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IDENTIFICATION OF *AGROBACTERIUM VITIS* AS A CAUSAL AGENT OF GRAPEVINE CROWN GALL IN SERBIA

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Abstract - In 2010, a serious outbreak of crown gall disease was observed on grapevines (*Vitis vinifera* L. cv. Cabernet Sauvignon) in several commercial vineyards located in the Vojvodina province, Serbia. Bacteria were isolated from the young tumor tissue on nonselective YMA medium and five representative strains were selected for further identification. Tumorigenic (Ti) plasmid was detected in all strains by PCR using primers designed to amplify the *virC* pathogenicity gene, producing a 414-bp PCR product. The strains were identified as *Agrobacterium vitis* using differential physiological and biochemical tests, and a multiplex PCR assay targeting 23S rRNA gene sequences. In the pathogenicity assay, all strains induced characteristic symptoms on inoculated tomato and grapevine plants. They were less virulent on tomato plants in comparison to the reference strains of *A. tumefaciens* and *A. vitis*.

Key words: Crown gall, grapevine, *Agrobacterium vitis*, tumor, pathogenicity, plasmid

INTRODUCTION

Grapevine crown gall, one of the most important and widespread bacterial diseases of grapevines (*Vitis vinifera* L.) throughout the world, is predominantly caused by tumorigenic strains of *Agrobacterium vitis* (Burr et al., 1998; Burr and Otten, 1999). Occasionally, tumorigenic strains of *A. tumefaciens* (Panagopoulos and Psallidas, 1973; Burr and Katz, 1983, 1984; Thies et al., 1991; Kawaguchi and Inoue, 2009) and *A. rhizogenes* (Panagopoulos et al., 1973; Süle, 1978; Lopez et al., 2008) may also occur on grapevine plants. Crown gall is a very destructive plant disease that reduces the vigor and yield of infected plants by up to 40% (Schroth et al., 1988). In the past few decades, the disease has been reported in China, Japan, South Africa, the Middle East, North and South America, and in several European countries (Burr et al., 1998). In 1962, it was observed for the first time in Serbia, in the Trstenik vine-growing region, on the cultivar Kardinal (Panić, 1973).

Typical symptoms of grapevine crown gall disease are tumors and tissue proliferation on the lower areas of the trunk. Initial symptoms usually remain unnoticed since the young tumors formed beneath the bark layer may be inconspicuous. Tumorigenic tissue can enlarge rapidly and completely girdle the trunk. Young tumors are soft, white, pale brown or pink in color. Later, the surface of the tumors becomes dark brown, dry and corky. *A. vitis* also causes root decay in infected grapevine plants (Burr et al, 1987).

A. vitis infects grapevines mainly through the wounds caused by freezing temperatures or grafting. Signal molecules released from the wounds attract bacteria that attach to the wound sites. Infection oc-

curs when tumorigenic plasmid fragment (T-DNA) transfers from the bacteria into the plant genome (Zhu et al., 2000). This leads to the synthesis of the plant hormones auxin and cytokinin, causing uncontrolled proliferation of plant cells and tumors formation. The transferred fragment also contains the genes responsible for the production of small molecules called opines, used by bacteria as an energy source (Burr et al., 1998; Burr and Otten, 1999). *A. vitis* can survive in soil, particularly in the vicinity of plant debris, in galls and diseased plants. An important characteristic of *A. vitis* is the systemic distribution within the grapevine plants (Lehoczky, 1968). Bacteria can latently survive in grapevine plants without causing visible disease symptoms until conditions favorable for infection, such as wounding, take place. For this reason, the pathogen is often disseminated in new areas by asymptomatic propagation plant material (Burr and Katz, 1984).

During 2010, a serious outbreak of grapevine crown gall disease was observed in vineyards located in the Vojvodina province. This disease was sporadically present in vineyards in Serbia in previous years, but incidence and severity were very high now. The objective of this research was to study the etiology of the disease and to identify the causal agent of the disease using standard biochemical and physiological tests, as well as molecular-based techniques.

MATERIALS AND METHODS

Isolation of bacteria

Samples were collected from two three-year-old commercial vineyards located in the Vršac vine-growing region, from the cultivar Cabernet Sauvignon grafted onto Kober 5BB rootstock. A high percentage of the plants showed typical symptoms of crown gall disease (Figs. 1a, b). Large aerial tumors formed above grafting points were removed from the trunks, placed in plastic bags and transported to the laboratory. After removing the necrotic tissue from the tumor surface using a sterile scalpel, fragments from the fresh tumor tissue were taken and incubated in sterile distilled water (SDW). After 2 h, loopfuls of tissue suspensions were streaked on yeast mannitol agar (YMA) medium. Plates were incubated at 28°C for 3-5 days. Representative colony types were purified and maintained on potato dextrose agar (PDA) for further testing. Prior to PCR amplifications, bacteria were grown on King's B medium at 28°C for 24-48 h. Control strains of A. *tumefaciens* (KFB 096/C58), *A. rhizogenes* (KFB 098/A4) and *A. vitis* (KFB 099/S4) were used in all tests.

Pathogenic plasmid detection

Selected strains were analyzed by polymerase chain reaction (PCR) using primers VCF3 (5' – GGC GGG CGY GCY GAA AGR AAR ACY T – 3') and VCR3 (5' – AAG AAC GYG GNA TGT TGC ATC TYA C – 3') that amplify the 414 bp fragment of the *virC* pathogenicity gene located on tumorigenic (Ti) or rhizogenic (Ri) plasmid (Suzaki et al., 2004). DNA templates were prepared by heating bacterial suspensions (approx. 108 CFU/ml) at 95°C for 10 min. Lysates were incubated on ice for 5 min and centrifuged for 5 min at 8000 rpm. Supernatants were used directly for PCR, or stored at -20°C.

The PCR reaction mixture consisted of 1×*Taq* buffer with KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, 0.5U *Taq* DNA polymerase (Fermentas, Lithuania) and 2 μl of template DNA. SDW was added to the final volume of 25 μl. PCR amplifications were performed in a 2720 Thermal Cycler (Applied Biosystems, USA) as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94° for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were separated by 1.5% agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized on a UV transilluminator (Vilber Lourmat, France) after staining in ethidium bromide (1 μg/ml) solution.

Differentiation to biovar/species level

The strains were analyzed using standard bacteriological and differential physiological and biochemi-

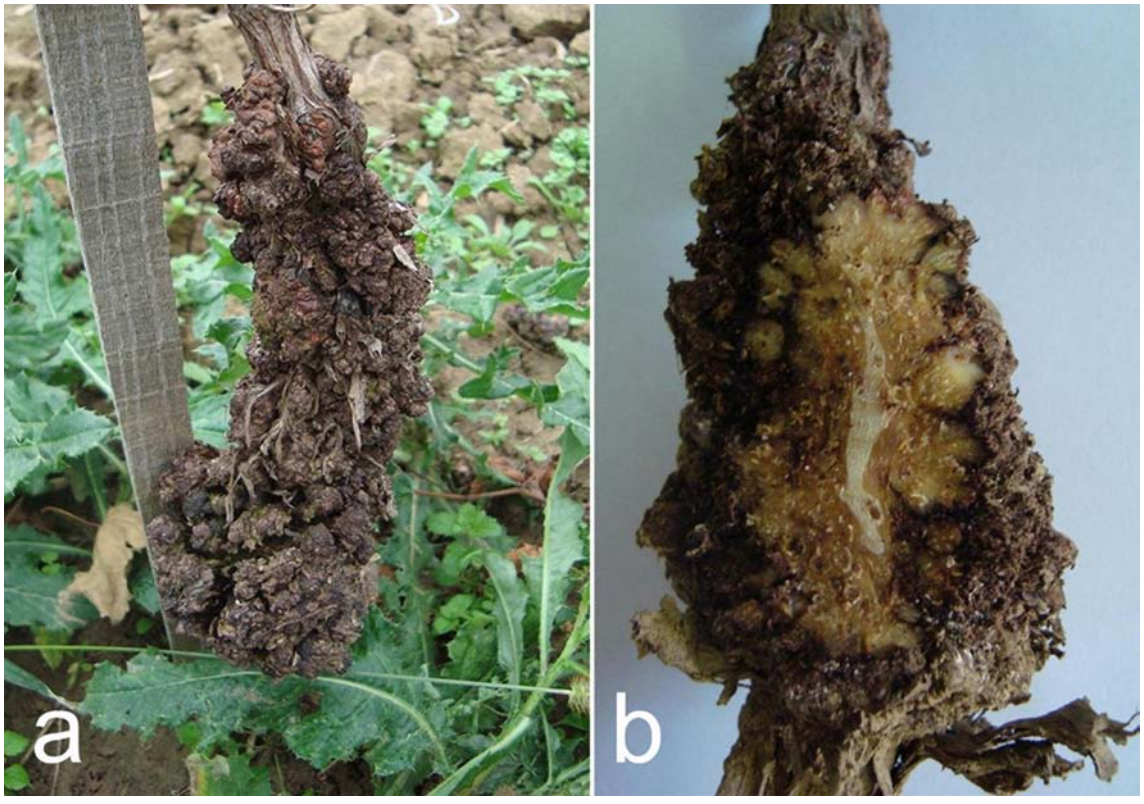

Fig. 1. Grapevine crown gall. Trunk of grapevine girdled by tumor (a). Tumor: Longitudinal section (b). Natural infection.

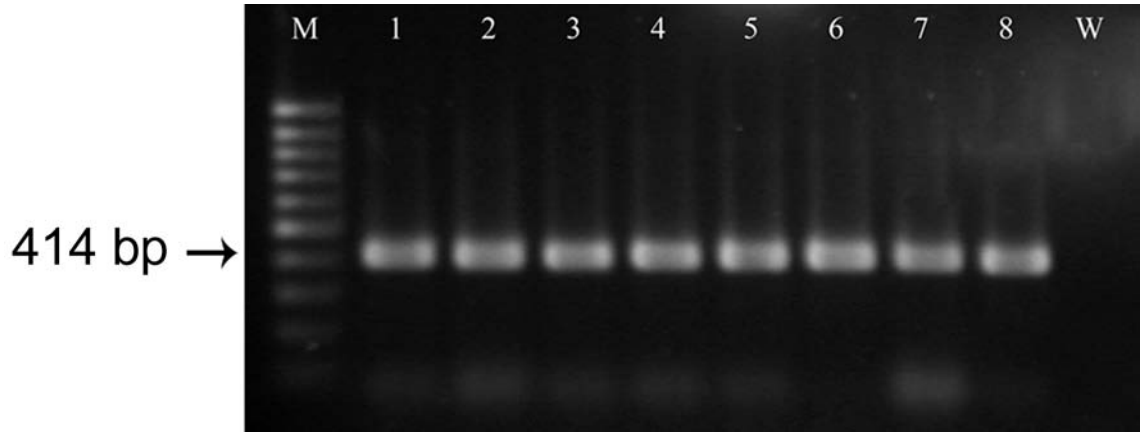

Fig. 2. PCR analysis of the strains. *virC* primer pair amplified 414-bp product from all studied and control strains. Lane M, marker (MassRuler Low Range DNA Ladder, Fermentas, Lithuania); Lane 1 - KFB 096/C58 (*A. tumefaciens* control strain); Lane 2 - KFB 098/A4 (*A. rhizogenes* control strain); Lane 3 - KFB 099/S4 (*A. vitis* control strain); Lane 4-8 - bacterial strains used in this study; lane W – SDW (negative control).

cal tests (Moore et al., 2003). The following tests were performed: fluorescence on King's B medium, Gram and oxidase reaction, growth in 2% NaCl at 35°C, 3-ketolactose production, acid clearing on PDA amended with CaCO3, ferric ammonium citrate test, motility at pH 7.0, pectolytic activity at pH 4.5, citrate utilization, production of acid from sucrose and alkali from tartarate.

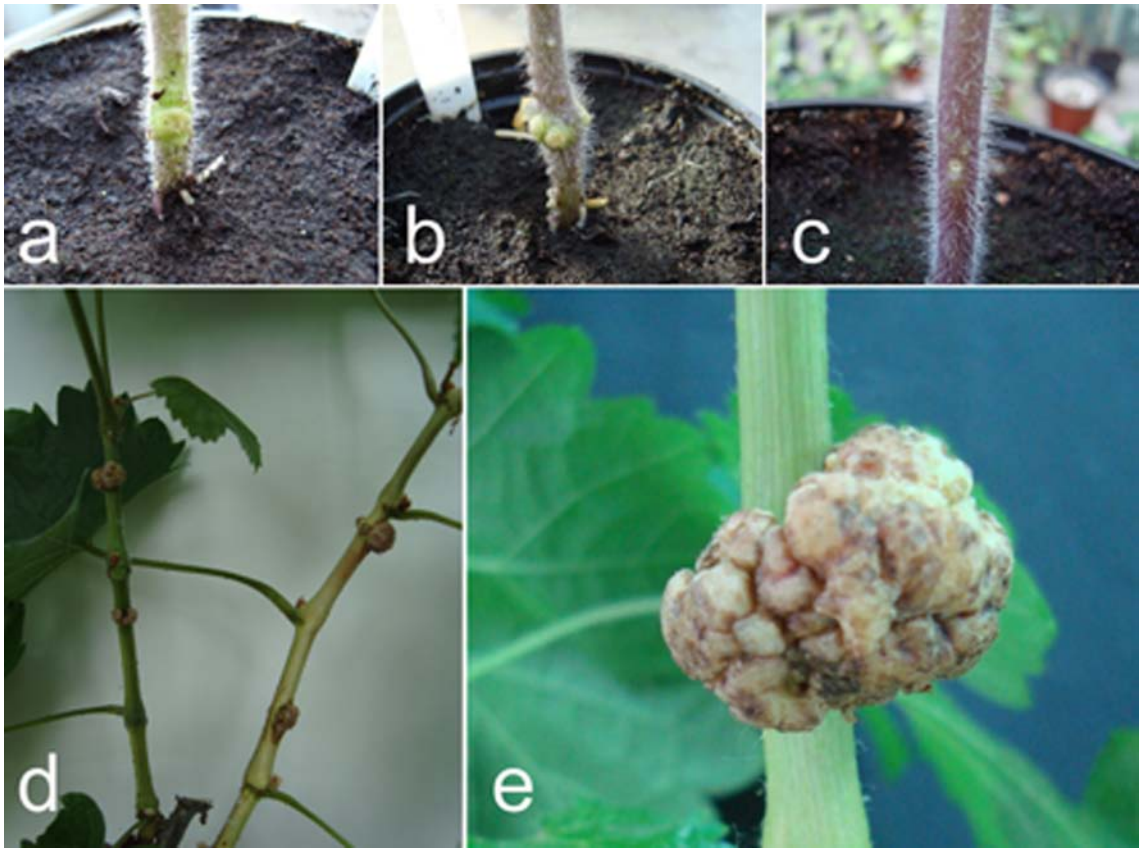

Fig. 3. Pathogenicity assay. Tomato plant inoculated with one of the studied strains (a), *A. vitis* control strain (b) and SDW (c). Tumor formation on grapevine plant inoculated with one of strains from this study (d,e).

The strains were also differentiated to the species/biovar level using a multiplex PCR assay targeting 23S rRNA gene sequences. Universal forward primer UF (5' – GTA AGA AGC GAA CGC AGG GAA CT – 3') and four reverse primers specific for *A. tumefaciens* (biovar I), B1R (5' – GAC AAT GACT GTT CTA CGC GTA A – 3'); *A. rhizogenes* (biovar II), B2R (5' – TCC GAT ACC TCC AGG GCC CCT CAC A – 3'); *A. vitis*, AvR (5' – AAC TAA CTC AAT CGC GCT ATT AAC – 3'); and *A. rubi*, ArR (5' – AAA ACA GCC ACT ACG ACT GTC TT – 3'), were used (Pulawska et al., 2006). Primer pairs UF/B1R, UF/B2R, UF/AvR and UF/ArR amplified the 184, 1066, 478 and 1006-bp fragments, respectively.

PCR amplifications were performed in a total volume of 15µl. The PCR reaction mixture consisted of 1×DreamTaq™ Green Buffer (includes 20 mM $MgCl_2$), 0.33 mM dNTPs, 0.66 µM of each primer, 0.3U DreamTaq™ DNA polymerase (Fermentas, Lithuania) and 1.5 µl of template DNA. The PCR conditions were as described by Pulawska et al. (2006): initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94° for 1 min, annealing at 67°C for 1 min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. Amplified products were visualized by agarose gel electrophoresis as described above.

Pathogenicity assay

The pathogenicity assay was performed by inoculation of the grapevine cv. Cabernet Franc and local tomato cultivar (*Solanum lycopersicum* L.). Three plants were inoculated for each bacterial strain. Bacterial suspensions (108 CFU/ml) were prepared

Table 1. Results of biochemical and physiological tests, PCR analysis and pathogenicity test.

	Strains			
	A. tumefaciens (KFB 096/C58)	*A. rhizogenes* (KFB 098/A4)	*A. vitis* (KFB 099/S4)	Studied strains
Physiological and biochemical tests				
Gram reaction	-	-	-	-
Oxidase reaction	+	-	+	+
Growth at 35°C	+	-	+	+
Growth in 2% NaCl	+	-	+	+
3-ketolactose production	+	-	-	-
Acid-clearing on PDA amended with CaCO3	-	+	-	-
Motility at pH 7.0	+	+	-	-
Pectolytic at pH 4.5	-	-	(+)	+
Ferric ammonium citrate	+	-	-	-
Citrate utilization	-	+	+	+
Acid production from sucrose	+	-	(+)	+
Alkali production from tartarate	-	+	+	+
PCR				
VCF3/VCR3	+	+	+	+
UF/B1R	+	-	-	-
UF/B2R	-	+	-	-
UF/AvR	-	-	+	+
UF/ArR	-	-	-	-
Pathogenicity assay				
Grapevine	+	NT	+	+
Tomato	+	NT	+	+

+, positive reaction; (+), weak positive reaction; -, negative reaction; NT, not tested

from 24-h-old cultures grown on PDA medium. The bacterial suspension was placed on the young stem of the test plants (30µl) and 3-5 needle pricks were made throughout a drop of the inoculum. SDW was used as a negative control. *A. tumefaciens* and *A. rhizogenes* control strains were used as controls in the pathogenicity test. The inoculated plants were maintained in a greenhouse at 24±3°C. Tumor formation was recorded on a weekly basis.

RESULTS

Several different types of bacterial colonies were isolated from the tumor tissue on YMA medium. The predominant colonies were white, circular and glistening, resembling the pigmentation and morphology of the *A. vitis* control strain. Five representative strains each originating from a different plant/tumor were selected for identification.

PCR assay was used for detection of the *virC* pathogenicity gene located on plasmid DNA. VCF3/VCR3 primers specific for the *virC* gene yielded a 414-bp fragment from allthe studied and three of the control strains, confirming the presence of pathogenic plasmid in the bacterial genome (Table 1, Fig. 2).

All studied strains were non-fluorescent, oxidase positive, grew at 35°C and in nutrient broth with 2% NaCl. They were negative in 3-ketolactose, acid clearing on PDA amended with CaCO3, and ferric ammonium citrate tests; non-motile at pH 7.0; pectolytic at pH 4.5; utilized citrate; produced acid from

sucrose and alkali from tartarate (Table 1). Based on the physiological and biochemical tests, the strains were identified as *A. vitis*.

Identification with classical tests was also confirmed by molecular analysis of the 23S rRNA gene (Table 1). In multiplex PCR, the 478-bp fragment, specific for the *A. vitis* 23S rRNA gene, was amplified from all studied strains, as well as from the control strain of *A. vitis*. The *A. tumefaciens* and *A. rhizogenes* control strains yielded amplification products of 184 and 1066-bp, respectively.

In the pathogenicity assay, all studied strains caused characteristic symptoms on the inoculated plants (Table 1). Typical tumors developed at the inoculation sites on the tomato plants 3 weeks after inoculation and on the grapevine plants after 6 weeks (Fig. 3). Tumors on the tomato plants were small in diameter compared with those induced by the control strains of *A. tumefaciens* and *A. vitis*. The A. tumefaciens control strain did not cause symptoms on grapevine, but was tumorigenic on tomato, while *A. vitis* was tumorigenic on both grapevine and tomato. No symptoms were observed on the plants inoculated with SDW.

DISCUSSION

Young, fresh and actively growing tumors are the most suitable plant material for the isolation of *Agrobacterium* spp. (Moore et al., 2003). Tumorigenic *Agrobacterium* species can also be isolated from soil samples collected near the trunks of diseased grapevines (Burr and Katz, 1983). Due to systemic survival in grapevine plants, *A. vitis* was isolated and detected from the xylem sap of infected symptomatic and asymptomatic plants (Burr and Katz, 1983, 1984; Szegedi and Bottka, 2002). For particular *Agrobacterium* species, semi-selective and selective media were developed (Moore et al., 2003). Selective media are required for the isolation of bacteria from soil samples. With great attention to sterile process, nonselective media may be used for isolation from tumor tissue, as was shown in this study.

The classification of the bacteria belonging to the genus *Agrobacterium* has been based on their plant pathogenic properties for many years. The genes responsible for pathogenicity are mostly located on tumorigenic (Ti) or rhizogenic (Ri) plasmids (Kerr et al., 1977). Considering the mobility of these genetic elements, classification of the genus *Agrobacterium* based on plant pathogenic properties is untenable (Kerr et al., 1977). Furthermore, various taxonomic studies have indicated that the genus *Agrobacterium* consists of several different groups/taxons based on stable and reliable characteristics (Young et al., 2005). In this study, strains were identified to the species level using a set of physiological and biochemical tests (Moore et al., 2003) and by genetic analysis of the 23S ribosomal gene (Pulawska et al., 2006).

PCR is a convenient method for the rapid determination of pathogenic *Agrobacterium* strains and their differentiation from nonpathogenic ones (Eastwell et al., 1995; Haas et al., 1995; Sawada et al., 1995; Szegedi and Bottka, 2002; Suzaki et al., 2004; Kawaguchi et al., 2005; Pulawska and Sobiczewski, 2005; Bini et al., 2008; Kumagai et al., 2009). However, significant genetic diversity among *Agrobacterium* strains decreases primer specificity. For this reason, the *virD2* specific primers developed by Haas et al. (1995) failed to amplify the corresponding sequences from some of the *A. vitis* strains (Bini et al., 1998; Kumagai et al., 2009). Similarly, *virC* specific primers (Sawada et al., 1995) did not detect various pathogenic *A. vitis* strains (Szegedi and Bottka, 2002). Improved *virC* primers (VCF3/VCR3) were able to detect a higher number of *A. vitis* strains (Kawaguchi et al., 2005; Kumagai et al., 2009). With this primer pair, a specific product was amplified from the tumorigenic *A. vitis* strains used in this study.

The pathogenicity of the studied strains was checked by inoculation of grapevine and tomato plants. Host-range differences between strains of *Agrobacterium* spp. have been previously reported (Anderson and Moore, 1979). However, some strains have limited host range and high host specifi-

city (Anderson and Moore, 1979; Panagopoulos and Psallidas, 1973). For this reason, it is important that no single plant species is used in the pathogenicity assay. The plant species from which the strain was isolated should also be included in the experiment. In this study, the size of the induced tumors on tomato plants differed between the studied and control strains of *A. vitis* and *A. tumefaciens*. The strains used in this study that caused smaller tumors may belong to the less virulent group. Interestingly, the control strain of *A. tumefaciens* was non-pathogenic on grapevine. This could be explained by the host-range differences, or incompatibility with the selected grapevine cultivar.

The high incidence of grapevine crown gall in Serbia in the last few years indicates a need for a more detailed examination of this disease and the causal agent. In this study, the isolated bacterial strains were determined as tumorigenic and identified as *A. vitis*, combining classical bacteriological and molecular methods. Reliable and rapid identification of the pathogen is a very important step in the prevention of further spreading of the disease and successful protection.

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