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Characterization of *Agrobacterium vitis* Isolates Obtained from Galled Grapevine Plants in Egypt

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Abstract: The incidence of crown gall disease recently has been gradually increased in Egyptian vineyards and grapevine nurseries. Twelve isolates of tumorigenic bacteria were isolated from grapevines galls which collected from different areas in Egypt. The isolates were identified as *Agrobacterium vitis* based on their reaction with a monoclonal antibody raised to *A. vitis*, their ability to induce galls and necrosis on grapevine, their reactions to standard biochemical and physiological tests and also by polymerase chain reaction amplification of specific Ti plasmid using polygalacturonase specific primers. All isolates were able to induce galls on grapevine. The isolates differed between itself in host range. All of these isolates except two were able to induce galls on kalanchoe, sunflower, datura, tobacco and chenopodium. Only 5 isolates produced galls on tomato. All isolates caused necrosis on grapevine explants. The genetic diversity was evaluated for these isolates by comparing DNA samples using multiplex PCR with 3 Specific primer pairs. They segregated into three main groups, the first group that is isolates carrying octopine type Ti plasmids; the second group that is isolates carrying vitopine Ti plasmids and the third group that is isolates carrying both octopine and vitopine type Ti plasmids. To our knowledge, this is the first report on the identification of *A. vitis* in Egypt.

1. Introduction

Crown gall is one of the most important bacterial diseases of grapevines worldwide and is especially debilitating on cultivars of *vitis vinifera* (**Burr et al 1998**). *Agrobacterium vitis* is the predominant species that causes the disease, although *A. tumefaciens* is occasionally isolated from infected vines (**Szegedi et al 2005**). Typically, infections are initiated at wound sites on trunks and canes that are caused by freezing temperatures or other cultural practices. In addition to crown gall disease, *A. vitis* induces electrolyte leakage and strong necrotic lesions on the roots of grapevine (**Burr et al 1998 and Stover et al 1997**).

Transmission of *A. vitis*, that is adapted to living in the vascular system of grapevine plants, occurs by vegetative propagation of infected cuttings (**Tarbah and Goodman, 1986**). Since vineyard soils have been excluded as a source of infection with *A. vitis*, spreading of the disease can be prevented by utilization of pathogen- free propagation material (**Tarbah and Goodman, 1986**).

Four major T-DNA structures have been characterized in strains of *A. vitis*. They differ by their numbers of T-DNAs (delineated by characteristic border sequences) and by gene composition (**Burr and Otten, 1999**). Strains are often referred to by the type of opine synthase gene or genes they carry on their Ti plasmids (i.e., nopaline [N], vitopine [V], or octopine and cucumopine [OCD. The OC strains are further grouped depending on whether their T-DNA TA region is large (OL) or small (OS). Phylogenetic models of *A. vitis* Ti plasmids have been developed by **Otten et al (1992)** that are based on T-DNA structures, homology of oncogenes to those on other Ti plasmids, and the pattern of insertion by various insertion sequence (IS) elements. It was discovered that the type of Ti plasmid that is carried by an *A. vitis* strain is highly correlated with the restriction fragment fingerprints

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derived from its intergenic spacer region (ITS) that lays between the 16s and 23s rRNA genes (**Otten et al 1996**). Similar correlation was shown between Ti plasmid type and restriction fragment fingerprints generated from the 5 region of the 23 gene (**Momol et al 1998**). The purposes of this study were to isolation of *Agrobacterium vitis* from infected grapevine and study the characteristic diversity between its isolates using conventional tests and PCR technique.

2. Materials and methods

2.1. Isolation of *Agrobacterium vitis*

Vineyards and nurseries of grapevine (*Vitis spp.*) were inspected for crown gall occurrence in field trips made to different areas in Egypt. Samples of newly developed galls from infected plants were collected, placed in plastic bags, and transported to the laboratory. Plant samples were washed under running tap water to remove adhering soil particles, surface-sterilized by dipping into 0.5% v:v sodium hypochlorite for 2 min, rinsed 3 times with sterile distilled water (SDW), and blotted dry on sterile filter paper. Small portions were aseptically removed from each sample and placed in few drops of SDW in a mortar and pestle for maceration. The resulting suspension was left to stand for 30 min, and then loopfuls of the gall suspensions were streaked on RS medium (**Roy and Sasser, 1983**). Inoculated plates were incubated at 28°C until bacterial growth developed. Colonies consistent with *A. vitis* morphology (opaque red center, domed, mucoid, white translucent margin) after 5 days on RS medium were streaked on potato dextrose agar (PDA). Isolates still resembling *A. vitis* were saved for further characterization. All isolates were maintained on PDA slants.

2.2. Identification of *Agrobacterium vitis*

2.2.1. Specific monoclonal antibody

Isolates that produced typical colony types on RS and PDA media were evaluated for their reaction to an *A. vitis*-specific monoclonal antibody designated AbF21-1D3G7C8 (**Bishop et al 1989**). Colonies grown for 48 hr on 523 medium (**Kado et al 1972**) were sampled by collecting a mass of cells on the flat end of a sterile toothpick and suspending the cells in 100 µl of sterile distilled water adjusted to A600 nm = 0.1, about 10^8 cfu /ml. These suspensions (4 µl each, applied as two superimposed 2-µl spots, 1 spot/cm^2) were air dried on nitrocellulose membranes. Membranes were blocked by slowly shaking in PBSTM (PBST + 5% nonfat dry milk) for 30 min. They then were incubated for 1 hr in 1 µg of AbF21-1D3G7C8 (Epitomics) per milliliter of PBSTM. The membrane was washed three times (3 min each) in PBSTM (100 µl /cm^2 of membrane) and then incubated 1 hr in goat antimouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:400 in PBSTM. Antibody incubations were carried out in heat-sealable plastic bags to minimize reagent volume. After washing in PBST three times, the membrane was incubated with enzyme substrate/dye solution of 330 µg of nitroblue tetrazolium and 167 µg 5-bromo-4-chloro-3-indolyl phosphate per milliliter of 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgC_{l2}, pH 9.5. After adequate color developed (10-20 min), the reaction was stopped by washing the membrane in 20 mM Tris-HCl, 0.5 mM Na_2EDTA, pH 7.5. A negative control was included, consisting of the same procedure as above but omitting the monoclonal antibody incubation.

2.2.2. Tumorigenicity

The ability of the bacterial isolates to induce galls was tested on stems of sunflower (*Helianthus annuu*, cv. Giza 1), datura (*Datura stramonium*), tobacco (*Nicotiana tabacum* cv. White Burley), kalanchoe (*Kalanchoe daigremontiana*), tomato (*Lycopersicon esculentum* cv. Castlerock), chenopodium (*Chenopodium album*) and grapevine (*Vitis vinifera* cv. Flame seedless) by the needle prick inoculation method. Inoculum was prepared by suspending a loopful of bacterial growth from a 28-hr-old PDA slant culture into 2 ml distilled water. Plants grown in a greenhouse were inoculated at 5-8 sites per internode by pricking through a drop of inoculum with a needle. The pathogenicity was determined 30 days after inoculation based on gall formation at the site of pricking. Regard to the severity of isolates, only one isolate was selected as a representative from the pathogenic ones derived from each diseased grapevine.

2.2.3. Ability to induction grape necrosis

The ability of isolates to cause necrosis (**Burr et al 1987**) was determined on grape shoot explants. Actively growing shoots from potted vines in the greenhouse were harvested, and then surface disinfected by submersion in 10% bleach for 10 min followed by rinsing thoroughly with sterile distilled water. Internodal sections (about 7 mm in length) were cut and supported vertically in 1 % water agar in Petri dishes. The exposed ends of the explants were inoculated with 2 µl of aqueous bacterial suspensions made to optical density (OD) 600 = 0.1 (about 10^8 CFU/ml). Three explants were used as a control, one untreated, one treated with sterile distilled water and one treated with *A. tumefaciens* (isolated from rose). The appearance of necrosis was recorded after 72 hr. Assays were repeated at least once.

2.2.4. Biochemical and physiological tests

Isolates that reacted positively with the antibody and produced galls on any of the indicator plants were further compared with a standard set of biochemical and physiological tests that differentiate tumorigenic *Agrobacterium spp.* (**Moore et al 2001**). These include evaluation of 3-ketolactose production, alkaline reaction in litmus milk, growth on 2 and 5% NaCl, growth at 36°C, acid production from erythritol and melezitose, alkali production from malonic acid and L-tartaric acid. Isolates were also evaluated for production of polygalacturonase (**Rodriguez et al 1991**) and endoglucanase (**Ophel et al 1990**). All experiments were repeated at least once.

2.2.5. Polymerase chain reaction (PCR)

The multiplex PCR was conducted with three different primer pairs that amplify characteristic fragment sizes from genes from *A. vitis*. The PGF/PGR primers amplifying the chromosomal polygalacturonase gene *pehA* (**Szegedi and Bottka, 2002**) were used to identify *A. vitis* isolates and to distinguish them from *A. tumefaciens* (**Table, 1**). The Opine types of each isolate were determined in parallel using primers designed according to the octopine, nopaline and vitopine synthase gene sequences of *A. vitis*. Specific primers $VIRFF_1$ / $VIRFR_2$ and $VIRD2S4F_{716}$ / $VIRD2S4R_{1036}$ designed by **Bini et al (2008)** on sequences of octopine, nopaline and vitopine synthase genes of *A. vitis* were used.

DNA extraction from bacterial cultures

All *Agrobacterium* isolates were routinely cultured on potato semi-synthetic agar medium **(Kawaguchi et al 2005)**, which contained (per liter of distilled water) 300 g of potato, 0.5g of $Ca(NO_3)_2 . 4H_2O$, 2g of $Na_2HPO_4 . 12H_2O$, 5g of peptone, 20g of sucrose, and 15g of agar (pH 6.8–7.0). Cells from colonies on this medium were suspended in 20µl sterile distilled water. The suspension was heated at 95°C for 10 min and cooled for 5 min on ice. The suspension was centrifuged at 12000 rpm for 2 min and the resulting supernatants were used as templates for PCR. All DNA samples were stored at -20°C.

PCR amplification

PCR amplification of the target sequences was performed in a reaction volume of 25 µl containing 1X buffer, 3 mM $MgCl_2$, 200 µM each dNTP, 0.3 µM for primers $VIRFF_1$/ $VIRFR_2$ and $VIRD2S4F_{716}$/$VIRD2S4R_{1036}$, 0.4 µM for primers PGF-PGR, 5% DMSO, 0.02U/µl DNA polymerase (GoTaq Flexi DNA polymerase, PROMEGA) and 5 µl of template DNA prepared in 0.1% Tween-20. PCR experiments were performed in a 9700 Perkin-Elmer thermal cycler according to the following conditions: pre-denaturation 94°C 1 min; 40 x 94°C 1 min, 60°C 1 min, 72°C 1 min; final extension 72°C 5 min.

Gel electrophoresis

Three µl of the PCR products, mixed with 7 µl of (6x) bromophenol blue as a loading dye, were separated by horizontal agarose electrophoresis in 1x TBE buffer (Promega), 2% (w:v) LE agarose (Promega, USA) containing 1 µg ml^{-1} ethidium bromide. The marker molecular weight was 100 bp ladder (Promega). The electrophoresis reaction was performed at 75 V for 90 min and the bands were visualized with a UV transilluminator and photographed.

3. Results

3.1. Isolation of *Agrobacterium vitis*

Typical colonies of *A. vitis* were recovered from almost vineyard and nurseries samples collected from various grapevine growing areas in Egypt. In all, a lot of isolates were recovered that resembled *A. vitis* by producing typical colonies on RS and PDA media. Colonies resembling *A. vitis* appeared after 4 days at 28°C as convex and slightly mucoid with red centers and white margin **(Fig. 1; left)**. Resembling *A. vitis* colonies were transferred to potato dextrose agar (PDA). Isolates exhibited typical growth of *A. vitis* on PDA (convex, glistening, circular with an entire edge, and white to beige in color) after 48 hr at 28°C **(Fig. 1; right)**.

3.2. Identification of *Agrobacterium vitis*

3.2.1. Specific monoclonal antibody

From several isolates that produced typical colonies on RS medium, only 18 isolates reacted positively with the *A. vitis* antibody. Positive response with the immunoblot procedure was indicated by a dark purple spot on nitrocellulose membranes. Negative responses gave either no spot or a faint spot not noticeably darker than the negative control.

3.2.2. Tumorigenicity

Of 18 isolates reacted positively with the *A. vitis*-specific antibody, only 12 isolates were able to induce tumor on inoculated hosts. All of the 12 isolates were found to be tumorigenic on grape **(Table, 2)**. Symptoms appeared on grape shoots 1 month after they were inoculated as swelling tumors having a light brown color started at the wound sites **(Fig. 2; A and B)** then by the time the tumors become solid and gradually turned to dark brown in color **(Fig. 2; C and** D). All of the isolates except Av8 and Av11 were found to be tumorigenic on sunflower **(Fig. 3; 1)**, datura **(Fig. 3; 2)**, tobacco **(Fig. 3; 3)**, kalanchoe **(Fig. 3; 4)** and Chenopodium **(Fig. 3; 6)** after 10-30 days **(Table, 2)**. Only the isolates named Av8, Av9, Av10, Av11 and Av12 produced galls on tomato **(Fig. 3; 5)**. The plants of *k. daigremontiana* respond relatively readily and rapidly to inoculation of *Agrobacterium* isolates by producing tumors on stem, leaves and detached leave in as few as 10 days. The causative organism was readily reisolated from the infected plants showing galls resulted from the pathogenicity tests.

3.2.3. Ability to induction grape necrosis

All isolates were identified that caused necrosis phenotypes on grape explants **(Fig. 4)**. Severity of necrosis differed between isolates which some isolates (Av: 1, 4, 6, 7, 8, 12) caused a black necrosis with ooze within 48 h, whereas the others caused less necrosis (brown in color with less or no ooze). No necrosis was occurring on the control explants. By streaking cut ends of the explants on RS medium, the standard colonies of *A. vitis* were readily appeared after 3 days.

3.2.4. Biochemical and physiological tests

Results of the tests used to characterize 12 isolates are shown in **(Table, 3)**. No variation among isolates was observed and all isolates conformed closely to establish set of biochemical and physiological tests results expected for *A. vitis*. These isolates produced polygalacturonase and endoglucanase, oxidized lactose to 3-ketolactose, produced acid as a result of erythritol and melezitose oxidation, grew on NA medium supplemented with 2% NaCl and did not with 5%, did not grow at 36°C and produced alkali from litmus milk, malonic acid, and L-tartaric acid.

3.2.5. PCR reactions

A. vitis was further identified by PCR using the *pehA* gene-specific PGF/PGR primer followed by identification of opine types (specific primers, $VIRFF_1$/$VIRFF_2$ and $VIRD2S4F_{716}$ / $VIRD2S4R_{1036}$). Multiplex PCR with primer pair $VIRFF_1$/$VIRFR_2$ detected octopine but did not detect vitopine isolates of *A. vitis*. Opposite results were obtained using $VIRD2S4F_{716}$/ $VIRD2S4R_{1036}$ primer that amplified the corresponding sequences from vitopine isolates only. All of the analysed isolates amplified a product of appropriate 466 bp with *pehA* gene-specific primers. Also, all isolates carried *vir* gene sequences according to the results of multiplex PCR **(Fig. 5)**. Of the 12 *vir* positive isolates, 4 isolates designated Av 1, Av 2, Av 9 and Av 10 had octopine-type and 6 isolates designated Av 3, Av 4, Av 6, Av 7, Av 11 and Av 12 had vitopine-type. Two isolates, named Av 5 and Av 8 was

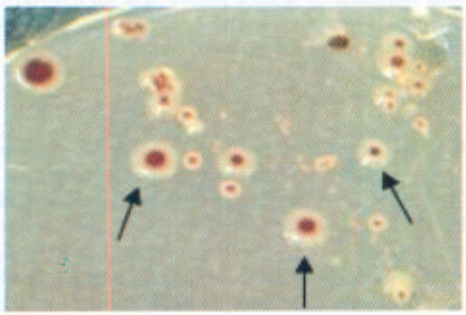
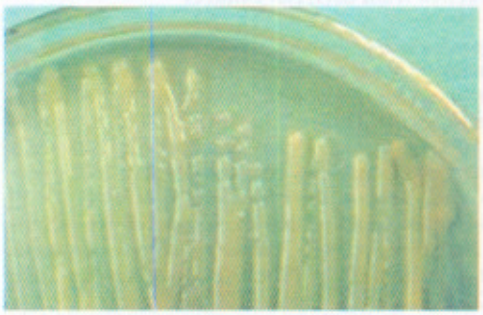


Fig. 1. Typical colonies of *A. vitis* (arrows) on RS medium



Typical growth of *A. vitis* on PDA medium

Fig. 2. Tumor progress on grape shoots after artificial inoculation: A, B, C and D = 30, 45, 60 and 90 days respectively

Fig. 3. Tumors on sunflower (1), datura (2), tobacco (3), kalanchoe (4), tomato (5) and Chenopodium (6) resulted from artificial inoculation by *A. vitis*

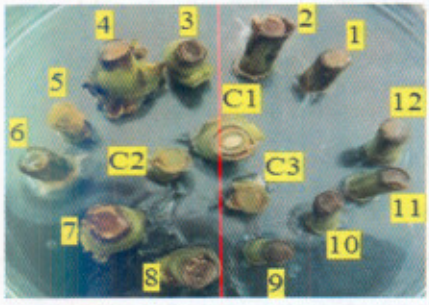


Fig. 4. Necrosis on grape explants resulted from artificial inoculation by 12 isolates of *A. vitis*. Control explants (center); C1= untreated C2= treated with sterile distilled water C3 = treated with *A. tumefaciens*

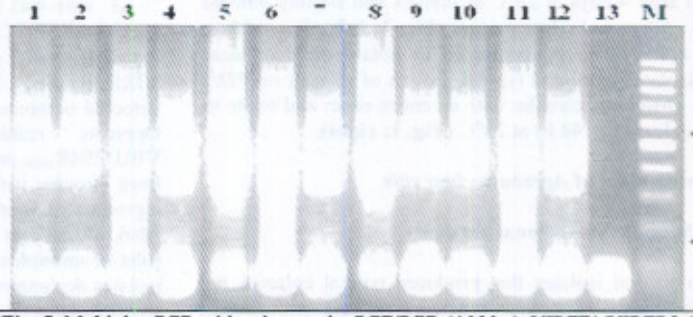


Fig. 5. Multiplex PCR with primer pairs PGF/PGR (466 bp), VIRFF1/VIRFR2 (382 bp) VIRD2S4F716/VIRD2S4R1036 (320 bp) using pure cultures of *A. vitis*. Lane 1: isolate Av1(octopine); lane 2: isolate Av2 (octopine); lane 3: isolate Av3 (vitopine); lane 4: Av43 (vitopine); lane 5: Av5 (octopine and vitopine); lane 6: Av6 (vitopine); lane 7: Av7 (vitopine); lane 8: Av8 (octopine and vitopine); lane 9: Av9 (octopine); lane10 Av10 (octopine); lane 11 Av11(vitopine); lane12 Av12(vitopine); lane 13: H2O, negative control. M: 100 bp ladder, PROMEGA

Table 1. Primers used for Comparison of *Agrobacterium vitis* isolates by PCR.

Name (forward/reverse)	Primer specificity	Sequence	Length of amplified fragment
PGF/ PGR	*pehA* Polygalacturonase gene	5'GGGGCAGGATGCGTTTTTGAG3' 5'GACGGCACTGGGGCTAAGGAT3'	466 bp
$VIRFF_1/VIRFR_2$	*virF* gene of *A. vitis* octopine and nopaline pTi	5' ATG AGA AAT TCG AGT TTG CAT GAT G 3' 5' TCG TGA TGG GTA TAC GCT ACG 3'	382 bp
$VIRD2S4F_{716}/VIRD2S4R_{1036}$	*virD2* gene of *A. vitis* vitopine pTi	5' GAC CGC AAA ACC TGC CAG 3' 5' GAG CCT GTA TTG ACG ATG TC 3'	320 bp

All primers were synthesized by UCDNA Services, University of Calgary, Albena, Canada

Table 2. Host rang of 12 *Agrobacterium vitis* isolates obtained from grapevine in Egypt

Host	isolate response											
	Av1	Av2	Av3	Av4	Av5	Av6	Av7	Av8	Av9	Av10	Av11	Av12
Grapevine	+	+	+	+	+	+	+	+	+	+	+	+
kalanchoe	+	+	+	+	+	+	+	-	+	+	-	+
Sunflower	+	+	+	+	+	+	+	-	+	+	-	+
Datura	+	+	+	+	+	+	+	-	+	+	-	+
Tobacco	+	+	+	+	+	+	+	-	+	+	-	+
Chenopodium	+	+	+	+	+	+	+	-	+	+	-	+
Tomato	-	-	-	-	-	-	-	+	+	+	+	+

+ = positive reaction. - = negative reaction.

Table 3. Physiological and Biochemical characteristic of 12 isolates of *A. vitis* isolated from grapevine in Egypt

Test	isolate											
	Av1	Av2	Av3	Av4	Av5	Av6	Av7	Av8	Av9	Av10	Av11	Av12
Polygalacturonase production	+	+	+	+	+	+	+	+	+	+	+	+
Endogluconase production	+	+	+	+	+	+	+	+	+	+	+	+
3-Ketolactose production	–	–	–	–	–	–	–	–	–	–	–	–
Acid from erythritol	–	–	–	–	–	–	–	–	–	–	–	–
Acid from melezitose	–	–	–	–	–	–	–	–	–	–	–	–
Alkaline on litmus milk	+	+	+	+	+	+	+	+	+	+	+	+
Alkali from malonic acid	+	+	+	+	+	+	+	+	+	+	+	+
Alkali from L-tartaric acid	+	+	+	+	+	+	+	+	+	+	+	+
Growth on 2% NaCl	+	+	+	+	+	+	+	+	+	+	+	+
Growth on 5% NaCl	–	–	–	–	–	–	–	–	–	–	–	–
Growth at 36°C	–	–	–	–	–	–	–	–	–	–	–	–

+ = positive reaction. - = negative reaction.

positive for the presence of both *virD2* and *virF* sequences and also for both octopine and vitopine synthases. None of all isolate had nopaline type indicates that Egyptian isolates probably do not carry nopaline type Ti plasmids.

4. DISCUSSION

Tumorigenic isolates of *A. vitis* were isolated from grapevine galls collected from commercial vineyards located in different areas in Egypt. The isolates produced typical colonies on RS medium, reacted positively with the *A. vitis* antibody, produced polygalacturonase and endoglucanase, and induced necrosis on grape explants. They also conformed closely to the established set of biochemical and physiological tests that are used to differentiate *A. vitis* from other *Agrobacterium spp.* In Bergey's Manual 1984 **(Kersters and De Ley, 1984)**, the genus *Agrobacterium* is divided into 4 species mainly on the basis of the pathogenicity and the types of symptoms induced on plants. Namely the genus consists of crown-gall-forming *A. tumefaciens*, hairy-root-inducing *A. rhizogenes*, non-pathogenic *A. radiobacter* and tumorigenic *A. rubi* which were isolated from *Rubus* spp. In addition, there are 2-3 genetically and phenotypically different groups in each species except for *A. rubi* **(Kersters et al 1973)**, which are assigned to biovars 1, 2 and 3. Thus two steps are required to identify agrobacteria: the first are to analyze the pathogenicity which distinguishes species, and the second to study the bacteriological properties that differentiate biovars **(Kersters and De Ley, 1984)**. The grapevine isolates were found to belong to *A. vitis;* since they were agrobacteria isolated from galls and produced galls on grapevine. As for their cultural, physiological and biochemical characteristics, they were different from the isolates belonging to *A. tumefaciens* biovars 1 and 2 in many characteristics, but gave similar reactions to those of biovar 3 (*A. vitis*) described in **Kersters and De Ley (1984)**.

In the previous reports on crown gall diseases, *A. tumefaciens* biovars 1 and 2 have already been detected in Egypt **El-Helaly et al (1969)**. However, this is the first report, to our knowledge, on the identification of biovar 3 (*A. vitis*) in Egypt. Although only *A. vitis* could be isolated from grapevines in the present work, *A. tumefaciens* biovar 1 or 2 as well as 3 (*A. vitis*) was recovered from the same host in Greece, Hungary, *etc.* **(Panagopoulos et al 1978)**. Further research is needed to determine whether causal agents of grapevine crown gall other than biovar 3 occur in Egypt.

The host range is not limited to grapevines in most cases but includes a variety of dicotyledonous plants. Strains may be tumorigenic or nontumorigenic, but to date no rhizogenic isolates have been described. All isolates that have been tested are also capable of causing the necrosis on grapevine described by **Burr and Otten (1999)**.

All of the isolates were found to be tumorigenic on grape but differs between itself in its pathogenicity to other hosts. This difference in host range is interesting and is one indication of the diversity among the isolates. Host range differences between *A. vitis* isolates have been previously reported and are known to be associated with Ti plasmid structures. For example, isolates carrying the type of Ti plasmid (with small TA region) have a limited host range compared with those with large TA regions **(Knauf et al 1984)**.

Other potential host specificity-related factors include the production of polygalacturonase (PG) and endoglucanase **(McGuire et al 1991)**. PG, which has been studied more extensively, is associated with induction of grape necrosis and was identified as a virulence factor since a PG-minus mutant was impaired in ability to induce tumors on grape and to attach to grape roots **(Rodriguez et al 1991)**. The *pehA* gene from *A. vitis*, which encodes for PG, was cloned and sequenced and the enzyme was compared to PGs from other microorganisms. The *A. vitis* enzyme is more similar to PGs produced by the plant pathogenic bacteria *Ralstonia solanacearum* and *Erwinia carotovora* than it is to those from *Aspergillus niger* and *Lycopersicum esculentum* **(Herlache et al 1997)** The *A. vitis* PG released dimers, trimers, and monomers from polygalacturonic acid and caused less electrolyte leakage from potato tubers than did PGs from *E. carotovora* and *R. solanacearum*.

A. vitis typically causes gall formation on grape trunks at or above graft unions. It is most interesting that galls are rarely observed on roots, but instead the bacterium induces a grape specific necrosis. Tumorigenic and nontumorigenic *A. vitis* strains induce necrosis within 24-48 h on roots of all *Vitis* species, but not on other plants that have been examined **(Burr et al 1987)**. Recently, it has been determined that necrosis is also induced on shoot explants and grape leaves, that induction is inoculum dependent, and that genes for necrosis are carried on the bacterial chromosome **(Burr and Otten, 1999)**. Therefore, *A. vitis* induced necrosis is different from the necrosis reported for certain *A. tumefaciens* strains, which appears to be related to hormone toxicity and is associated with different Ti plasmid genes **(Pu and Goodman, 1992)**.

A. vitis strains are classified into three taxonomical groups based on Ti plasmid-encoded opine markers, as octopine-, nopaline-, and vitopine-type strains. Of these, octopine strains are found most commonly in grapevine accounting for 60-75 % of isolates **(Burr et al 1998)**. Since the introduction of thermostable DNA polymerase and automated thermocyclers **(Saiki et al 1988)**, PCR has rapidly become a basic diagnostic and identification protocol in plant pathology as well **(Louws et al 1999)**. Early studies to identify *Agrobacterium* with PCR used pure bacterial cultures to determine the suitability of primers which were usually designed on the basis of Ti plasmid vir region-, or T-DNA sequences **(Haas et al 1995)**. The *pehA* specific PGF and PGR primers **(Szegedi and Bottka, 2002)** were combined with primers designed for *virF* and *virD2* sequences **(Bini et al 2008)**. The *pehA*-specific sequences allowed distinguishing *A. vitis* from *A. tumefaciens* **(Szegedi and Bottka, 2002)**. The *virF* gene is a host range factor that occurs on the octopine-type Ti plasmids of *A. tumefaciens* and octopine and nopaline-type Ti plasmids of *A. vitis* **(Schrammeuer et al 1998)**. The protein product (*VirF*) of this gene is transferred into plant cell during transformation and contributes to the deproteinization of T-DNANirE2 complex prior to its integration into the plant nuclear DNA **(Tzfira et al 2004)**. Multiplex PCR with these primers proved to be suitable to unambiguously detect all the assayed *A. vitis* strains and to partially discriminate among their different pTi plasmids, since *virF*-specific primers detected all octopine and nopaline strains whereas *virD2* gene primers detected

all vitopine strains. PCR results with opine synthase-specific primers showed that four (33.3 %) of the *A. vitis* isolates belonged to the octopine and six (50%) belonged to the vitopine group. Nopaline type isolates were not found. These results are not in accordance with previously published data for strains **(Burr et al 1998)** and field tumors **(Szegedi, 2003)**. Formerly octopine-types were reported to be the most prevalent opine-type in *A. vitis* populations (~60 %), and nopaline types were also reported (~30%) to exceed vitopine ones (~10 %). Interestingly, two *A. vitis* isolates (Av5 and Av8) were found to be positive for both *virD2* and *virF* genes, as well as for vitopine and octopine synthase genes. **Bini et al (2008)** founded as this result with the strain IBV-BO 5372 and they hypothesized that this strain might harbor two distinct Ti plasmids, one coding for vitopine and the other for octopine-markers. The dual opine character of these isolates can be explained by the different incompatibility vitopine pTis **(Szegedi et al 1996)**.

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حوليـــات العلـــوم الزراعية
جامعة عين شمس ، القاهرة
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توصيف عزلات من المسبب المرضى *Agrobacterium vitis* المعزولة من العنب فى مصر [١٤]

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الموجـــز

يعتبر مرض التـدرن التـاجى المتـسبب عـن البكتيريـوم *Agrobacterium vitis* أهـم الأمـراض البكتيرية التى تصيب العنب على مستوى العالم وينتج عنه خسائر كبيرة سواء فى البساتين أو المشاتل.

هذا وقد لوحظ فى الأونة الأخيرة الإنتشار المتزايد لهذا المرض فى مناطق زراعة العنب فى مصر.

لذا أجريت هذه الدراسة بهدف عـزل وتعريـف المسبب المرضى ودراسة بعض الخصائص المختلفة لعزلات هذا المـسبب المرضـى المنتـشرة تحـت الظروف المصرية.

وكانت نقاط الدراسة وأهم نتائجها فيما يلى

أولاً: عزل المسبب المرضى

تم تجميع عينات عنب مصابة من مناطق مختلفـة فى مصر وبإجراء العزل منها باسـتخدام بيئـة (RS) المتخصصة لعزل المسبب المرضى تم الحصول على العديد من العـزلات المطابقـة مـن حيـث الـشكل المورفولوجى على بيئة البكتريـوم *Agrobacterium vitis* .

ثانياً: تعريف المسبب المرضى

١- بأستخدام الـ Antibody المتخصص لهذا المسبب المرضى كانت هناك ١٨ عزلة فقط هـى التـى تفاعلت إيجابياً معه.

٢- بأجراء العدوى الصناعية على العنب و عديد من العوائل المشخصة كانت هناك ١٢ عزلة فقط من أصل ١٨ كانت لها القدرة على أحـداث الأورام على هذه العوائل حيث أن جميعها أعطـت أورام على العنب وتباينت فيما بينها فى قـدرتها علـى إحداث أورام علـى الكالانـشو و دوار الـشمس والداتوراه و الدخان والزربيح و الطماطم.

٣- بإختبار قدرة العزلات على أحداث موت وتحلـل موضعى على القطع النباتية الغضة المأخوذة مـن نباتات العنب كانت جميعها لها القدرة على أحداث هذ ا العرض مع وجود تباين فيما بينها فى شـدة هذا العرض حيث أن بعض العزلات أعطت موت وتحلل مصحوباً بإفرازات بكتيرية على موضـع الأصابة والبعض الآخر لم يعطى هذه الإفرازات.

٤- بأجراء الاختبارات الفـسيولوجية والكيموحيويـة المشخصة لهذا المسبب المرضى أعطـت جميـع العزلات نتائج متطابقة تماما مع النتائج المذكورة فى المراجع العلمية.

ثالثاً: المقارنة بين العزلات بإستخدام تفاعل البلمـرة (PCR)

بإستخدام ثلاث بـوادئ متخصـصة (Specific Primers) إحداها خاص بتعريف هذه البكتيريا إعتماداً على أختلافاتها الجينية الخاصة بإنتاج أنـزيم البـولى جلاكتورنيز والآخران متخصصان فى التفريق بـين العزلات من حيث نوع الأوبين (Opine type) كانـت جميع هذه العزلات إيجابية مع البادىء الأول وتباينت فيما بينها فى أستجابتها للبادئين الآخرين حيث أمكـن تقسيمها إلى ثلاث مجموعات رئيسية أحدهما تحمـل البلازميد الخاص بالاكتوبين والثانية تحمل البلازميـد الخاص بالفيتوبين والثالثة التى تحمل الإثنين معا.

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