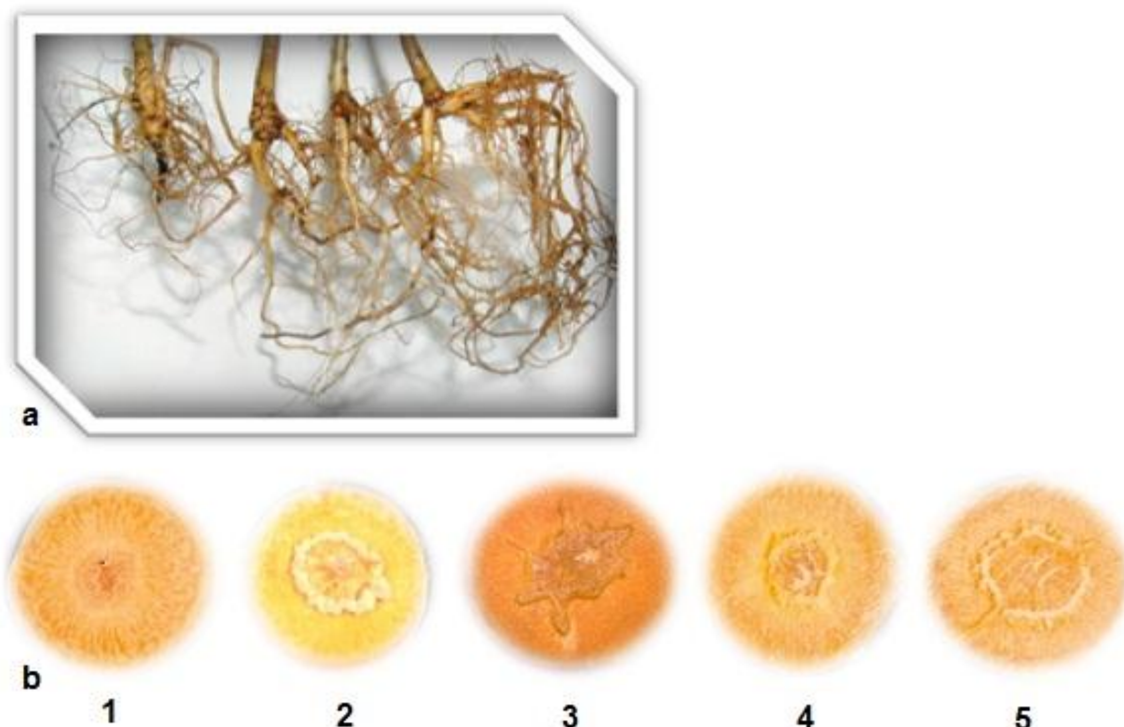

Fig. 1.a) the naturally infected olive seedlings with *Agrobacterium* showing galls on twigs and roots and b) 1; control and 2, 3, 4, 5; *Agrobacterium* isolates exhibited white small tumors induced on the surface of carrot discs

Multigenic identification

Vir and *tms* region of the Ti-plasmid

The PCR result of the virD2 region in all tested *Agrobacterium* isolates and the reference strain C58 also were positive. In Fig. 2, the primer pairs virD2A/C and virD2A/E amplified specific fragments of 224 and 338bp respectively. These results were in the same way of further phenotypic and tumorgenicity tests identity of the present isolates as *A. tumefaciens*, which was in agreement of previous reports (Saiki *et al*., 1988; Bini *et al*., 2008; Tolba and Soliman, 2014; Kuzmanović *et al*., 2016). Interestingly, all isolates gave positive amplification signal with virD2A/C and virD2A/E primer pairs were the same as that giving strong amplification signal with primer pair tms2Fl/tms2B. All primer pairs studied provided reliable identification of all tumorigenic isolates of *A. tumefaciens*, as in line with Kuzmanović *et al*., 2016. For a practical point of view, PCR detection of sequences in Ti-plasmid is sufficient for diagnosis of *Agrobacterium* isolated but not for further species identification or differentiation, therefor the 16SrRNA gene sequencing were used.

The bacterial 16S rRNA partial gene identification

The region of the 16S rRNA gene (1550bp) was amplified for all the bacteria isolates utilizing the universal primers P0 and P6. The sequences obtained from Macrogen, inc, Korea, used in the BLAST ncbi tool and revealed that, all isolates were identical to those of *Agrobacterium tumefaciens* and showed homology with *Agrobacterium tumefaciens* GenBank isolates reached to 99% or 100%. The nucleotide sequences were GenBank accessioned with numbers illustrated in Table 2.

Phylogenetic analysis

The phylogenetic tree confirmed the sequence comparison analysis since the four 16S- rRNA sequences of *A. tumefaciens* clustered in different phylogenetic branch each other's (Fig.3). In any case, many efforts could be forward towards the development of more specific and sensitive real-time PCR assay as more sufficient detectable tool than the conventional PCR and of course more primers are needed to deep characterize agrobacteria strains.

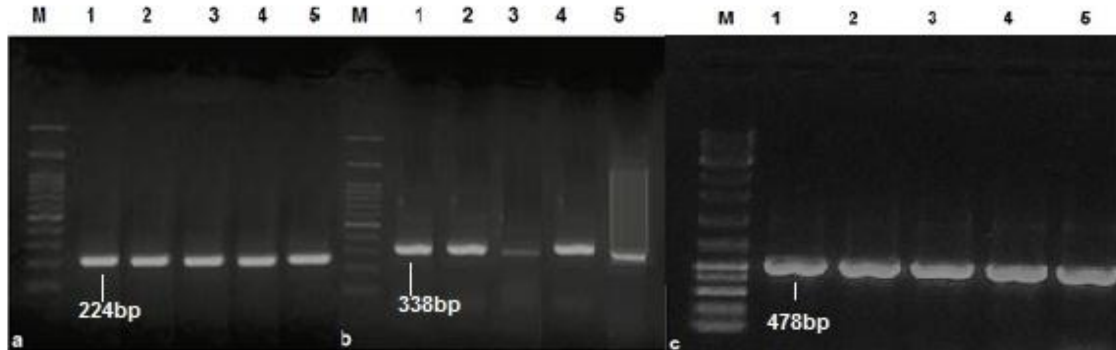

Fig.2. Specific amplified pattern on agarose gels, a) 224bp amplicon amplified by primer pair virD2A/C ,in, b) 338bp amplicon amplified by virD2A/E, in, c) 478bp amplicon amplified by tms2F1/B detecting *tms2* gene and M; 100bp DNA Ladder

Table 2. Primers used in this study

Primer	Target gene	Sequence 5` to 3`
virD2A		ATGCCCGATCGAGCTCAAGT
virD2C	*virD2*	TCGTCTGGCTGACTTTCGTCATAA
virD2E		CCTGACCCAAACATCTCGGCTGCCCA
tms2F1	*aaH(tms2)*	TTTCAGCTGCTAGGGCCACATCAG
tms2B		GGAGCACTGCCGGGTGCCTCGGGA
P0	16S rRNA	GAAGAGTTTGATCCTGGCTCAG
P6		CTACGGCTACCTTGTTACGA

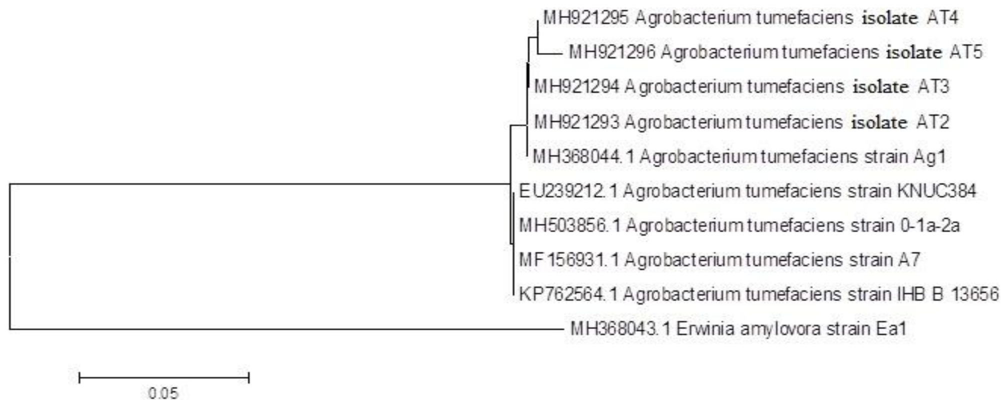

Fig.3. A rooted molecular phylogenetic tree using Maximum Likelihood method. Bootstrap values above 500 are shown. Five closely related nucleotide sequences from the *A. tumefaciens* isolates. The sequences of four *A. tumefaciens* isolates having code (AT2,AT3,AT4 and AT5) from olive obtained in this work are in distinct cluster. On the left of the branch the GenBank accession number of the isolates employed. The bacterial strain *Erwinia amylovora* was used as out group to root the tree.

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الملخص العربي

التوصيف الجزيئى والقدرة الامراضية لبكتيريا الأجروباكتيريم تيوميفاشنز المعزولة من شتلات الزيتون

نجية محمد جادالله ، سعيد ابراهيم عبدالله بحيرى و محمد ادم

تم التعرف على البكتيرات المعزولة من عرض التدرن التاجى فى شتلات الزيتون بالشكل المظهرى والطرق البيوكيميائية وكذلك الجينات الكاشفة للامراضية و جين الـ 16SrRNA على أنها بكتيرة الأجروباكتيريم تيوميفاشنز – لأول مرة يتم تسجيلها على شتلات الزيتون فى ليبيا – وكشرط للوقاية من أصابة النباتات بالاجروباكتيريم لابد من تحديث طريقة للكشف السريع عن البكتيرات التى لها القدرة على احداث العدوى والاصابة بالتورمات، لذا فقد ركزت تلك الدراسة على رصد جنيين محمولين على بلازميد التورم وهما جين virD2 الخاص بالقدرة الامراضية وجين tms2 الخاص بتخليق الاوكسينات. وتبين من الدراسة وجود علاقة بين قدرة البكتيرات على الامراضية ووجود هذه الجينات المحمولة على Ti-plasmid.