JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

GRAPEVINE CROWN GALL: CURRENT DATA AND RESEARCH PERSPECTIVES

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https://doi.org/10.55251/jmbfs.10198

ARTICLE INFO

Received 23. 5. 2023
Revised 4. 8. 2023
Accepted 7. 8. 2023
Published 1. 12. 2023

Review

OPEN ACCESS

ABSTRACT

Grapevine (*Vitis vinifera* L.) is one of the most widespread and economically important fruit crops in the world based on its capacity to produce high yields of quality fruit, hectares cultivated and its ability to grow in a wide range of climates and soils. However, it is greatly exposed to a wide variety of pathogens, affecting production and fruit quality. Among the potential threats, *Allorhizobium vitis*, the causal agent of grapevine crown gall represents a limiting factor in grape production worldwide. It causes vine decline and mortality especially in young vineyards and orchards with important economic losses. Owing of the systemic survival of *Allorhizobium vitis* in grapevine, copper bactericides and antibiotics are ineffective, and they are able to kill only the bacterium on contact. Therefore, the knowledge of pathogen, effective control and prevention strategies, and sensitive detection methods of pathogen are needed to improve the management of the disease. This review highlights the current state of research and the major acquisitions in this field and provides efficient procedures for isolating from tumors and soil. In addition, this paper discusses the different strategies used for the management of grapevine crown gall along with their drawbacks. Moreover, detection methods for rapid and proper identification of the disease bacteria were provided to enhance the efficiency of control measures and prevent the spread of the pathogen.

Keywords: *Allorhizobium vitis*, grapevine crown gall, control and prevention strategies, detection methods

INTRODUCTION

Grapevine crown gall has been recognized as a serious worldwide problem in viticulture for many years (**Kuzmanović *et al.*, 2018**). The disease weakens vines and is responsible for significant reductions in yield and vigor and, in the worst case, leading to partial or complete plant death all over the world (Figure 1) (**Nguyen-Huu *et al.*, 2020**). The causal agent, *Allorhizobium vitis*, is a widely distributed pathogen and associated almost exclusively with grapevine (**Kuzmanović *et al.*, 2018; Habbadi *et al.*, 2019**). *All. vitis* causes crown gall disease by transforming plant cells into autonomously proliferating cells using a tumor-inducing (*Ti*) plasmid (**Noutoshi *et al.*, 2020**). Virulent strains of All. vitis induce the development of tumorigenic structures at the crown of the plant; hence, the name crown gall. They can also cause necrotic lesions on grapevine roots (**Kawaguchi *et al.*, 2017**), and "galls" or tumors to develop at the perennial stems where wounds are caused because of grafting or injury by farm implements or freezing temperatures (Figure 2) (**Gan *et al.*, 2019**). These tumors eventually block the vascular connection between roots and aerial parts of the plant. In young vineyards, infected vines developing crown gall at their graft unions often die, or they may be stunted with reduced growth and production (**Habbadi *et al.*, 2017**). Moreover, grapevine crown gall in nurseries results in huge losses due to unsaleable symptomatic plants and may lead to the spread of the pathogen in asymptomatic plants. Economic losses caused by grapevine crown gall are associated with reduced productivity and costs of vine replacement, because the causal agent can survive longer in infected roots, decaying grape and soil (**Kuzmanović *et al.*, 2018**), even after vines have been removed, therefore, *All. vitis* cells remain active and viable in the soil and could infect the new planting material (**Vizitiu *et al.*, 2012**).

To date, the most successful strategy is disease prevention by planting material free of the pathogen into non-infected and clean soil (**Voegel *et al.*, 2018**). However, systemic survival of *All. vitis* in symptomless vines results in difficulty in producing clean grapevine stock (**Burr *et al.*, 2016**), and the pathogen is often disseminated to new areas through the vegetative propagation of infected symptomless vines (**Yepes *et al.*, 2019**). In addition, due to this ability of *All. vitis* to live systemically within vines, there is no product able to significantly control grapevine crown gall (**Burr *et al.*, 2016**). Furthermore, copper bactericides and antibiotics are ineffective on *All. vitis* cells inside plant tissues (**Yepes *et al.*, 2019**). For effective control and prevention measures of diseases, a guiding principle is that when key inoculum sources for a given disease are known, appropriate and effective management strategies should be implemented to prevent further spread and subsequent disease outbreaks (**Alvarez *et al.*, 2004**). Therefore, the best way to prevent crown gall in the vineyard is to prevent the site from being contaminated with infected plants from the beginning (**Vizitiu *et al.*, 2012**). For that purpose, highly sensitive and rapid methods are required for detecting the pathogen in infected symptomless grapevines and soil. Furthermore, because control measures are limited and ineffective, new, and sustainable methods of biocontrol are required, including the use of bacteriophages, endophytic bacteria and medicinal and aromatic plants (MAP) extracts as potential biological agents against grapevine crown gall (**Habbadi *et al.*, 2017; Sabri *et al.*, 2021; Habbadi *et al.*, 2021**).

Figure 1 Distribution of grapevine crown gall disease in the world.

Figure 2 Symptoms of grapevine crown gall. Tumors on trunk of Moroccan vines (INRA-Meknes).

PATHOGEN BIOLOGY

Allorhizobium vitis, formerly known as *Agrobacterium vitis* or *Agrobacterium tumefaciens* biovar *3*, is a member of the genus of *Allorhizobium*, the family of *Rhizobiaceae*, the order of Rhizobiales, and the class of *Alphaproteobacteria* (**Kuzmanović *et al.*, 2020**). The taxonomy position of *All. vitis* has undergone several modifications since their first isolation in the vineyard by **Fabre *et al.*, 1853**. At first, it was classified as a *Bacterium tumefaciens* based on pathogenicity tests (**Smith *et al*, 1907; Reker *et al*, 1926**). After the creation of the genus *Agrobacterium* in 1942 by **Conn, Hooykaas *et al.*, 1977** and **Genetello *et al.*, 1977** have shown that bacteria belonging to this genus possess the *Ti* plasmid associated with the ability to induce crown gall. The bacterium was subsequently classified as an atypical strain that does not belong to *Agrobacterium tumefaciens* biovar 1 and 2 (**Panagopoulos *et al.*, 1973**). It has been designated *Agrobacterium tumefaciens* biovar *3* (**Kerr *et al.*, 1977**). In 1990, Multilocus Sequence Analysis (MLSA) a made it possible to distinguish it as a new species named *Agrobacterium vitis* (**Ophel *et al.*, 1990**). Subsequently, **Young *et al.*, 2001** proposed a new amendment in the genus *Agrobacterium* and *Rhizobium* classification and defined *Agrobacterium vitis* as a species belonging to the genus *Rhizobium* called *Rhizobium vitis.* Finally, **Mousavi *et al.*, (2014, 2015)** suggested transferring it to the genus *Allorhizobium* after phylogenetic analysis of 114 strains per MLSA using six housekeeping genes. However, *Agrobacterium vitis* remains the most common name in the scientific community despite improvement trials.

All. vitis grows aerobically and optimally at 25–28 °C, is a Gram-negative, rod-shaped, non-spore-forming, soil-borne bacterium that is specific to *vitis* spp. (**Thies *et al.*, 1991**). The bacterium is motile, having one to six peritrichous flagella (**Canik *et al.*, 2016**). Unlike other members of the genus *Agrobacterium* which is characterized by the presence of a linear chromosome and a circular chromosome (**Ramirez-Bahena *et al.*, 2014**). *All. vitis* has two circular chromosomes and a variable number of plasmids (**Tanaka *et al.*, 2006; Habbadi *et al.*, 2019**), Tumorigenic strains of *All. vitis* may contain two to five plasmids, one of which carries the genes responsible for tumor induction and is known as the *Ti* (*Tumour Inducing*) plasmid (**Buchholz *et al.*,1984, Schierstaedt *et al.*, 2019**). The *Ti* plasmid also harbors the host-range genes that determine symptoms, which the infection will produce. The bacterium is identified as non-virulent in the absence of this Ti plasmid and will not be able to cause disease on the grapevine (**Vizitiu *et al.*, 2011**). Besides the Ti plasmid, *All. vitis* associated exclusively with grapevine may also harbor other ecologically important plasmids that enhance the competitiveness of this pathogen on the grapevine, such as tartrate-catabolic plasmids responsible for the utilization of tartrate which is an abundant compound in grapevines (**Schierstaedt *et al.*, 2019**), and opine-catabolic plasmids, which contain genes encoding uptake and catabolism of small molecules called opines (**Kuzmanović *et al.*, 2018**). Opines are specific conjugates amino acids and α-ketoacids or sugars, they serve as nutrients, nitrogen and carbon source, and specific substances that increase the pathogenicity of the bacteria. However, opine-catabolic plasmids do not contain *vir* genes and T-DNA required for pathogenicity (**Wetzel *et al.*, 2014; Schierstaedt *et al.*, 2019**).

The crown gall disease is strictly linked to the presence of the *Ti* plasmid, it is an essential determinant of pathogenesis not only for *All. vitis* but also for some strains of *Agrobacterium* (**Burr *et al.*, 1987**). pTi and T-DNA were described in **1984 by Buchholz and Thomashow**; it is a plasmid of significant molecular weight (200kb), comprising several genes involved in the virulence of the bacteria which can be distinguished in two groups of genes according to the functional plane. Thus, the first group corresponds to genes expressed in the bacteria and whose regions are not transferable to the plant cell (region *ori*: origin of replication, genes *tra*: conjugal transfer, genes *vir*: virulence gene, etc.). The second group distinguishes the transferable genes expressed in the plant (T-DNA).

The five regions of the pTi are cited in the Figure 3 (**Nesme *et al.*, 1995; Szegedi *et al.*, 1998; Zhu *et al.*,2000; Burtin, 2008)**: T region (T-DNA), *vir* region, locus *tra* and *trb*, Rep region, and *opc* or *occ* region "Acquisition and catabolism of opines.

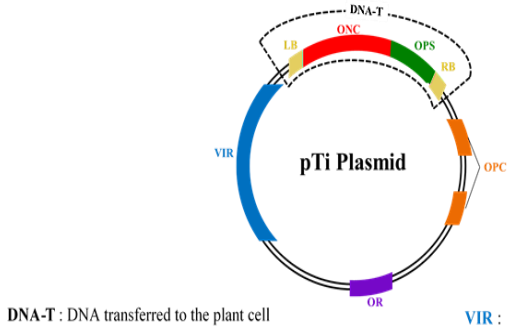

Figure 3 Different regions of plasmid *Ti* involved in the virulence of *Allorhizobium vitis*.

INFECTION PROCESS

The natural infection process by *All. vitis* is derived from a conjugal transfer that includes several stages and involves several genes located in different places in the DNA. All these genes are grouped in two: genes *chv* (chromosomal virulence) present on the bacterial chromosome, and which are responsible for the attachment of the bacteria to the plant cell and the genes of the region *vir* located in the pTi which are at the origin of the transfer of the T-DNA into the genome of the host cell (**Zhu *et al.*, 2000; Burtin, 2008)**. These genes are expressed in response to chemical signals released by the host plant. The virulence mechanism takes place in seven stages (Figure 4) (**Kemper *et al.*, 1985; Cangelosi *et al.*, 1989; Sanders *et al.*, 1991; Burr *et al.*, 1998; Lai *et al.*, 2000; Levin *et al.*, 2000; Portier, 2004; Tzfira *et al.*, 2004; Pitzschke *et al.*, 2010 ; Gelvin, 2012; Liang *et al.*, 2013**).

Table 1 Selective media for the isolation and purification of *Allorhizobium vitis* strains.

Media	Composition	Characteristics
Roy and Sasser (Roy, 1983)	Adonitol, 4g ; H_3BO_3, 1g ; yeast extract, 0.14g ; $MgSO_4$ x $7H_2O$, 0.2g ; KH_2PO_4, 0.7g ; K_2HPO_4, 0.9g ; NaCl, 0.2g ; Agar, 20g ; distilled water, 1000ml; the pH is adjusted to 7.2. After autoclaving: - Triphenyl tetrazolium chloride 0.8 g 2% Cycloheximide 1 ml.	• Specific to *All. vitis*, • Colonies are convex and slightly mucoid and have red centres with a narrow white margin after 4 days at 28°C.
3DG (Brisbane *et al.*, 1983)	**Solution A:** Na Tartrate-$2H_2O$, 5.75g; $NaH2PO_4$-$2H_2O$, 6.24g; NaCl, 5.84g; sodium Taurocholate, 0.29g; Congo red (1%), 2.5ml; D-glutamic Acid (4%), 15ml; Na_2HPO_4, 4.26g; $MgSO_4$ x $7H_2O$, 0.25g; yeast extract (1%), 1ml; distilled water 500ml. **Solution B:** $MnSO_4$ x $4H_2O$, 1.12g; Agar, 15g; water, 500ml. Dispense solutions A and B separately in 50 ml lots and autoclave at 120°C for 15 min. Before pouring, add, per 50 ml solution B, actidione (2% aqueous), 1.0 ml; Na_2SeO_3-$5H_20$ (1% aqueous), 0.5 ml; solution A (at 50°C), 50 ml. A precipitate form and is redistributed by recapping the bottle and inverting several times.	• Specific to *All. vitis* and *Agrobacterium larrymoorei*, • Colonies are convex with a white color.
MG-Te (Brisbane *et al.*, 1983)	D-mannitol, 5g; L-glutamic acid, 2g; KH_2PO_4, 0.5g; NaCl, 0.2g; $MgSO_4$ x $7H_2O$, 0.2g; yeast extract, 0.5g; pH 7; Agar, 15g; distilled water 1000ml; the pH is adjusted to 7.2. After autoclaving: - K_2TeO_3 0.2, 2% Cycloheximide 1 ml	• Specific to *Agrobacteium* spp. and *All. vitis*, • Typical circular glistening morphologies *with* back color with and metallic shine.
1A-Te (Brisbane *et al.*, 1983)	L-arabitol, 3.04g; NH_4NO_3, 0.16g; KH_2PO_4, 0.54g; K_2HPO_4, 1.04g; $MgSO_4$ x $7H_2O$, 0.25g; sodium Taurocholate, 0.29g; Crystal violet (0.1%), 2ml; Agar, 15g; distilled water 1000ml. After autoclaving: - K_2TeO_3 , 0.08g, 2% Cycloheximide 1 ml	• Specific to *Agrobacteium* spp. and *All. vitis*, • Colonies have a typical circular morphology plus a characteristic black color with a metallic shine.

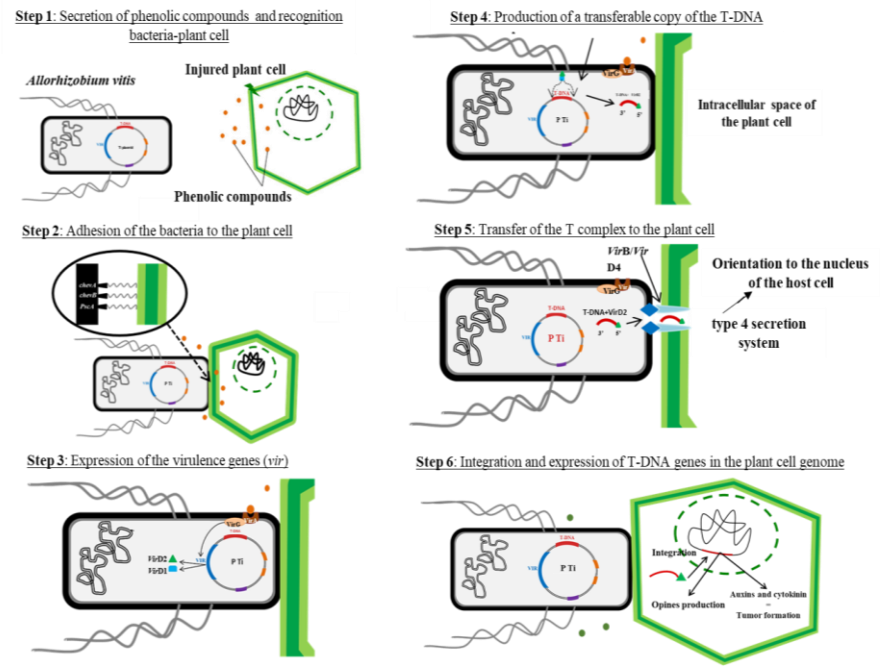

Figure 4 Schematic representation of the different stages of infection with *Allorhizobium vitis* reported by several studies.

METHODS FOR ISOLATION AND IDENTIFICATION OF *ALLORHIZOBIUM VITIS*

Isolation of *All. vitis* from complex environments

For efficient purification of *All. vitis* strains from complex environments (tumors and soil), appropriate selective media was used to obtain pure cultures. Table 1 shows four specific media (3DG, Roy and Sasser, 1A-Te and MG-Te) usually used to isolate *All. vitis*. These media are based on the ability of *All. vitis* to resist and use some specific compounds (**Mougel *et al.*, 2001**). As a carbon source, 3DG medium and Roy and Sasser medium use sodium L-tartrate which is important in the selectivity of these media (**Shams *et al.*, 2012**). The selectivity of medium 3DG also depends on the use of D-glutamic acid as nitrogen source. Most organisms, including the troublesome *Pseudomonas* fluorescent, cannot utilize it and it is toxic to *Rhizobium rhizogenes* strains (**Brisbane *et al.*, 1983**). Because of the resistance of *All. vitis* strains to potassium tellurite (K2TeO3), the selectivity of 3DG medium and Roy and Sasser medium could be improved by the addition of this compound (**Mougel *et al.*, 2001**). *All. vitis* can also be isolated on 1A-Te medium or MG-Te medium. These two media (1A-Te and MG-Te) are superior because they are easy to prepare and support faster growth of *All. vitis* (**Brisbane *et al.*, 1983**).

Sensitive and reliable methods for detection of *All. vitis*

After the isolation steps of the phytopathogenic agents, the characterization and the identification is conducted by molecular phenotypic techniques. This step is essential to understand the variability and genetic diversity within populations of *All. vitis* in order to get an idea on their origin and effectively detect the bacteria to manage the disease. It also gives a clear vision of the different genes involved in the infection process.

Currently, there are several methods of characterization and identification of all species and genomic groups of *All. vitis* and they vary according to the objectives. Biochemical characterization and pathogenicity tests are the most traditional methods used in the past, but they are still widely used. Additionally, PCR-based molecular techniques that target *All. vitis*-specific genes have been developed along with the technological revolution (Table 2 and S1). The main genes used are those encoding for the virulence factors, in particular *virC*, and *virD2*, the *pehA* gene coding for polygalacturonase specific for *All. vitis*, and the genes coding for opine degradation enzymes. Furthermore, **Shams *et al.*, 2013** developed *recA* gene primers that allow rapid identification and assessment of the genetic diversity of *All. vitis* populations.

Phylogenetic analysis of housekeeping genes such as *recA*, *rpoB*, *mutS*, *gyrB*, *glgC*, *chvA*, and *ampC*, which evolve rapidly, by multi-locus sequencing (MLSA) offers a very high level of characterization of *Allorhizobium* populations (**Costechareyre *et al.*, 2010; Kuzmanović *et al.*, 2015**). Furthermore, sequencing the region separating the transcribed sequences (ITS) from the highly variable 16S-23S rRNA genes is desirable for the estimation of genetic diversity. In contrast, RAPD is a widely used technique for distinguishing *All. vitis* genomic groups because of its simplicity and the degree of diversity it reveals in *All. vitis* populations (**Momol *at al.*, 1998; Kuzmanović *et al.*, 2015**). **Orel *et al.*, 2017** also used pulsed-field gel electrophoresis (PFGE) to separate restriction fragments generated by PmeI in order to distinguish between different genomic groups in Turkey. The polymorphism related to the number of plasmids can be used to distinguish between the different groups of *All. vitis*.

The characterization of *All. vitis* populations carried out in different countries have shown great diversity. Numerous genomic groups have been identified in different countries: Morocco (**Habbadi *et al.*, 2019**), Japan (**Kawaguchi *et al.*, 2008**), Turkey (**Orel *et al.*, 2017; Argun *et al.*, 2002**), Iran (**Rouhrazi *et al.*, 2012**), USA (**Irelan *et al.*, 1996; Otten *et al.*, 1996; Momol *et al.*, 1998; Burr *et al.*, 1999**), Serbia (**Kuzmanović *et al.*, 2014 ; Kuzmanović *et al.*, 2015**), Spain (**Palacio-Bielsa *et al.*, 2009**), Bulgaria (**Genov *et al.*, 2006; Genov *et al.*, 2015**), Germany (**Schulz *et al.*, 1993**), Australia (**Gillings *et al.*, 1995**), Korea (**Kim *et al.*, 2007**).

The Molecular characterization of Moroccan strains of *All. vitis* was carried in our previous study (**Habbadi *et al.*, 2019**) using specific-PCR targeted *recA* and *rpoB* genes. The results showed a high genetic diversity with the identification of 4 genomic groups of *All. vitis* (*Avi-1*, *Avi-2*, *Avi-3* and *Avi-8*), 3 of *A. tumefaciens* (*G1*, *G4* and G7), and *R. rhizogenes*. For all the characterized isolates, only *All. vitis* isolates were found pathogenic, possessing the pTi and were able to cause tumors on stems of inoculated tomato, a hypersensibility reaction (HR) on tobacco leaf, and necrosis on grapevine explants. All the genomic groups of *All. vitis* present the opine genes on their pTi coding for synthesis of octopine and vitopine; which are used as carbon, nitrogen, and energy sources. The study also showed that all characterized *All. vitis* possess genes coding to the tartrate utilization as a source of carbon.

Table 2 Molecular methods for detecting and quantifying of *All. vitis*.

Detection techniques	Sample	Extraction methods	Gene or molecules target	Sensitivity	References
Conventional PCR	Pure culture	High temperature	*Tm4 ipt*	-	**(Schulz *et al.*, 1993)**
Conventional PCR	soil	Phenol-chloroform	*virD2*	10 Cell/mg	**(Haas *et al.*, 1995)**
Conventional PCR	Pure culture	InstaGene DNA purification matrix	*virC*	-	**(Sawada *et al.*, 1995)**
Immunocapture-PCR	Grapevine	High temperature	Octopine 6b gene	-	**(Kauffmann *et al.*, 1996)**
Tartrique medium-PCR	Grapevine	High temperature	*pehA*	-	**(Szegedi *et al.*, 2002)**
Semi-nested PCR	Soil	High temperature et protéase K	*tms2*	1 à 2 cell/g	**(Pulawska *et al.*, 2005)**

Continue Table 2

Multiplex PCR	Pure culture	High temperature	16S rDNA and *virC*	-	**(Kawaguchi *et al*., 2005)**
RT-PCR	Grapevine	High temperature and Tween 20	*virD2*	104 à 10^6 ufc/ml	**(Bini *et al*., 2008)**
Multiplex BioPCR	Grapevine	Tampon d'extraction	*pehA* and *virC*	-	**(Kumagai *et al*., 2008)**
Nested PCR	Soil	Extraction Kit Omega	*pAVS3*	2 ufc/ml	**(Lim *et al*., 2009)**
Electronic nose	Grapevine	-	Styrene	83.3%	**(Blasioli *et al*., 2010)**
Magnetic Capture Hybridization Real-Time PCR	Grapevine	Magnetic Capture Hybridization	*virD2*	10 ufc/ml	**(Johnson *et al*., 2013)**
Multiplex PCR	Pure culture	Alkaline	*virC* and *pehA*	-	**(Lamovsek *et al*., 2014)**
Multiplex PCR	Pure culture	High temperature	*virC* and *pehA*	-	**(Kuzmanović *et al*., 2015)**
BioPCR	Tomato	High temperature and Triton or Tween 20	*virC*	10^3 ufc/ml	**(Habbadi *et al*., 2017)**
Droplet digital PCR	Grapevine and soil	MoBio Powersoil DNA extraction kit	*virA*	-	**(Duplay, 2008)**

SUSTAINABLE STRATEGIES FOR THE PROTECTION OF GRAPEVINES AGAINST *ALL. VITIS*

Crown gall is a very difficult disease to be controlled because it is a systemic bacterium, and once the infectious process is triggered, it is almost impossible to eliminate the disease or stop the development of tumors. To date, no effective treatment is available for the control of crown gall. Generally, the control of *All. A vitis* is based mainly on cultural, chemical and biological prevention techniques.

Cultural practices

In order to reduce the risk of attacks on vines by *All. vitis*, traditional, simple and environmentally friendly methods are used which consists of adapting and properly managing cultivation practices before and after planting. First, it is important to use clean plant material; several approaches have been developed to produce vines that are not contaminated with *All. vitis* (**Burr *et al*., 1999**). The first approach is to immerse vine cuttings in warm water (50-60 °C) for 30-60 min; this treatment showed a significant reduction in *All. vitis* infection rates (**Burr *et al*., 1998**). However, this method is not highly recommended as a means of removing the bacterium from cuttings and is no longer used by nurserymen due to the inconsistency of treatment which sometimes leads to poor implantation, late growth, and even bud death in some cases (**Burr *et al*., 1999**). On the other hand, it is possible to produce healthy vines by initiating the plants from the tips of the shoot *in vitro*; since *All. vitis* are never detected in the tips of grape shoots; vines propagated from these are free from the bacteria (**Szegedi *et al*., 2005; Otten *et al*.,2008**). Therefore, vines that are free of the pathogen can be planted in mother blocks as a source of breeding material. To date, this approach has been successful in three different studies to provide sources of pathogen-free propagation material (**Burr *et al*., 1998; Burr, 2004**).

Other disease control strategies include preventing winter injuries by selecting planting sites with good airflow and water flow to reduce low-temperature injuries that attract the bacterium (**Burr, 2004**). It is preferable that the planting of healthy vines be done in non-viticultural soils since *All. vitis* has never been detected in soils from non-viticultural sites (**Burr *et al*., 1998; Burr, 2004; Burr *et al*., 1995; Elwin *et al*., 2006**). The use of multi-trunk vines is a widely used technique that allows farmers to remove diseased trunks in the case of the onset of the disease, which helps keep the disease at tolerable levels. In the most severe cases, it is advisable to remove the diseased plants and dispose of them (**Odile *et al*., 2006**). The good management of cultural practices is the most important strategy for preventing the disease, since it is a bacterium able to move easily, frequent disinfection of work tools is, therefore, necessary to avoid contamination of healthy areas and to avoid runoff of contaminated water between plots (**Burr *et al*., 1998; Burr *et al*., 1995; Burr, 2004; Lacroix *et al*., 2006**).

Chemical control

Currently, there are no effective chemical options to control crown gall on grapevine in the field (**Tolba *et al*., 2013**). Chemical control of this disease is generally limited to the use of disinfectants and some antibiotics. However, these treatments can kill bacteria on the surfaces of galls, but fail to control the pathogen residing systematically in the vascular tissue of the vine (**Burr *et al*.,1998; Burr *et al*., 1999; Otten *et al*., 2008; Burr *et al*., 1995; Filo *et al*., 2013; Szegedi *et al*., 1996**). Some petroleum-based products (e.g. Gallex: Ag BioChem, Inc. Orinda, CA) have been used on individual galls, inducing temporary gall reduction but not total pathogen elimination. This treatment is very expensive and must be reapplied periodically once the disease appears and after removing most of the tumor from the vine. It can also be applied to the root system after removing all the soil surrounding the roots; it is necessary that the parts to be treated are dry (**Hartman *et al*., 2004**). In addition, *All. vitis* and some *A. tumefaciens* are known to be sensitive to industrial antibiotics such as rifampicin, streptomycin, and kanamycin (**Deblaere *et al*., 1985; Tarbah *et al*., 1986; Bishop *et al*., 1989; Deeba *et al*., 2014; Zäuner *et al*., 2006**).

Alternative methods

Alternative methods for the control of crown gall are imperative for the development of sustainable viticulture (**Burr, 2004**). They have the advantage of limiting the use of chemicals harmful to the environment as well as the emergence of pathogenic strains resistant to active molecules (**Tolba *et al*., 2013**). Several studies have been carried out to develop alternatives to chemical control of the causal agent of crown gall (**Herlache *et al*., 2002; Eastwell *et al*., 2006; Chen *et al*., 2007; Chen *et al*., 2009; Tolba *et al*., 2013**). These approaches are generally based on the use of beneficial microorganisms and natural plant-based molecules, as well as the stimulation of host plant defense mechanisms.

Selection and creation of resistant varieties

The choice of varieties and rootstocks resistant to *All. vitis* is considered an effective management method to prevent the spread of crown gall agent (**Burr *et al*., 1999**). The resistance of many cultivated or wild varieties, which respond differently to the pathogenic strains of *All. vitis*, can be natural or introduced (**Burr, 2004**). These considerations must be taken into account when evaluating breeding material in order to install a new vineyard.

Naturally, all *Vitis vinifera* are very sensitive compared to *Vitis labrusca* and hybrids (**Torregrosa *et al*., 2002**). However, hybrids are highly resistant to several pathogens, which is considered an effective way to prevent and manage crown gall. The vine rootstocks most resistant to *All. vitis* are couferc 3309 and Mgt 101-14 Richter (**Burr *et al*., 1998**). The study carried by **Burr *et al*., 2003** revealed that *Vitis riparia* cv. *portalis*, *V. riparia* cv. *gloire* of Montpellier and *V. amurensis*, as well as their hybrids, tolerates the presence of *All. vitis* better. In these varieties, the tumors were developed after the infection but the multiplication of *All. vitis* was made at the same rate in them as in the sensitive cultivars of *V. vinifera*. The transfer of resistance to *All. vitis* from *V. amurensis* to *V. vinifera* by interspecific recombination has shown that it is controlled by a single dominant gene located in the Rcg1 locus, and a molecular marker has been developed for this gene (**Kuczmog *et al*., 2012**). This marker may be useful for monitoring resistance to *All. vitis* and selection of resistant varieties. In addition, resistant rootstock grafts such as NAZ4, NAZ6, C3309, or 101-14 MGT reduce the severity of the disease, but do not prevent the infection (**Jackson, 2014**).

Genetic engineering is an attractive approach to develop vine cultivars resistant to *All. vitis* pathogenic strains. Another alternative means of control is using genetically modified plants to introduce resistance to one or more diseases by expressing defense genes from other organisms or by expressing the defense genes already present in the host plant (**Gilbert *et al*., 2009**). At the vine level, the use of transgenesis has provided protection against various diseases (**Kikkert *et al*., 1997**). However, public opinion is generally against the idea of genetically modified plants in the absence of certainty on the effect of the consumption of these plants on human health (**Domingo *et al*., 2011**).

Some transgenic varieties resistant to crown gall have been developed by the integration of the *virE2* gene of *A. tumefaciens* (**Xue *et al*., 1999; Krastanova *et al*., 2010**). Other varieties, transformed by the integration of the *iaaM* and *ipt* oncogene sequences, have also shown resistance to some strains of *All. vitis*, but sensitiveness to other pathogenic strains. This suggests that the transgenic plant approach is limited by the genetic variability within *All. vitis* populations (to establish crown gall-resistant lines, somatic proembryos of *Vitis berlandieri* × *V. rupestris* cv. 'Richter 110' rootstock were transformed with an oncogene-silencing transgene based on *iaaM* and *ipt* oncogene sequences from octopine-type, tumor-inducing (Ti) plasmid pTiA6. Twenty-one transgenic lines were selected, and their transgenic nature was confirmed by polymerase chain reaction (PCR). These lines were inoculated with two *A. tumefaciens* and three *All. vitis* strains. Eight lines showed resistance to octopine-type *A. tumefaciens* A348) (**Galambos *et al*., 2013**).

Physical control

Among the alternative methods used for the prevention of crown gall infection, physical practices are one of the effective methods for the control of *All. vitis* (**Burr *et al*., 1998**). This pathogen is sensitive to some physical parameters that may alter one or more functions of the bacterium, temperature in particular (**Dillen *et al*., 1997**). In fact, the solarization technique is one of the most widely used methods, which consists of covering the ground with a dark plastic tarp during the warm period of the year. Long-term exposure to the sun increases soil temperature, resulting in chemical, physical, and biological changes to the soil. Under these conditions, the bacterium loses its pTi and consequently its pathogenicity (**Katan *et al*., 1991**).

Biological control agents and plant extracts

All. vitis is a phytopathogenic agent that is difficult to control because it is a systemic bacterium. It uses the wounds, caused by the fall of the temperature during the winter or by the agricultural machinery, to gain access to the vegetable tissue. As a result, biological control appears to be a promising alternative to protect vines against *All. vitis* (**Burr *et al*., 1998; Burr *et al*., 2005**) and to develop sustainable viticulture. The use of biological agents and natural extracts to prevent and limit the damage caused by All. vitis has made the goal of several current studies, given the lack of means for effective control.

The use of non-tumorigenic strains of *All. vitis* has been shown to be effective in controlling grapevine crown gall as they colonize the vascular system of the vine where they act on pathogenic strains (**Burr *et al*., 1997**). The strain *Agrobacterium radiobacter* K84 (**Kerr, 197**7) is the first model used to control crown gall. It has been used for the development of a pesticide marketed under the name Dygall. This biocontrol agent acts by antibiosis by producing agrocin, which blocks the penetration of opines into pathogenic strains, by the presence of a specific plasmid for this species (pAg K84) (**Kerr *et al*., 1977**). Because of the limited effect of this bacterium on *All. vitis* (**Kerr *et al*., 1977**), it is necessary to find other endophytic bacteria capable of colonizing the vascular system of the vine. Other antagonistic bacteria have shown the ability to inhibit the formation of tumors on the vine such as *A. radiobacter* HLB-2 (Pu, 1992), M115 (**Xuemei *et al*., 1993**), *A. tumefaciens* J73 (**Webster *et al*., 1986**), *All. vitis* E26 (**Liang *et al*.,2001**), F2/5(**Staphorst *et al*., 1985**), VAR03-1 (**Kawaguchi *et al*., 2005**), strains belonging to the genus *Pseudomonas* (**Sholberg *et al*. 1995; Bell *et al*., 1995; Khmel *et al*., 1998; Eastwell *et al*., 2006**), *Bacillus subtilis* SR63 (**Ferrigi *et al*., 2017**), *Pantoea agglomerans* 2066.7, *Rahnella aquatilis* 2332.A1 (**Habbadi *et al*., 2017**) and *Rahnella aquatilis* HX2 (**Bell *et al*., 1995**). In addition, treatment with certain bacteria producing the enzyme 1-aminocyclopropane-1-carboxylate deaminase reduces tumor formation induced by *All. vitis* S4 on tomato (**Toklikishvili *et al*., 2010**). Trichoderma asperellum T1 is the only fungal species that has been suggested to inhibit tumor formation (**Ferrigi *et al*., 2017**).

The essential oils of Origanum compactum and Thymus vulgaris have antimicrobial activity against *All. vitis in vitro* and also reduce the development of tumors in planta (**Habbadi *et al*., 2017**). Other studies have demonstrated antimicrobial activity against *All. vitis in vitro* and *in vivo* using extracts of Vicia villosa and Lolium perenne (**Islam *et al*., 2013; Islam *et al*., 2012**).

***All. vitis* F2/5:** After the control failure of *All. vitis* by *A. radiobacter* K84, **Staphorst *et al*., 1995** evaluated 16 strains including *All. vitis* F2/5 which inhibited the majority of *All. vitis* strains *in vitro* and reduced the incidence of crown gall in greenhouse conditions (27°C and 70% RH). This has encouraged other researchers to further study this strain. **Burr and Reid, 1994** reported that this inhibitory effect is mainly due to agrocin production. However, some strains susceptible to this agrocin such as *All. vitis* (CG78) produced the symptoms on vines treated with *All. vitis* F2/5. In contrast, the competition hypothesis at the attachment site was rejected by **Burr *et al*., 1997. Szegedi *et al*., 1999** reported the presence of plasmids encoding tartaric acid catabolism and octopine in *All. vitis* F2/5, which are not associated with tumor inhibition, but are likely to facilitate colonization of vine tissues. In order to improve the effect of *All. vitis* F2/5, **Herlache & Triplett, 2002** showed that the integration of a stable plasmid encoding for the production of trifolitoxin reduces the appearance of galls on the vine.

Although, the action mechanism of *All. vitis* F2/5 is not yet clear, **Creasap *et al*., 2005** suggested that it inhibits the normal healing of pleas by producing necrosis at the level of cambium. Another study has demonstrated the role of clp system including *clpA* and *clpP1* genes of *All. vitis* F2/5, which is involved in the degradation of intercellular proteins, are indispensable in the control of *All. Vitis* (**Kaewnum *et al*., 2013**). Recently, two other genes encoding nonribosomal synthetase peptide (*F-avi3342* and *F-avi5730*) and polyketide synthetase have been identified as indispensable in the *All. vitis* F2/5 biocontrol mechanism (**Zheng *et al*., 2016**).

***All. vitis E26*:** The non-pathogenic strain *All. vitis* E26 is one of the most studied biocontrol agents against vine crown gall. **Liang *et al*., 2001** demonstrated the efficacy of this bacterium to control *All. vitis*, *A. tumefaciens* and *A. radiobacter*. The genetic characterization of this biocontrol agent has shown that it lacks virulence genes such as *virA* and *virG* (**Wei *et al*., 2009**). The inhibitory power of this strain is associated with the production of an Ar26 antimicrobial compound with a molecular weight of 761 Da (**Wang *et al*.,2003**). This compound exerts a bactericidal effect on *All. vitis* by the inhibition of DNA, RNA and protein synthesis (**Li *et al*., 2009**). In addition, the chemotaxis of *All. vitis* E26 is a limiting factor in the control of *All. vitis* since it allows the attachment and colonization of the tissues of the vine (**Yang *et al*., 2009**). Considering the importance of this strain, a PCR-based method was developed to follow the evolution of *All. vitis* E26 in the environment using a SCAR primer pair designated 740F/R (**Akgul *et al*., 2018**).

***All. vitis* VAR03-1:** From a non-pathogenic *All. vitis* collection, **Kawaguchi *et al*., 2005** isolated a strain with inhibitory activities against crown gall on the vine. This strain designated *All. vitis* VAR03-1 has the ability to reduce the incidence of disease and gall size with 84-100%. Soaking the vines and tomato roots in the bacterial suspension for 24 hours before planting in a soil infected with pathogen and reduces the formation of tumors (**Kawaguchi *et al*., 2007**). In comparison with *A. radiobacter* K84, *All. vitis* VAR03-1 has an identical effect on gall formation on tomatoes and rose, but it is more effective on grapes (**Kawaguchi *et al*., 2008**). In order to understand the mechanism of *All. vitis* VAR03-1, Saito *et al*., 2018 revealed that the supernatant of this ABC inhibits *virE2* gene induction, intervenes in the protection of single-stranded T-DNA, and prevents the growth of pathogenic strains of *All. vitis*. They also showed that the active ingredient of this bacterium has a molecular weight greater than 100 kDa.

***All. vitis* ARK-1: Kawaguchi *et al*., 2018** referred to *All. vitis* ARK-1 as a promising new agent for biocontrol against grapevine crown gall. This bacterium inhibits *All. vitis* by a different mechanism from the one demonstrated in *All. vitis* VAR03-1. *All. vitis* ARK-1 limits the development of vine crown gall with a risk factor of 0.15 against 0.24 for *All. vitis* VAR03-1 while *A. vitis* VAR03-1 has scored 0.24 in the risk factor. It colonizes and persists inside the roots without causing the necrosis of the vine explants (**Kawaguchi, 2013**). Kawaguchi, 2014 suggested that this ABC does not work by antibiosis. On the other hand, the expression of *virD2* and *virE2* virulence genes of the pathogenic agent is affected following the treatment with *All. vitis* ARK-1, whereas the cellular filtrate has no effect. This suggests that the inhibitory power of ABC is associated with the suppression of the expression of virulence genes of the pathogen (**Kawaguchi *et al*, 2017**). This ABC has also shown efficacy against crown gall in other plants such as apple, Japanese pear, peach, rose and tomato with risk factors of 0.38; 0.16; 0.20; 0.29 and 0.16, respectively (**Kawaguchi, 2015**).

***Rahnella aquatilis* HX2:** In addition to the *Allorhizobium* antagonist strains, *Rahnella aquatilis* HX2 has been reported as a potential ABC capable of inhibiting the formation of grapevine crown galls (**Chen *et al*., 2007**). This antagonist produces a thermostable and alkali-sensitive antimicrobial substance that has a bactericidal effect on *All. vitis*, as well as other phytopathogenic bacteria by the inhibition of RNA and protein synthesis (**Chen *et al*., 2009**). **Li et al., 2014** reported that it is a gluconic acid and it requires pyrroloquinoline quinone, cofactor of aldose and alcohol dehydrogenase. This mechanism is also used by *R. aquatilis* HX2 for the solubilization of mineral phosphate (**Li *et al*., 2014**). Recently, Mei *et al*., 2017 demonstrated the regulatory activity of untranslated CsrB RNA by the BarA-dependent pathway on antimicrobial principle production and on the antagonistic effect of *R. aquatilis* HX2 against *All. vitis*.

The genomic structure of this bacterium was determined by **Guo *et al*., 2012**, it consists of a circular chromosome, two plasmids designated pRA1 and pRA2 and a fragment named pR22. The sequences of these components are available on GenBenk under accession numbers CP003403, CP003404, CP003405 and CP003406, respectively.

Essential oils: In phytopathology, essential oils (EOs) and plant extracts, from aromatic and medicinal plants, have been used to control phytopathogens such as *Erwinia amylovora*; *Pseudomonas* spp., *Bacillus* spp., *A. tumefaciens*, and *Xanthomonas* (**Kokoskowa *et al*., 2011; Mikicinski *et al*., 2012**). They are endowed with a strong antimicrobial activity due to their richness in phenolic compounds such as eugenol, carvacrol and thymol. These hydrophobic compounds integrate into the cell membrane and cause lysis of the cell (**Carson *et al*., 2002; Sikkema *et al*. 1994; Ultee *et al*., 2002**).

In our recent works (**Habbadi *et al*., 2017; 2021; 2022**), we showed that EOs of *Origanum compactum* and *Thymus vulgaris* are the most effective EOs in controlling *All. vitis* S4 *in vitro*. These two EOs are also able to prevent the development of tumors on tomato plant and vine. On the other hand, the synergistic effect of these two EOs proved to be more effective (*in vitro* and *in planta*) than the separate use of both treatments. They alter the wall and the cell membrane of *All. vitis* (Figure 5), which causes the death of the bacteria (**Habbadi *et al*., 2017**). However, the use of EOs in the field is not desired considering their volatile properties at medium temperatures. Hence the need to develop formulations that protect the effect of these EOs. In this sense, several encapsulation methods have been developed to control certain phytopathogenic agents while avoiding the volatilization of the active components of the EOs. The clay-based formulation (**Nguemtchouin *et al*., 2010**), modified agar (**Gašić *et al*., 2013**) and gelatin-gum arabic complex appear to be a good protection of the EOs effect against *All. vitis* in field.

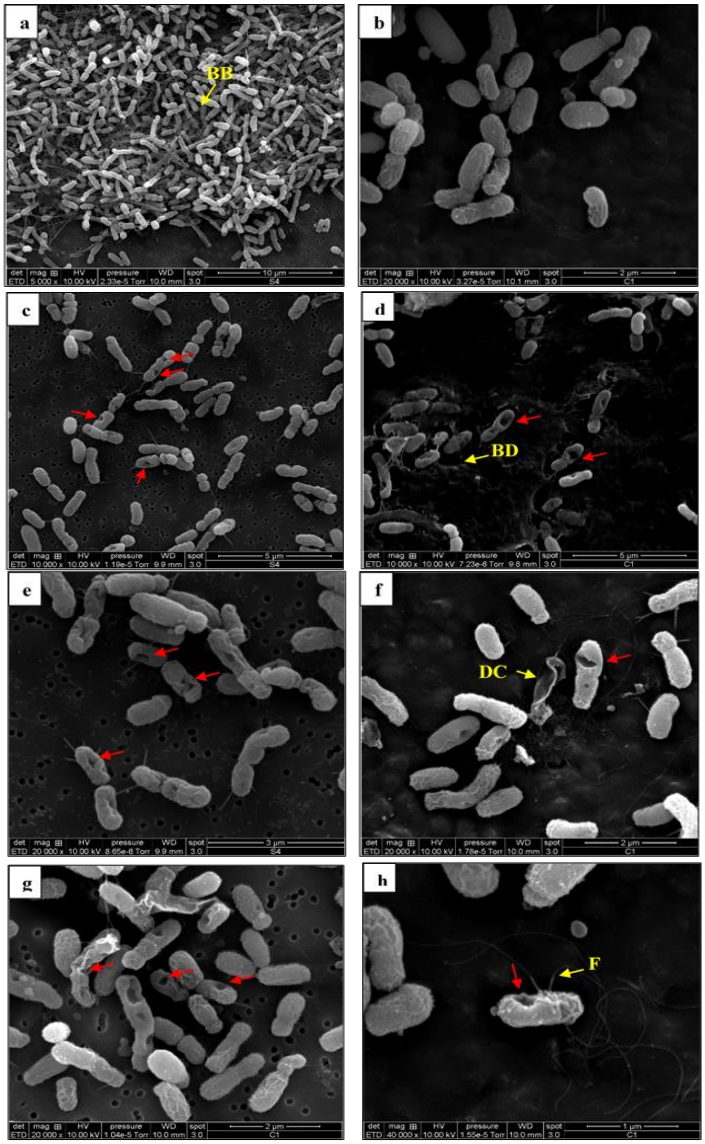

Figure 5 Scanning electron micrographs of *All. vitis* S4 cells.
a and **b**: controls (untreated cells), **c** to **h**: cells treated with the mixture of oregano and thyme EOs at 0.3mg/ml (1:1). **BB**: Bacterial Biofilm, **DC**: beginning of polar cell division, **BD**: Biofilm degradation, **DC**: Dead Cell, **F**: Flagella.
Cell wall and plasma membrane alterations are indicated by red arrows

CONCLUSION AND RESEARCH PERSPECTIVES

In phytopathology, grapevine crown gall has a special importance due to the lack of control means and some specificity related to *All. vitis*, the causal agent of this disease. This problem has been the subject of several studies and especially during the last years when the number of publications has increased in a considerable way. This research has addressed the different aspects of this problem. The genetic, phenotypic characterization, the study of the diversity within *All. vitis* populations and the development of biocontrol means are the most studied axes.
After several years of research on *All. vitis*, basic elements such as genetic, phenotypic, taxonomic position and infection mechanisms of this pathogen is currently known. The study of the diversity and distribution of the pathogen agent has been carried out in the majority of affected countries.
The development of an effective control strategy and a standard diagnostic technique is the major success of various research projects in phytopathology. Regarding the grapevine crown gall, several projects have been launched for a long time to achieve these goals. Despite the efforts made, there are still gaps that slow down solving the issue related to *All. vitis*. The standardization of diagnostic methods, to detect *All. vitis* in breeding material of grapevine, and the establishment of strict control means in the frontiers is an effective strategy to limit the spread of *All. vitis* to other non-affected regions. More than 28 publications about detection and identification methods of *All. vitis* are being developed. However, these techniques are limited within research laboratories and are not recognized by viticulture professionals. Collaborations between the various actors in the field and research laboratories are necessary in order to begin this essential step to solving this problem.
Similarly, the lack of coordination between the laboratories also generates other problems related to the *All. vitis* nomenclature. Within the scientific community, every living organism has a common name. While, the nomenclature of *All. vitis* has undergone several changes. Currently, this pathogen is designated by different names according to the laboratories. *Agrobacterium vitis*, *Rhizobium vitis* and *Allorhizobium vitis* are the three names used in recent years. The unification of this nomenclature will facilitate the search for publications and information about the *All. vitis*.
In order to develop methods to control the disease, the use of resistant varieties is a partial solution to reduce the severity, but it is limited by the variability of *All. vitis* strains.
In the context of the development of biocontrol agents against crown gall, several publications have been published. However, they focus on *in vitro*, *in planta* and in greenhouse conditions. In the field, scientific researchers should develop a biopesticide with the same efficacy in controlled conditions. Among the articles published on *All. vitis*, none deals with topics related to the mass production and the formulation of ABCs developed against grapevine crown gall. This final step in the process of developing a marketable product is of particular importance since it is a study of all factors that can alter the viability and antagonistic power of ABC in the field and in storage in order to develop a protective matrix.
Biological control using antagonistic bacteria and plant essential oils has proven to be an alternative solution. Several ABCs are in characterization and development phase. The use of *R. aquatilis* HX2, which has a positive effect on plant growth in addition to its inhibitory effect against grapevine crown gall, seems to be a promising solution to limit this disease and ensure sustainable viticulture.

Acknowledgments: We thank all INRA team for their contribution on this study.

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Supplementary material:

Table S1. Primers used in the molecular characterization of *All. vitis* and *A. tumefaciens*.

Gene	Primer		Sequence	Fragment (pb)	Hybridation (°C)	Amplified Taxon	Reference
16S	***1***	F809PA	AGAGTTTGATCCTGGCTCAG	1477	59	Universal	*
		F810PH	AAGGAGGTGATCCAGCCGCA				
23S	***2***	UF f	GTAAGAAGCGAACGCAGGGAACT	478	67	*All. vitis*	(Pulawska *et al*., 2005)
		AvR r	AACTAACTCAATCGCGCTATTAAC				
rrs	***3***	F667pA	AGAGTTTGATCCTGGCTCAG	*	*	Universal	(Bruce *et al*., 1992)
		F668pH	AAGGAGGTGATCCAGCCGCA				
16S-23S	***4***	FGPS1490-72	TGCGGCTGGATCCCCTCCTT	*	55	Universal	(Normand *et a*l., 1992)
		FGPL132'	CCGGGTTTCCCCATTCGG				
recA	***5***	F7386 F	AGCAAGGCACTGGAAGCGG	779	52	*Rhizobiaceae*	(Shams *et al*., 2013)
		F7387 R	CCATACATGATGTCGAATTC				
	6	F2898recA-T7F	TAATACGACTCACTATAGGGTCTTTGCGKCTCGTAGAGGAYA	1068	58		
		F2899recA-T3R	ATTAACCCTCACTAAAGGGATGCAGGAAGCGGTCGGCRATSAG				
	7	F8360	AGCTCGGTTCCAATGAAA	453	52	*Agrobacterium* spp.	
		F8361	GCTTGCGCAGCGCCTGGCT				
	8	G0004	GATATCGCGCTCGGCATTGGT	329	55	*All. vitis*	
		G0005	CCTTCGATTTCAGCTTTCG				
chvA	***9***	F2044chvA-F T3	ATTAACCCTCACTAAAGGGATTCGGCCGWATCATYGACGC	1497	58	*Rhizobiaceae*	*
		F2047chvA-R T7	TAATACGACTCACTATAGGGCGATGATGAAGGTCGTCC				
mutS	**10**	F2895mutS-T7F	TAATACGACTCACTATAGGGCTGATGTCGCCAYTGACCGAYC	1430	62	Universal	(Costechareyre *et al*., 2010)
		F2896mutS-T3R	ATTAACCCTCACTAAAGGGACTTCCCAYTCCTTCACSCGCAT				
gyrB	**11**	F3136gyrB-F	GAAGTCATCATGACCCAGCTTCATGCSGGCGGNAAATTCGA	686	58		
		F3139gyrB-R	CCYTCRCGGCAGTCYTCRCC				
	12	F3138gyrB-F2	GTGCTNTGYTTYACCAACAAC	734	56		
		F1014gyrB-R2	AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT				
gltD	**13**	F3277gltD-T7F	TAATACGACTCACTATAGGGCGGCAGGTGGCAAGTAYCAGC	1329	62		
		F3279gltD-T3R	ATTAACCCTCACTAAAGGGAGAGGTCCTGTAGTCSGTTTCGTT				
glgC	**14**	F4581glgC-F	TTGGCGCGTGATGCMATGG	1183	57		
		F4586glgC-R	TGCGGCGGAAGCGYTTGGC				
ampC	**15**	F4427ampC-F	ATCGCAGACATATCGCACTG	1044	60		
		F4426ampC-R	TCGGTATGAACGCCACATAA				
	16	F4427ampC-F	ATCGCAGACATATCGCACTG	1008	57		
		F5280ampC-R3	CGGAGCCGGTCTTGTTGATG				
	17	F5278ampC-F2	GACGRGCCTGKTCACGCAG	974	57		
		F5279ampC-R2	CGGAGCCGGTCTTGTTGATG				
	18	F5278ampC-F2	GACGRGCCTGKTCACGCAG	974	75		
		F5280ampC-R3	CGGAGCCGGTCTTGTTGATG				
rpoB	**19**	G0953	TCGTTTCGCAGATGCACCG		55		
		G0954	TAGGCGCCAACATTGACGTG				
virC	**20**	VCF3	GGCGGGCGYGCYGAAAGRAARACYT	414	57	Pathogenic species of *All. vitis* and *Agrobacterium* spp.	(Swada *et al*., 1995)
		VCR3	AAGAACGYGGNATGTTGCATCTYAC				
vir	**21**	F14	GAACGTGTTTCAACGGTTCA	432	57		*
		F749	GCTAGCTTGGAAGATCGCAC				
tms2	**22**	tms2F1	TTTCAGCTGCTAGGGCCACATCAG	617	63		(Pulawska *et al*., 2005)
		tms2R2	TCGCCA TGGAAACGCCGGAGTAGG				
	23	tms2F1	TTTCAGCTGCTAGGGCCACATCAG	458			
		tms2B	GGAGCACTGCCGGGTGCCTCGGGA				
tms1	**24**	iaaH-F2	ACATGCATGAGTTATCGTTTGGAAT3	420	54		(Bini *et al*., 2008)
		iaaH-R1	GCATCAAGGTCATCGTAAAAGTAGGT				

	25	iaaH-F10	GGAAACATGCATGAGTTATCGTT	425	54		
		iaaH-R10	CCACATCAGCATCAAGGTCATC				
	26	S4iaaM5	CGCGTCCCCGTTTACACTA	561	54		
		S4iaaM3	CGAGATCGCGCTTCAAGAT				
virB/virG	**27**	F14	GAACGTGTTTCAACGGTTCA		55		(Nesme *et al*., 1990)
		F749	GCTAGCTTGGAAGATCGCAC				
virD2	**28**	A	ATGCCCGATCGAGCTCAAGT	224	50		(Haas *et al*., 1995)
		C'	TCGTCTGGCTGACTTTCGTCATAA				
	29	A	ATGCCCGATCGAGCTCAAGT	338			
		E'	CCTGACCCAAACATCTCGGCTGCCCA				
	30	virFF	ATG AGA AAT TCG AGT TTG CAT GAT G	382	60	*All. vitis* (octopine and nopaline)	
		vrFR	TCG TGA TGG GTA TAC GCT ACG				
	31	VirD2S4F716	GAC CGC AAA ACC TGC CAG	320	60	*All. vitis* (pTi vitopine)	
		VirD2S4R1036	GAG CCT GTA TTG ACG ATG TC				
ipt	**32**	CTY	GATCG(G/C)GTCCAATG(C/T)TGT	427	*	*All. vitis* and *Agrobacterium* spp.	
		CTY'	GATATCCATCGATC(T/C)CTT				
pehA	**33**	PGF	GGGGCAGGATGCGTTTTTGAG	466	54	*All. vitis* (polygalacturonase gene)	(Szegedi *et al*., 2002)
		PGR	GACGGCACTGGGGCTAAGGAT				
ops	**34**	TF	TGGCCGAAATTGTTTACTTCCACCC	520	58	*All. vitis* (pTi Octopine)	(Suzaki *et al*., 2004)
		TR	CTATGCCGAAAGACGGCTTGACCCT3				
	35	NF	TTAACCCAAATGAGTACGATGACGA	570	54	*All. vitis* (pTi nopaline)	
		NR	TTATTTCGGTACTGGATGATATTAG				
	36	visF	CCGGCCACTTCTGCTATCTGA	561	54	*All. vitis* (pTi vitopinne)	(Canaday *et al*., 1992)
		visR	CCATTCACCCGTTGCTGTTATT				
	37	OCTF	GAA TAT GAG AAA TCC GTC TCG	475	50 or 52	*All. vitis* (pTi octopine)	(Bini *et al*., 2008)
		OCTR	ACT CAG AGC TCG TGG CCT TG				
	38	NOPF	GCA AAC GTA AGT GTT GGA TC	394	50 or 52	*All. vitis* (pTi nopaline)	(Bini *et al*., 2008)
		NOPR	CAA GCG AAT ACT CGA GAC G				
	39	SF	TGGCGGTACCGAGATGGGCTGTTCG	620	62	*All. vitis* (pTi vitopine)	(Bini *et al*., 2008)
		SR	TTAAGCAGAATTAGGACATGAGCCC				