Prevalence and Genetic Diversity of Grapevine Virus D in Tunisia

Ilhem Selmi, Laboratoire de Protection des Végétaux LR16INRAT04, Institut National de la Recherche Agronomique de Tunisie, Université de Carthage, Rue Hedi Karray, 1004 ElMenzah, Tunis, Tunisia, **Toufic Elbeaino,** Istituto Agronomico Mediterraneo di Bari, Via Ceglie 9, 70100 Valenzano, Bari, Italy, **Lehad Arezki,** Laboratoire de Phytopathologie et Biologie Moléculaire, Ecole Nationale Supérieure d'Agronomie, Rue Hacen Badi, Belfort, El Harrach, 16000 Alger, Algeria, **Manel El Air,** Laboratoire de Protection des Végétaux LR16INRAT04, Institut National de la Recherche Agronomique de Tunisie, Université de Carthage, Rue Hedi Karray, 1004 ElMenzah, Tunis, Tunisia, **Michele Digiaro,** Istituto Agronomico Mediterraneo di Bari, Via Ceglie 9, 70100 Valenzano, Bari, Italy, **and Naima Mahfoudhi,** Laboratoire de Protection des Végétaux LR16INRAT04, Institut National de la Recherche Agronomique de Tunisie, Université de Carthage, Rue Hedi Karray, 1004 ElMenzah, Tunis, Tunisia **(Tunisia / Italy / *Algeria*)**

https://doi.org/10.52543/tjpp.16.2.1

ABSTRACT

Selmi, I., Elbeaino, T., Arezki, L., El Air, M., Digiaro, M., and Mahfoudhi, N. 2021. Prevalence and genetic diversity of grapevine virus D in Tunisia. Tunisian Journal of Plant Protection 16 (2): 19-27.

The prevalence and the genetic diversity of *g*rapevine virus D (GVD) isolates from rootstocks, wine and table grape varieties grown in Tunisia were studied. RT-PCR assays performed on the coat protein gene (CP) showed the presence of GVD in 31.5% of the 403 samples tested. The highest rate of infection was found in table grapes (56.5%), followed by autochthonous table grapes (24.1%), wine grapes (20.8%) and rootstocks (12.5%). Sequences and phylogenetic analyses of the partial CP genes of 14 GVD isolates showed nucleotide identities that ranged from 84% to 99%. The Tunisian GVD-isolates segregated in 3 phylogenetic groups together with international isolates reported in GenBank. The present study extends our knowledge of the presence of GVD in Tunisian vines and on its genetic diversity, which is useful for developing broad-spectrum molecular diagnostics (RT-PCR) capable of detecting the different isolates infecting vines.

Keywords: Grapevine, phylogenetic analysis, RT-PCR, sequences, vitiviruses

Rugose wood (RW) represents one of the most important graft-transmissible diseases affecting *Vitis* species worldwide (Martelli and Boudon-Padieu 2006). Four disorders distinguishable by grafting onto woody indicators plants are associated with this complex disease: stem pitting on *Vitis rupestris*, stem grooving on Kober 5BB, corky bark and stem grooving on LN33 (Martelli 2014). Some of these syndromes are associated with vitiviruses of the family *Betaflexiviridae*. Grapevine virus A (GVA) is associated with stem grooving

Corresponding author: Naima Mahfoudhi
Email: nmahfoudhi@yahoo.fr

Accepted for publication 25 December 2021

on Kober 5BB (Digiaro et al. 1994; Garau et al. 1994; Chevalier et al. 1995), grapevine virus B (GVB) is recognized as the causal agent of corky bark (Bonavia et al. 1996) and grapevine virus D (GVD) is associated to the growth reduction of Freedom rootstock (AbouGhanem et al. 1997). Additionally, these vitiviruses are frequently detected in coinfection with grapevine leafroll viruses, resulting in synergetic interactions that can lead to lethal effects in several scion and rootstocks combinations (Rowhani et al. 2018). In recent years, several new vitiviruses have been isolated from grapevine (GVE, GVF, GVG, GVH, GVI, GVJ, GVL and GVM) but their potential pathogenic role in RW disease is still unknown (Nakaune et al. 2008; Al Rwahnih et al. 2012; Blouin et al. 2018a and 2018b; Candresse et al. 2018; Diaz-Lara et al. 2018; Alabi et al. 2019; Debat et al. 2019).

GVD was detected for the first time in south Italy in vines with corky rugose wood symptoms (AbouGhanemet al. 1997). The virus is serologically distantly related to GVA and GVB (Choueiri et al. 1997), but it closely resembles GVA in biological behaviour, particle size and morphology, cytopathology, dsRNA pattern, size of RNA and organization of the 3' terminal genomic region. Virions are filamentous particles of about 825 x 12 nm. The presence of GVD was assessed in Tunisian vineyards in a previous study (Mahfoudhi et al. 2014), but no information was provided about the genetic diversity of its isolates. Therefore, a new investigation has been carried out to determine the genetic population structure of GVD isolates in Tunisia.

MATERIALS AND METHODS

Field surveys and sample collection.

Field surveys were carried out in the main Tunisian grapevine growing areas to assess the GVD occurrence in the vineyards. Mature canes were collected from a total of 403 vine samples, including 141 vines of autochthonous varieties maintained in a germplasm collection plot established at INRAT (Tunis), 115 samples of wine and 115 of table grape varieties from commercial vineyards, and 32 rootstocks from mother tree plots. The number of samples collected was relative to the importance, type and origin of cultivars in Tunisia. Mature canes were randomly collected in winter and stored at 4°C, until laboratory testing.

Total nucleic acids extraction.

Total nucleic acids (TNA) were extracted according to Foissac et al. (2001). Two hundred mg of phloem tissues (cortical scrapings) from each sample were ground in 1 ml of extraction buffer (4 M guanidine thiocyanate, 0.2 M Sodium acetate pH 5.2, 25 mM EDTA, 1.0 M potassium acetate pH 5.0 and 2.5% w/v PVP-40) and mixed with 2% sodium metabisulfite as antioxidant. The mixture was transferred into an Eppendorf tube containing 100 µl N-Lauroylsarcosine sodium salt (NLS 10%), incubated at 70°C for 10 min, and then placed on ice for 5 min. After centrifugation at 10,000 g for 10 min, 300 µl of supernatant were transferred to an Eppendorf tube to which 150 µl absolute ethanol, 300 µl sodium iodide (6M) and 50 µl of silicon dioxide (SiO_2) were added. The mixture was stirred for 30 min at room temperature and then centrifuged at 5,000 g for 1 min. After washing with 500 µl of washing buffer (50% STE 1X, 50% absolute ethanol), the pellet was re-suspended in 120 µl of sterile distilled water, incubated for 3 min at 70 °C and then centrifuged at 10,000 g for 3 min. The supernatant containing the total

nucleic acids was transferred to a new Eppendorf tube and stored at -20°C.

Reverse transcription and Polymerase chain reaction (RT-PCR).

Five hundred ng of TNAs were mixed with 0.5 µg random primers and 1.5 µl of sterile water and denatured at 95°C for 5 min. Reverse transcription was performed for 1 h at 39°C using 200 units of *Moloney Murine Leukemia Virus* (Invitrogen Corporation, USA), 4 µl buffer (5x First-strand Fs), 2 µl DTT (0.1 M) and 0.5 µl dNTPs (10 mM) and adjusted to a final volume of 20 µl with sterile distilled water. A volume of 2.5 µl of the synthetized cDNA was submitted to PCR amplification using a mixture containing 2.5 µl 10X Taq polymerase buffer, 1 µl $MgCl_2$ (50 mM), 1 µl dNTPs (10 mM), 0.5 µl of each primer (20 µM) [GVD-CP7V (5'-CTTAGGACGCTCTTCGGGTACA-3') and GVD-CP471C (5'-CTGCTCTCCAACCGACGACT-3')] (AbouGhanem et al. 1997) and 0.25 µl Taq polymerase (5 u/µl) (Invitrogen Corporation, CA, USA), and adjusted to a final volume of 25 µl with sterile distilled water. PCR reactions consisted of one cycle at 94° C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 sec, annealing at 54°C for 45 sec and elongation at 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR products were analyzed by electrophoresis in 1.2% agarose gel in 1X TBE buffer and visualized under UV light after staining with ethidium bromide.

Sequencing and sequence analysis.

Fourteen Tunisian GVD isolates from the major cultivated table and wine grape cultivars as well as from autochthonous grapevines from different regions were chosen for the genetic diversity study of this virus. RT-PCR amplicons of all selected isolates were ligated into Strataclone™ PCR cloning vector PSCA (Stratagene, California, USA), sub-cloned into *Escherichia coli* DH5α cells and sequenced.

Sequences of Tunisian GVD-isolates obtained from this study were compared to those reported in GenBank. The phylogenetic tree was constructed using the Neighbour-joining (NJ) method (with 1000 bootstrap replicates) under MEGA7 software (Kumar et al. 2016), and the inter- and intra-group genomic identities were calculated.

RESULTS

Detection of GVD in Tunisian vineyards.

RT-PCR assays successfully amplified the expected 474 bp product from 127 out of 403 tested samples (31.5%). The highest GVD infection rates were detected in table grapes (56.5%) and wine grapes (20.8%) collected from commercial vineyards, while a significant lower level of GVD-infection was found in rootstock samples collected from mother plant plots (12.5%). The autochthonous vines grown in the collection plots, which were dominantly table grapes, showed a level of GVD-infection of 24.1%.

Among table grapes, the highest infection rates were observed in cvs. Italia and Rich Baba Sam (88.8%), followed by cv. Dabouki (73.3%), whereas, among wine grapes, in cvs. Alicante Boushet (25%), Grenache and Carignan (21.4%).

Out of 141 autochthonous vine samples tested, 34 were GVD-positive. Among these vines, significant difference in GVD infection rate was observed in samples coming from north of Tunisia (32.8%) compared to those collected from the south (15.5%). In the rootstocks, GVD was present in 30% of 1103P and 11.1% of 140Ru, while no infection was detected in 110R.

Genetic diversity analysis.

The partial CP sequences of 14 Tunisian GVD isolates obtained in this study from different infected Tunisian grapevine accessions (autochthonous, table and wine grape cultivars) were deposited into the European Nucleotide Archive (ENA) database and assigned accession numbers (Table 1). The sequences were compared with each other and with those of GVD isolates present in the database to obtain more information on the genetic diversity of this virus in Tunisian vineyards.

Table 1. List and identifiers of the coat protein genes of grapevine virus D isolates sequenced and analyzed in this study

Isolate	Cultivar	Origin	Accession number
Tn4	Carignan	Takelsa	LT908458
Tn6	Dabouki	Takelsa	LT908459
Tn7	Italia	Essaada	LT908460
Tn8	Italia	Essaada	LT908461
Tn9	Marsaoui*	Raf Raf	LT908462
Tn10	Asli*	Sfax	LT908463
Tn11	Testouri*	Djebba	LT908464
Tn12	Dabouki	Takelsa	LT908465
Tn13	Italia	Takelsa	LT908466
Tn14	Italia	Takelsa	LT908467
Tn15	Jerbi*	Degueche	LT908468
TnM1	Meski	Raf Raf	LT908457
TnG5	Rich Baba Sam	Bousalem	LT908454
TnI-3	Italia	Takelsa	LT908455
TnI-5	Italia	Takelsa	LT908456

* Autochthonous varieties

Sequence analyses showed that the Tunisian isolates share nt identities comprised between 84% to 99 % (Table 2). Isolates Tn11, Tn12, Tn13, Tn15 and TnI3 of the same cluster II-a shared 91% to 96% nt identities among them, except for the isolate Tn11 that shared 87% to 92% identities (Table 2). Isolate Tn4 of Group II-b showed a high divergence from all the other Tunisian isolates, with nt identities ranging from 87% to 92% (Table 2). Isolates TnG5, Tn6, Tn7, Tn8, Tn9, Tn14 and TnI5 of the Group I-a showed 91% to 100% of nt identities. The two Tunisian isolates TnM1 and Tn10 of the Group II-c were distant from all other Tunisian isolates and shared 97% nt identity with each other. The clones TnI-3 and TnI-5 obtained from the same isolate TnI showed 90% nt identity between them.

The "intra mean distance" revealed 94% nt identity for Group II-a, 98% for Group II-b, 94% for Group I-b, 95% for Group I-a and 97% for Group II-c. The "inter mean distance" between the Groups I and II ranged from 90% to 91% at nt level. The sole isolate BM-RM of the Group III showed a great divergence at nt level from the other two groups, with identity values varying between 83% and 84% (Tables 3 and 4).

Table 2. Matrix of nucleotide homologies among the CP sequences of 14 GVD Tunisian isolates

Isolate	TnG5	TnI3	TnI5	TnM1	Tn4	Tn6	Tn7	Tn8	Tn9	Tn10	Tn11	Tn12	Tn13	Tn14	Tn15
TnG5	100														
TnI3	86	100													
TnI5	93	90	100												
TnM1	87	89	91	100											
Tn4	88	92	92	91	100										
Tn6	93	90	99	91	92	100									
Tn7	94	90	98	91	92	99	100								
Tn8	91	89	95	87	89	95	95	100							
Tn9	94	90	99	91	92	99	99	96	100						
Tn10	88	91	91	97	92	91	91	88	91	100					
Tn11	84	88	88	87	87	87	87	86	87	87	100				
Tn12	87	92	92	89	91	92	92	90	92	91	91	100			
Tn13	87	91	92	91	92	92	92	90	93	92	92	95	100		
Tn14	94	90	99	91	92	99	99	96	100	91	87	92	93	100	
Tn15	87	91	91	90	90	91	91	90	92	91	91	95	96	92	100

Table 3. Intramean distance identity between different subgroups of GVD isolates

I	94%
II	92%
I-a	95%
I-b	94%
II-a	94%
II-c	97%
II-b	98%

Table 4. Intermean distance identity between different subgroups of GVD isolates

Subgroup	I-a	I-b	II-a	II-c	II-b	III
I-a	100%					
I-b	91%	100%				
II-a	90%	91%	100%			
II-c	90%	90%	90%	100%		
II-b	91%	91%	91%	89%	100%	
III	84%	83%	83%	84%	84%	100%

Phylogenetic analysis.

The phylogenetic tree constructed on sequences of the Tunisian and GenBank isolates revealed three groups (GI-III) for GVD, with GI and GII further subdivided into 2 (a, b) and 3 subgroups (a-c), respectively (Fig. 1). Most of Tunisian isolates clustered into the Group I (TnG5, Tn6, Tn7, Tn8, Tn9, Tn14 and TnI5) and Group II (Tn11, Tn12, Tn13, Tn15, TnI3, Tn4, TnM1 and Tn10), in particular in the subgroups GI-a and GII-a, together with Italian isolates whose plant material is reported to be originated from Tunisia (Elbeaino et al. 2019). Interesting was the allocation of the Brazilian isolate RM-BR that was highly divergent and distant from all other GVD-isolates.

Dissecting the allocations of Tunisian isolates within the phylogenetic tree, isolates from the table cultivars clustered into Group I-a (TnG5, Tn6, Tn7, Tn8, Tn14 and TnI5), II-a (Tn12, Tn13 and TnI3) and II-c (TnM1). In the same three subgroups of isolates clustered all the GVD isolates from autochthonous accessions: Tn9 (Group I-a), Tn11 and Tn15 (Group II-a) and Tn10 (Group II-c). Tn4 isolate of cv. Carignan was the sole Tunisian isolate to cluster into Group II-b. The two clones TnI3 and TnI5, from the same grapevine sample (TnI), clustered into Group II-a and Group I-a respectively, indicating a putative co-existence of mixture of GVD genetic variants in the same vine sample.

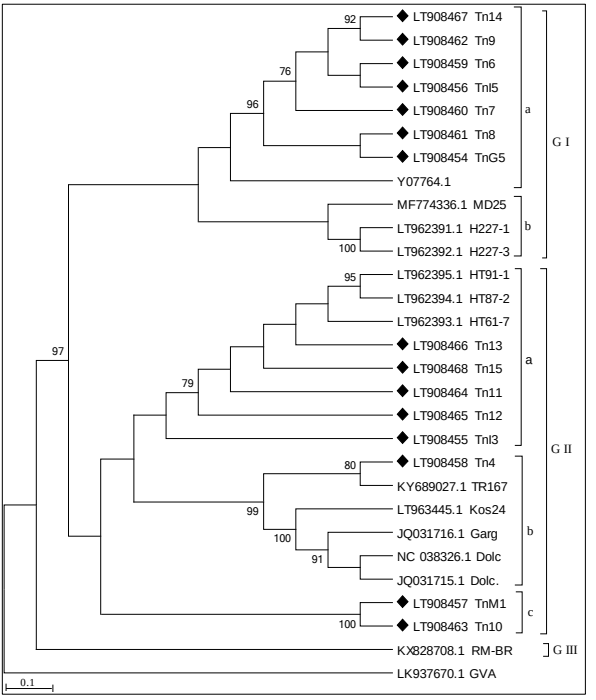

Fig. 1. Phylogenetic tree constructed based on nucleotide sequences of partial CP genes of GVD isolates obtained in this study (indicated with a diamond) and those available in GenBank.The percentage support of bootstrap (>75%) from 1000 replicates. Accessions numbers are followed by isolates names. G: Group.

DISCUSSION

The present study has shown a high prevalence (31.5%) of GVD infections in the Tunisian vineyards, a value relatively similar to that of 41.4% reported in a previous survey (Mahfoudhi et al. 2014).

The high infection rate of GVD, especially in table grapes, together with the high incidence of other viruses in the Tunisian vineyards (Mahfoudhi et al. 1998) reveal an alarming situation for viticulture in the country. GVD is the least studied among all the rugose wood-associated viruses, and its prevalence has only been assessed in a few countries. A similar GVD-infection rate (31%) was reported in grapevines from Italy (Boscia et al. 2001), whereas it was significantly lower (9%) in Turkey (Buzkan et al. 2017).

Despite the natural vector of GVD is still unknown, the high rate of infection by this virus could be due to the presence of a still unidentified vector, especially since the mealybugs, i.e. *Planococcus ficus* and *Planococcus citri* known to be involved in the dissemination of other vitiviruses associated with rugose wood disease are very widespread in Tunisia (Mahfoudhi and Dhouibi 2009).

This work provides information on the genetic diversity of GVD in the Tunisian grapevines based on the sequence comparison of a fragment of the CP gene. In the phylogenetic analysis, the Tunisian isolates clustered into two groups, and in particular in Group II (8 isolates) and Group I (7 isolates). It is worth noting that most of these isolates were grouped into two subgroups (I-a and II-a), thus suggesting the existence of a large variability among Tunisian GVD-isolates.

The high divergence found between GVD-isolates, based on the sequence analysis of CP gene, with the presence of six GVD-clusters (considering groups and subgroups), confirms the high genetic diversity of this virus as that reported for other vitiviruses (Elbeaino et al. 2019).

Furthermore, the segregation of Tunisian GVD isolates into different phylogenetic groups regardless of the grapevine plant material type, cultivars, but not the geographical origin as is the case of Tunisian and Italian isolates found close to each other in the phylogenetic tree, suggests that GVD isolates from Tunisia and Italy had a common origin (Elbeaino et al. 2019).

This study extends the knowledge on the incidence of GVD in the Tunisian vineyards and provides the first molecular information on the high sequence variability that could be exploited to develop broad-spectrum molecular diagnostics (RT-PCR) for the detection of different GVD isolates infecting grapevine in nature.

ACKNOWLEDGEMENTS

This work was financed in part by the Tunisian-Algerian Cooperation, as part of the INNOVITIS project "Valorisation et innovations dans l'utilisation des vignes autochtones nord-africaines".

RESUME

Selmi I., Elbeaino T., Arezki L., El Air M., Digiaro M. et Mahfoudhi N. 2021. Prévalence et diversité génétique du virus D de la vigne en Tunisie. Tunisian Journal of Plant Protection 16 (2): 19-27.

La prévalence et la diversité génétique des isolats du virus D de la vigne (GVD) provenant de porte-greffes, de cépages de cuve et de variétés de table (introduites et autochtones) cultivés en Tunisie ont été étudiées. Les tests RT-PCR effectués sur le gène de la protéine de la capside

(CP) ont montré la présence de GVD dans 31,5% des 403 échantillons testés. Le taux d'infection le plus élevé a été trouvé dans les variétés de table introduites (56,5 %), suivis par les variétés de table autochtones (24,1 %), les cépages de cuve (20,8 %) et les porte-greffes (12,5 %). Les séquences et les analyses phylogénétiques des gènes partiels du CP de 14 isolats de GVD ont montré des identités nucléotidiques allant de 84 % à 99 %. Les isolats tunisiens du GVD ont été groupés en 3 groupes phylogénétiques avec les isolats internationaux inscrits dans le GenBank. La présente étude élargie nos connaissances sur la présence de GVD dans les vignes tunisiennes et sur sa diversité génétique, ce qui est utile pour développer le diagnostic moléculaire à large spectre capables de détecter les différents isolats infectant la vigne.

Mots clés: Vigne, analyses phylogénétiques, RT-PCR, séquences, vitivirus

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ملخص

سالمي، إلهام وتوفيق البعينو ولحاد أرزقي ومنال العير وميكال ديجيارو ونعيمة محفوظي. 2021. الانتشار والتنوع الجيني لفيروس كروم العنب D في تونس. Tunisian Journal of Plant Protection 16 (2): 19-27.

تمت دراسة انتشار والتنوع الجيني لفيروس كروم العنب D (GVD) المعزول من حوامل الطعوم ومن أصناف عنب النبيذ ومن عنب المائدة، المزروعة في تونس. أظهر تفاعل البوليمراز المتسلسل مع النسخ العكسي (RT-PCR) التي أُجري على بروتين الغلاف وجود الفيروس في 31.5% من العينات المختبرة البالغ عددها 403. أعلى نسبة إصابة كانت لدى أصناف عنب المائدة الأجنبية (56.5%)، يليها الأصناف المحلية (24.1%) ثم أصناف عنب النبيذ (20.8%) وحوامل الطعوم (12.5%). أظهرت المتواليات والتحليلات الجينية للجينات الجزئية لـ 14 عزلة أن هويات النوكليوتيدات تراوحت بين 84% إلى 99%. تم فصل العزلات التونسية في 3 مجموعات نسجية مع عزلات دولية مسجلة في بنك الجينات. تُوسّع الدراسة الحالية معرفتنا بوجود فيروس العنب D بالكروم التونسية وبتنوعه الوراثي، وهو أمر مفيد لتطوير تشخيصات جزيئية واسعة النطاق قادرة على اكتشاف العزلات المختلفة التي تصيب كروم العنب.

كلمات مفتاحية: تحاليل جينية، تسلسل، كروم العنب، RT-PCR، vitivirus

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