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Comparative study of diagnostic methods used for monitoring of common grape vine (*Vitis vinifera* L.) crown gall (*Agrobacterium vitis* Ophel & Kerr*)* in Slovenia

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ABSTRACT

Agrobacterium vitis causes common grape vine (*Vitis vinifera* L.) crown gall disease that destroyed a lot of Slovenian vineyards more than a decade ago. Eighty isolates of *Agrobacterium* spp. collected during monitoring in 2006 were identified as *A. vitis* and *A. tumefacies* by *peh*A and multiplex PCR method. Tumor-inducing capacity of these strains was assessed on test plants and with PCR methods for detection of the Ti plasmid responsible for tumor induction. With VCF3/VCR3 primer pair six false negatives and no false positives were detected. The high genetic diversity of pathogenic *Agrobacterium* spp. strains affects the performance of molecular methods, thus biological test should be performed where results from molecular methods are doubtful.

Key words: *Agrobacterium vitis*, common grape vine, host plants, *peh*A, multiplex PCR, VCF3/VCR3

IZVLEČEK

PRIMERJAVA DIAGNOSTIČNIH METOD SPREMLJANJA POJAVA RAKA (*Agrobacterium vitis* Ophel & Kerr) ŽLAHTNE VINSKE TRTE (*Vitis vinifera* L.) V SLOVENIJI

Bakterija *Agrobacterium vitis* je povzročitelj raka žlahtne vinske trte (*Vitis vinifera* L). Ta bolezen je uničila mnogo slovenskih vinogradov pred več kot desetimi leti. V sklopu spremljanja pojava bolezni smo leta 2006 izolirali 80 izolatov, ki smo jih s PCR metodama *peh*A in multipleks določili kot vrsti *A. vitis* in *A. tumefaciens*. Sposobnost sevov, da izzovejo nastanek tumorjev, smo ocenjevali na gostiteljskih rastlinah. Z molekularnimi metodami pa smo določali prisotnost plazmida Ti, povzročitelja nastanka tumorjev. Z metodo PCR smo ugotovili šest lažno negativnih patogenih sevov in nobenega lažno pozitivnega. Velika genetska raznolikost patogenih sevov *Agrobacterium* spp. vpliva na zanesljivost določanja z molekularnimi metodami, zato se v primeru dvomljivih rezultatov priporoča dodatna izvedba bioloških testov na rastlinah.

Ključne besede: *Agrobacterium vitis*, vinska trta, gostiteljske rastline, *peh*A, multipleks PCR, VCF3/VCR3

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1 INTRODUCTION

Crown gall disease occurs worldwide and causes major economical losses in fruit and grapevine production (De Cleene and De Ley, 1976; Kennedy and Alcorn, 1980; Pulawska, 2010). The major part of income loss is attributed to crown gall on young grafted plants in nurseries. The disease is characterized by a tumor which is usually formed on a plant stem just above the ground. Still, the disease is rarely fatal. Mainly young or stressed plants develop more pronounced symptoms: loss of plant vigour, reduction in crop yield, or even plant death (Poncet *et al.*, 1996; Epstein *et al.,* 2008). The disease is problematic on perennial horticultural crops, such as grapevines, stone and pome fruit trees, and ornamental plants, where tumors weaken the plant year after year. The causal agents of the disease are pathogenic *Agrobacterium* spp. carrying the Ti plasmid (pTi) (Van Larebeke *et al.*, 1974; Watson *et al.*, 1975). The ability to cause tumors is encoded on a portion of the pTi (T-DNA) that integrates into the host genome. Upon expression, the T-DNA genes alter the level of plant hormones resulting in uncontrolled plant cell proliferation and tumor formation (reviewed in Escobar and Dandekar, 2003).

Traditional identification of *Agrobacterium* spp. is based on biochemical tests (Holt *et al.*, 1994). The INCO-DC European program ERBIC18CT970198, "Integrated Control of Crown Gall in Mediterranean Countries" has presented an identification scheme for agrobacteria with minimal biochemical tests (reviewed in Shams *et al.*, 2012). Additionally, accurate identification can be achieved by molecular methods. Eastwell *et al.* (1995) developed a PCR method for detecting *A. vitis* (Ophel and Kerr, 1990) – causative agent of crown gall of grapevines. The method targets chromosomal polygalacturonase gene that is found in *A. vitis*, but not in *A. tumefaciens* (Smith & Townsend, 1907) Conn 1942 or *A. rhizogenes* (Riker *et al.* 1930) Conn 1942, which are rarely found in grapevine tumors. A decade later, Pulawska *et al*. (2006) developed a multiplex PCR for classification of *Agrobacterium* strains into *A. tumefaciens*, *A. rhizogenes* and *A.vitis*. This method amplifies the specific fragment on 23S rRNA and enables rapid diagnosis of *Agrobacterium* species. These molecular techniques are based on bacterial DNA and are more specific, sensitive, rapid and suitable for diagnostics. The genetic diversity within genus *Agrobacterium* has recently led to reclassification of *A. rhizogenes* into genus *Rhizobium* (Young *et al.*, 2006). On the other hand, *A. tumefaciens* and *A. vitis* were reported to differ from the members of the genus *Rhizobium* and therefore can remain in the same genus (Farrand *et al.*, 2003, Lindström and Young, 2011). Additionally, genetically variable strains of *A. tumefaciens*, now termed *A. tumefaciens* species complex group, were clustered into genomospecies that will progressively be reclassified into new species (Mougel *et al.,* 2002¸ Portier *et al.,* 2006; Lindström and Young, 2011; Pulawska and Kalužna, 2012).

Effective detection of tumor-inducing agrobacteria in plant material is crucial in propagating material and efficient management of crown gall disease. Traditionally, bacteria are isolated from plant or soil material by cultivation on selective media followed by testing their tumor-inducing capacity on test plants. According to Schroth *et al*. (1971), this protocol is not sensitive or robust enough in comparison to molecular methods. Most molecular methods for detection of tumorigenic isolates target tumorigenicity genes on a conserved *vir* region on the pTi. Sawada *et al.* (1995) developed VCF/VCR primers that target pTi-encoded *vir*C1 and *vir*C2 genes. Suzaki *et al*. (2004) improved the specificity of the primers (VCF3/VCR3) for pathogenic *Agrobacterium* strains from apple seedlings, but the primers work on *A. vitis* strains as well (Kumagai and Fabritius, 2008).

Grape crown gall caused substantial damage to vineyards in the winegrowing regions of Slovenia in 1999 (Šabec-Paradiž *et al.,* 2002). Much of the following *Agrobacterium*-based research in Slovenia was dedicated to control and prevention of *A. vitis* and *A. tumefaciens* infections on grapevine plants and propagating material, and also to characterization of *A. vitis* isolates in Slovenia (Fabjančič and Milevoj, 2003). In the present study we compared the *Agrobacterium* identification methods used for grape crown gall disease monitoring in Slovenia.

2 MATERIALS AND METHODS

2.1 Bacterial strains and isolates

A crown gall monitoring was conducted in 2006. Eighty-seven symptomatic grapevine grafts (Figure 1) were collected from nurseries and vineyards from across various winegrowing regions of Slovenia. Eighty isolates of *Agrobacterium* spp. were obtained from plant material on 3DG medium semi-selective for *A. vitis* (Brisbane and Kerr, 1983). All 80 strains were subcultured on King's B medium (KB), pure cultures preserved in meat peptone broth with glycerol, and stored at -80 °C until further use. All 80 strains were analysed in the diagnostic laboratory at Agricultural Institute of Slovenia.

Reference *A. tumefaciens* C58 (INRA, France), *A. vitis* 339-26 (IVIA, Spain) and *Rhizobium rhizogenes* K84 (IVIA, Spain) strains were used as controls in molecular and biological diagnosis.

2.1.2 Preparation of bacterial DNA

The bacterial DNA used in PCR reactions was extracted from 24 h-old colonies grown on KB medium at 27 °C. We used a standard alkaline lysis method (Sambrook *et al*., 1989), diluted the DNA (1:1000) in sterile distilled water and stored it at -20 °C.

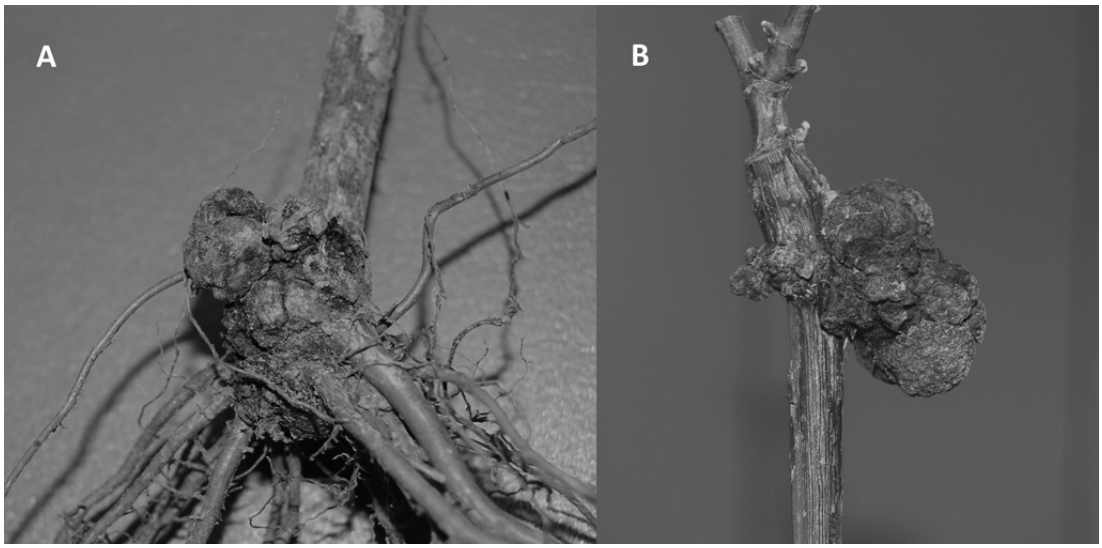

Figure 1: Grapevine grafts showing crown gall symptoms on a heel (A) and on a graft union (B) (Photos: I. Zidarič).

2.2 Identification of *A. vitis*

A. vitis isolates were identified based on polygalacturonase gene amplification (*peh*A) and multiplex PCR (Pulawska *et al*., 2006). Repeatability of the PCR results on the same DNA samples was verified in 2013.

2.2.1 Polygalacturonase gene amplification

The *peh*A PCR was performed in a total volume of 25 µl applying the protocol of Eastwell *et al*. (1995). For the PCR reaction, 1 µl of bacterial DNA template was used for PCR amplification in 1× PCR Buffer (Promega), 2.0 mM $MgCl_2$, 0.1 µM each *peh*A primer (Table 1), 0.2 mM dNTPs, 0.25 U GoTaq Flexi DNA Polymerase (Promega). The thermal cycler was programmed for an initial denaturation at 95 °C for 3 min followed by 40 cycles of amplification (95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min) with 5 min of final elongation at 72 °C. The amplified fragments of 205 bp were visualized on 2 % agarose gel.

2.2.2 Multiplex PCR

The multiplex PCR was performed in a 15 µl reaction volume applying the protocol of Pulawska *et al*. (2006). All reactions were performed in 1× PCR buffer (Promega), 1.5 mM $MgCl_2$, 1 µM each primer (UF, B1R, B2R and AvR) (Table 1), 0.2 mM dNTPs and 1.0 U GoTaq Flexi DNA

Polymerase (Promega). The amplification conditions comprised an initial denaturation at 95 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 67 °C for 1 min, extension at 72 °C for 1.5 min and a final extension step at 72 °C for 10 min. The amplified PCR fragments were visualized on a 2 % agarose gel. Strains belonging to *A. tumefaciens* gave a 184 bp product and those belonging to *A. vitis* gave a 478 bp product (Figure 2).

2.3 Assessing tumor-inducing capacity

Diagnosis of pathogenic strains of *Agrobacterium* spp. is carried out biologicaly on wounded test plants and molecularly through detection of bacterial tumour-inducing plasmid (pTi) responsible for uncontrolled plant cell growth.

2.3.1 Pathogenicity tests

The pathogenicity of *Agrobacterium* strains was determined on tomato, sunflower and kalanchoe plants. Young, four-week-old seedlings were punctured three times in the stem using a sterile entomological needle dipped in pure culture colonies grown on KB medium for 24 hours at 27 °C. Tests were performed in triplicates. Inoculated seedlings were maintained in a glasshouse at 20 – 30 °C with natural lighting conditions. In the period of 3 to 6 weeks post inoculation, the plants were visually inspected for tumor formations every few days. The strains C58 and 339-26 were used as positive controls; strain K84 and water served as negative controls. The test was completed in 2007.

2.3.2 pTi detection

The PCR was performed in a 25 µl reaction volume applying the protocol of Suzaki *et al*. (2004). For the PCR reaction, 2 µl of bacterial DNA template (diluted 1000 ×) were used for PCR amplification in 1× PCR Buffer (Promega), 1.5 mM $MgCl_2$, 0.5 µM VCF3 and VCR3 primers (Table 1), 0.2 mM dNTPs, 0.5 U GoTaq Flexi DNA Polymerase (Promega). The thermal cycler was programmed with an initial denaturation at 94 °C for 5 min followed by 35 cycles of amplification (94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min) with 5 min of final elongation at 72 °C. The amplified fragments of 414 bp were visualized on 2 % agarose gel (Figure 2).

Table 1: Primers pair sequences used in our study.

Primer	Sequence	Reference
pehAF	5'-CGATGGCGGCGAGGATTT-3'	Eastwell *et al*., 1995
pehAR	5'-ATCGGGCGTGAAACAAGT-3'	
UF f	5'-GTAAGAAGCGAACGCAGGGAACT-3'	Pulawska *et al*., 2006
B1R r	5'-GACAATGACTGTTCTACGCGTAA-3'	
B2R r	5'-TCCGATACCTCCAGGGCCCCTCACA-3'	
AvR r	5'-AACTAACTCAATCGCGCTATTAAC-3'	
VCF3	5'-GGCGGGCGYGCYGAAAGRAARACYT-3'	Suzaki *et al*., 2004
VCR3	5'-AAGAACGYGGNATGTTGCATCTYAC-3'	

2.4 Data analysis

Agreement between PCR and pathogenicity test was evaluated by calculating positive and negative percent agreement with respect to imperfect reference standard, in our case the *peh*A method and the biological pathogenicity test. The agreement indices were calculated from two-dimensional contingency table shown in Table 2.

Table 2: Two-dimensional contingency table for calculating agreement indices between two methods.

	Standard method A	
Method B	positive	negative
positive	a	b
negative	c	d
Total	(a+c)	(b+d)

Positive percent agreement with respect to imperfect reference standard was calculated according to equation (1) and was interpreted as sensitivity (Se) of the method. Similarly, negative percent agreement with respect to imperfect standard was calculated according to equation (2) and was interpreted as specificity (Sp) of the method. For estimation of confidence limits the 95 % confidence interval (CI) was calculated where appropriate.

$$\mathrm{Se} = \left(a/a+c\right)\cdot 100\% \quad (1)$$

$$\mathrm{Sp} = \left(d/b+d\right)\cdot 100\% \quad (2)$$

3 RESULTS AND DISCUSSION

3.1 Identification of *A. vitis* by *peh*A or multiplex PCR method

Morphologically, most rhizobial colonies appeared similar to one another on a general media. Therefore, it is imperative to use selective media for isolation of *A. vitis*. On 3DG medium, *A. vitis* colonies were visible sooner (after 3 days at 27 °C) than colonies of *A. tumefaciens* and *R. rhizogenes,* which also had different colony morphologies on 3DG medium. Where no typical *A. vitis* colonies were found, we selected for colonies that predominated on 3DG medium.

From 87 grapevine grafts we obtained 80 *Agrobacterium* isolates. According to multiplex PCR (Pulawska *et al.*, 2006) 75 isolates were identified as *A. vitis* and five as *A. tumefaciens*. The number of identified *A. vitis* strains was compared to the number of *peh*A positive (*A. vitis*) strains. There was a perfect agreement (100 %) between the two methods. All *peh*A positive isolates had an *A.vitis*-diagnostic band of 478 bp in multiplex PCR. The results were verified in 2013 on the same DNA samples stored at - 20 °C.

Our diagnostic laboratory has completely replaced the *peh*A identification method with multiplex PCR as it distinguishes between *A. vitis* and *A. tumefaciens* and differentiates them from other rhizobia in the Rhizobiaceae family in one reaction.

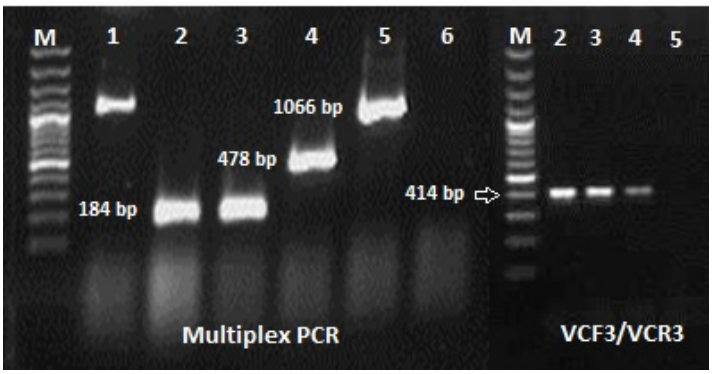

Figure 2: Agarose gel electrophoresis of diagnostic fragments from multiplex and VCF3/VCR3 PCR; M (ladder), 1 (unknown soil isolate), 2 (grapevine isolate), 3 (C58, *A. tumefaciens*), 4 (339-26, *A. vitis*), 5 (K84, *R. rhizogenes*), and 6 (water).

3.2 Pathogenicity status and pTi detection

All 80 isolates were tested for pathogenicity and abilitiy to cause tumors on stems of inoculated plants. This is a standard method for diagnosis of tumor-inducing strains and detection of latent infections (Janse, 2005). Almost 70 % of the strains were found pathogenic. One pathogenic strain was identified as *A. tumefaciens* causing tumors on all three test plants. In 2013 we analysed the same strains for the presence of pTi. We used a PCR method for identification of pathogenic and non-pathogenic strains of agrobacteria using primers VCF3/VCR3 with improved specificity (Suzuki *et al.*, 2004). All strains with detected pTi were identified as *A. vitis* by multiplex PCR. The agreement between results from pathogenicity tests and pTi detection method was not exact (Table 3). The sensitivity of VCF3/VCR3 primer pair was 89.1 % with six false negatives (Table 4). The only pathogenic *A. tumefaciens* strain was one of them. However, the specificity was 100 % with no false positives. The VCF3/VCR3 results were most compatible with pathogenicity assessment on kalanchoe and tomato test plants, though specificity was higher on kalanchoe plants (Table 4).

Table 3: Summary of results by two methods for *Agrobacterium spp.* pathogenicity assessment.

	Pathogenicity test		
VCF3/VCR3	+	-	Total
+	49	0	49
-	6	25	31
Total	55	25	80

It is not uncommon for *A. vitis* strains to have different host range even with identical physiological and biochemical characteristics (Tolba and Zaki, 2011). Interestingly, results from Tolba and Zaki (2011) indicate tomato as an unreliable test plant giving positive results on pathogenic *A. vitis* strains in only 5 of 12 isolates. At the same time, test on tomato proved specific for certain strains that caused tumors only on grapevines. In our case, none of the grapevine strains caused tumors specifically on tomato. On the other hand, six strains caused tumors solely on sunflower, and two solely on kalanchoe test plants. The presence of pTi was diagnosed on only half of these strains (three and one). One possible explanation is the sensitivity of the primers. These might be affected by high genetic diversity within pathogenic agrobacteria which could result in false negatives.The use of a set of three plants proved crucial in pathogenicity determination, as few of the pathogenic strains exhibited preference toward one host plant. If we had used only tomato or sunflower test plants, we would have observed fewer pathogenic strains and determined lower specificity of the VCF3/VCR3 PCR method in comparison to pathogenicity test results (Table 4).

Table 4: Sensitivity (Se) and specificity (Sp) of VCF3/VCR3 primer pair with respect to pathogenicity test on test plants.

VCF3/VCR3	Test plant tomato	sunflower	kalanchoe	Overall
Se	95.1	89.1	95.7	89.1 CI [82.27; 95.93]
Sp	74.4	76.5	85.3	100

The biological pathogenicity test is laborious and time-consuming. Pathogenicity is affected by environmental factors like temperature (Hamilton and Fall, 1971) and plant age (Binns and Thomashow, 1988). Also, the absence of tumors does not necessarily imply the absence of pTi. This is where molecular methods provide an additional confirmation. We repeated pathogenicity tests three times in three different seasons (spring, summer and autumn) using young to mature plants (results not shown). The most consistent results were obtained on young plants in late spring and early autumn when the average air temperature in the greenhouse was around 25 °C. Also, the interpretation can be doubtful when the PCR shows the absence of pTi, but the test plants develop tumors. Although PCR techniques for simultaneous identification of pathogenic and non-pathogenic *A. vitis* are available (Kawaguchi *et al.*, 2005), the traditional pathogenicity test is still a standard technique in strain pathogenicity determination.

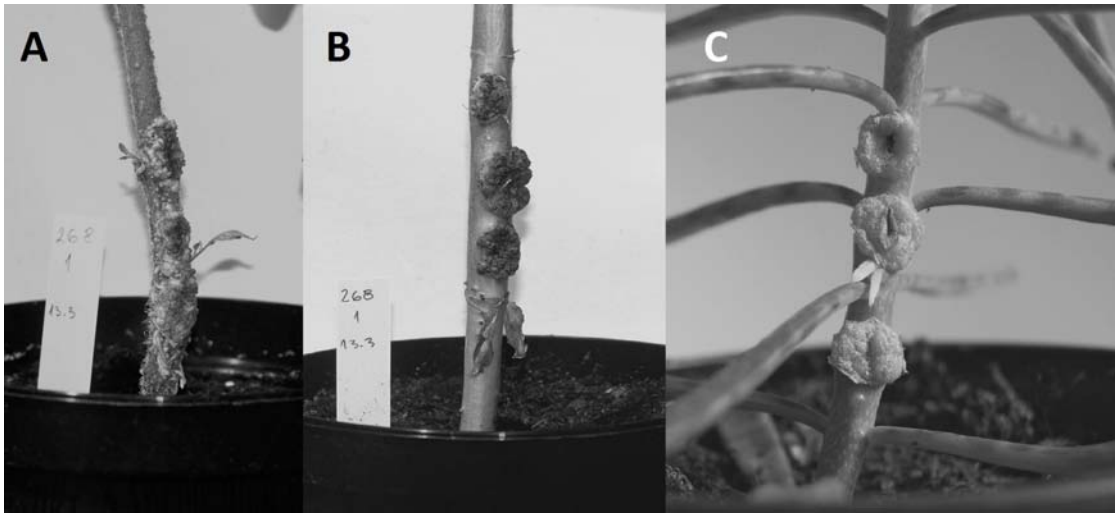

Figure 3: Pathogenicity tests on tomato (A), sunflower (B) and kalanchoe (C) plants (Photos: I. Zidarič).

4 CONCLUSION

Pathogenic *A. vitis* strains predominated among isolates of *A. vitis* from Slovenian grapevine grafts. Only one strain of *A. tumefaciens* was found pathogenic. In identification of *A. vitis* we obtained matching results using *peh*A or multiplex PCR primers. Therefore, we suggest using multiplex PCR (Pulawska *et al.*, 2006) for reliable identification of *A. vitis* and *A. tumefaciens* on grapevine. Further, we detected most of the pathogenic strains with VCF3/VCR3 primers. Based on our results, one might conclude that VCF3/VCR3 PCR could replace pathogenicity tests, but due to the false negatives, we conclude that biological pathogenicity test is still an invaluable tool in plant bacteriology.

5 ACKNOWLEDGEMENTS

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