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CROWN GALL OF GRAPEVINE IN SLOVENIA

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ABSTRACT

42 samples of grapevine plants and propagating material were taken in 2 Slovene vine growing districts and tested for the presence of *Agrobacterium* sp. 31 samples had suspicious symptoms, the other 11 did not have them. The samples were analysed using 4 methods: growth of agrobacteria on 4 semiselective media, detection of T-DNA carrying oncogenes *tmr, acs* and *vis/6b* by PCR, detection of opines in tumor tissue and pathogenicity testing on tomato and *Kalanchoe daigremontiana* plants. 20 samples with symptoms were proved to be infected with *Agrobacterium vitis* by means of molecular method and 19 by detection of opines. *A. vitis* was also proved in 3 samples without symptoms. 19 isolates induced tumor formation on tested plants. This is the first evidence of *A.vitis* in Slovenia.

Key words: *Agrobacterium vitis*, Slovenia, opines, PCR, pathogenicity testing

IZVLEČEK

BAKTERIJSKI RAK KORENINSKEGA VRATU NA VINSKI TRTI V SLOVENIJI

Na prisotnost bakterij *Agrobacterium* sp. smo preiskali 42 vzorcev trsov in cepljenk, ki smo jih zbrali v 2 slovenskih vinorodnih rajonih. 31 vzorcev je imelo sumljive rakaste izrastke, preostalih 11 pa ne. Vzorce smo preiskovali s 4 metodami: z rastjo agrobakterij na 4 polselektivnih gojiščih, z dokazovanjem prisotnosti onkogenov *tmr*, *acs* in *vis/6b* na T-DNA z verižno reakcijo s polimerazo, z določanjem opinov v tumornem tkivu in s testiranjem patogenosti izolatov na paradižniku in *Kalanchoe daigremontiana*. Pri 20 vzorcih z znamenji bolezni smo dokazali okuženost z *Agrobacterium vitis* z molekularno metodo, pri 19 pa smo dokazali opine. Okuženost z *A. vitis* smo dokazali tudi pri 3 vzorcih brez bolezenskih znamenj. 19 izolatov je induciralo nastanek tumorjev na testnih rastlinah. To je prvi dokaz *A. vitis* v Sloveniji.

Ključne besede: *Agrobacterium vitis*, Slovenija, opini, verižna reakcija s polimerazo, testiranje patogenosti

1 INTRODUCTION

Crown gall of grapevine can be induced by 3 biovars of *Agrobacterium tumefaciens*. While bv. 1 and bv. 2 have a wide host range, bv. 3 causes damage only on grapevine (Knauf *et al.*, 1982). *A. tumefaciens* bv. 3 was given the new species name *A. vitis* (Ophel and Kerr, 1990).

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Sympoms of the disease on grapevine appear early in the summer and consist of tumours that may be several cm in diameter. At first they develop at the crown, the graft union or around wounds caused by cultural practice or frost injuries. On rootstocks the tumors are rarely found. On older grapevines the tumors can spread and cover the whole aerial part of the plant. Parts above the tumors become weakened and the whole plant may die. When vine grafts in nurseries are affected by the disease, it is often difficult to distinguish the tumorous tissue from the callus one. Grapevines can be infected latently: *Agrobacterium* survives in the grapevine and no symptoms are induced until injuring of the plant occurs. (Bauer *et al.*, 1997; Bazzi and Piazza, 1987; Burr *et al.* 1998)

It is not possible to distinguish whether a plant is infected by *A. tumefaciens* or *A. vitis* on the basis of visual symptoms. The tumorous tissue can be distinguished from callus by the presence of opines in the sap from macerated suspicious tissue (Szegedi *et al.*, 1988). The existance of *Agrobacterium* sp. in plants with or without symptoms can be proved by the isolation of causal agents on semiselective media and determination of their morphological, biochemical and physiological characteristics or determination of their whole cell fatty acid patterns (Bouzar *et al.*, 1993; Holt *et al.*, 1994; Jäger *et al.*, 1989; Kersters and De Ley, 1984; Spies, 1979). Serological methods were described for rapid detection of Agrobacterium, but gained no wide use in practice because of methodological difficulties (Bazzi and Piazza,1987). Pathogenicity of isolates can be determined by testing on plants and by detection of specific T-DNA sequences with the PCR by which it is possible to determine also the species of causal agent (Dong *et al.*, 1992; Moore *et al.*, 1994; Schultz *et al.*, 1993; Oliveira *et al.* 1999).

Crown gall of grapevine, caused by *Agrobacterium*, occurs in many continents and countries (Burr *et al.*, 1998). Hyperplasias and dying of affected plants were found in vineyards and nurseries in Slovenia in the last years (Sebenik and Zemljič, 1998). Their appearance excited suspicion of beeing caused by *Agrobacterium*.

The objective of our research was to collect samples with hyperplastic alterations suspicious for crown gall and without them in the Posavski and Podravski vine growing districts, to find out whether these are tumors, to isolate eventual causal agents and differentiate them.

2 MATERIALS AND METHODS

2.1 Materials

42 samples to be used for laboratory analyses were taken in 12 vineyards and 6 nurseries in the Posavski and Podravski vine growing district and in 1 mother plant vineyard situated in this district in autumn 1998 and spring 1999. All of 22 grapevine plants had tumors and so had half of 18 vine graft samples, 2 samples from mother plant vineyard were without them.

2.2 Methods

The samples were examined for the presence of pathogenic agrobacteria using 4 methods: detection of opines in the tumor tissue, growth of causal agent on semiselective media, detection of specific T-DNA sequences, and pathogenicity testing. *A. vitis* vitopine strains

NW233 and NWA 405, octopine strains NCPPB 2562 and GmE 321 and nopaline strain NW 310 were used as positive control (Jäger *et al.*, 1991; Schultz, 1992).

For the detection of opines the altered tissues were macerated and analysed using high voltage paper electrophoresis. The opines octopine, nopaline and vitopine were detected as described by Otten and Schilperoort (1978) and Szegedi *et al.* (1988).

For the isolation of agrobacteria the samples were prepared according to methods described by Burr and Katz (1983), Moore *et al.* (1988) and Schultz (1992) and inoculated on 4 semiselective culture media: medium 1A for *A. tumefaciens* bv. 1, medium 2E for *A. tumefaciens* bv. 2 and media 3DG and RS for *A. vitis* (Brisbane and Kerr, 1983; Burr and Katz, 1983; Moore *et al.*,1994; Roy and Sasser, 1983).

For the detection of specific sequences on the Ti plasmid the obtained isolates were analysed by the polymerase chain reaction as described by Schultz (1993). 3 oligonukleotide primers which amplify specific fragments on T-DNA were purchased from Vitolab (Lauffen a.N., Germany). The primer X targets a conserved region of *A. tumefaciens* bv. 1 and bv. 2 strains by amplifying a fragment from the *tmr* gene 170 bp in size. The primers Ia and VIIb target *A. vitis* genes: Tm4 *acs* gene of nopaline/octopine strains and *vis/6b* region of vitopine strains, amplifying fragments with the size of 421 bp and 571 bp, respectively.

The pathogenicity of the isolates was tested by artificial infection of 3 weeks old tomato and *Kalanchoe daigremontiana* plants as described by Anderson and Moore (1979). The plants were infected in November and July, kept in greenhouse and observed for tumor formation through 3 months and some of them even after 10 months.

3 RESULTS

Not any sample was detected to be infected with *A. tumefaciens* bv. 1 or bv. 2. Typical colonies on semiselective media for these biovars were not formed. Suspicious colonies that grew from two samples were tested by PCR. The fragment of the *tmr* gene did not amplify.

The colonies that grew from mother plant samples were not suspicious for *Agrobacterium* sp. and were not tested further.

On semiselective media 3DG and RS colonies typical for *A. vitis* were isolated from all samples taken in vineyards and from 14 samples taken in nurseries. Pure cultures were tested by PCR, using the primers that amplify fragments of Tm4 *acs* and *vis/6b* genes and by pathogenicity on plants.

Particulars on samples and the results of analyses of the grapevines and vine grafts are presented in Table 1. The samples from the same vineyard or nurserie are grouped together and divided with horizontal lines.

plasmids with *acs* genes and 1 isolate also with the *vis/6b* gene. Opines were detected in 19 from 21 tested grapevines with symptoms. 13 samples contained octopine, 4 samples nopaline, 1 sample octopine and nopaline and 1 sample octopine and vitopine. 14 isolates were tested for pathogenicity on *K. daigremontiana* plants in November. Tumor formation was observed only on 1 plant and even on this one only after 10 months. 26 isolates were tested for pathogenicity on plants in July. 12 of them were tumorogenic on tomato and *K. daigremontiana*, 3 only on tomato and 4 only on *K. daigremontiana*. 7 isolates did not induce tumor formation 3 months after artificial infection.

4 DISCUSSION

In this research it was demonstrated that all of 22 grapevine samples were infected with *A.vitis* since all of the isolates grew on media selective for this bacteria. The infection of 6 samples was demonstrated by 3 methods: the presence of opines, specific T-DNA sequences and pathogenicity testing. 13 samples were proved to be infected using 2 methods: 6 samples by detection of opines and marker sequences on Ti plasmid, 6 samples by detection of opines and tumor induction on at least one test plant and 1 sample by detection of marker sequence and tumor induction detection. 3 samples were confirmed to be infected with pathogenic agrobacteria only by means of one of the methods mentioned.

Our results show that the isolation of causal agent followed by PCR method and/or pathogenicity testing is a suitable approach for the detection of latent infections with *Agrobacterium* sp. and are in accordance with Schultz *et al.*(1993) and Tarbach and Goodman (1986). The infection of 7 vine grafts was detected, on 3 among them no clear symtoms were observed.

19 from 26 isolates checked for pathogenicity induced tumor formation on at least one of the inoculated plants after 3 months. Our observation that not all of the isolates were pathogenic to both of the plant species used may be attributed to their host specificity (Anderson and Moore, 1979). It is also possible that inoculum concentration or environmental conditions may have reduced gall formation (Stover *et al.*, 1997). The latter is supported by the observation that by many of the strains, even the positive controls, no tumors were formed, when the plants were inoculated in November, but were formed when they were tested in July. Aditionally, the tumors developed only after 10 moths when the plants were infected in November while when infected in July the tumors developed already after 2 months. It is interesting that Olivera *et al.* (1999) report that avirulent strains are often isolated from tumors.

Samples, where the sap can be obtained from tumors, can be proved to be infected with *Agrobacterium* sp. by detection of opine presence. Opines are specific substances, produced only by *Agrobacterium* transformed plant cells. While octopine and nopaline can be produced in tumors induced by *A. tumefaciens* and *A. vitis*, vitopine is produced only in tumors induced by *A. vitis* (Canaday *et al.*, 1992; Szegedi *et al.*, 1988). The species of causing agent can be determined by the growth of bacteria on culture media selective for the particular biovar and determination of its characteristics. Also, the latent infection of a plant can only be determined if the

causing agent is isolated. The isolated bacteria were identified as *A. vitis* by the primers used in PCR. Since these primers target specific sequences on T-DNA that can be inserted into the plant cell genome and induce tumor formation, it was shown that the isolated bacteria possessed Ti plasmids carrying oncogenes (Chilton *et al.*, 1977; Dong *et al.*, 1992; Schultz, 1992; Schultz *et al.*, 1998).

This is the first evidence of crown gall in Slovene vineyards and nurseries. The tumors were found on Kerner, Sauvignon, Welsh Riezling, Rhine rizling, Chardonnay, Pinot Blanc, Pinot Gris, Šipon (Furmint) and Žametovka (Kölner Blauer) cultivars and on one Kober 5BB rootstock.

The fact that by means of the analysis performed only *A. vitis* was found in our plantations may reflect the state in the world, where this is the predominant bacterial species causing crown gall on grapevine (Bauer *et al.*, 1994; Burr and Katz, 1983; Burr *et al.*, 1998; Stover *et al.*, 1997).

With further research the distribution of crown gall in Slovene vineyards and nurseries and the methods for reducing *A. vitis* population levels in grape that could serve for the introduction of preventive measures against the spread of the disease should be investigated.

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