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DTBA AND ELISA METHODS IN DETECTION OF GRAPEVINE LEAFROLL – 1 VIRUS

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Grapevine leafroll syndrome is caused by a complex of up to nine different *Grapevine leafroll-associated viruses* (GLRaV-1–9). Many methods, including indexing, serological and molecular procedures, have been developed for the detection of GLRaV. However, due to the low concentration of the virus in plants a method with improved sensitivity and with the capacity to detect is required. In this study plants were tested for *Grapevine leafroll virus* (GLRaV) by visual examination, enzyme-linked immunosorbent assay (ELISA) and direct tissue-blotting assay (DTBA). Tissues were homogenized for use in a double-antibody sandwich ELISA system and then blotted on a nitrocellulose membrane for DTBA. Comparison of DTBA with ELISA and with visual plant symptoms suggest that DTBA can be used with the same accuracy as ELISA for detecting GLRaV-1 in leaf tissue.

Key words: grapevine, viruses, ELISA, tissue blot

INTRODUCTION

Grapevine leafroll (GLRaV) disease has been associated with yield losses of as much as 20–40% in grapevine (*Vitis vinifera*) (Goheen, 1988; Woodrum et al., 1984). This disease causes poor color development and non-uniform maturation of fruit in grapes (Goheen and Cook, 1959). Additional symptoms included downward rolling of basal leaves followed by rolling of the leaves near the shoot tips, interveinal reddening in red grape varieties and chlorosis in white grape varieties and phloem disruption (Hoefort and Gifford, 1967; Weber et al., 1993). Necrotic areas can develop in the interveinal tissue when the leaf is heavily infected (Martelli, 1993; Emmett and Hamilton, 1994). The symptoms of grapevine leafroll disease can resemble those caused by mechanical damage to the trunk,

other diseases of the phloem tissue, and arthropod damage, thereby complicating visual diagnosis (Weber et al., 1993).

The spread of grapevine leafroll disease has been assumed to occur only through infected plant material, mainly of asymptomatic American grapevine rootstocks, but during the last 20 years several pseudococcids (*Planococcus citri, P. ficus, Pseudococcus longispinus, P. affinis, P. calceolaria, P. comstocki, P. maritimus, P. viburni, Heliococcus bohemicus, Phenacoccus aceris*) and coccids (*Parthenolecanium corni, Pulvinaria vitis*) were found to be vectors of several GLRaV (Gugerli, 2003). The mechanisms underlying transmission of closteroviruses by mealybugs are still largely unknown. No alternate hosts are known and leafroll-associated closteroviruses have not been identified in any wild or cultivated plant species other than Vitis species (Martelli, 1993).

The etiology of GLRaV seems to be very complex and has not been definitively resolved. With recent advances in molecular characterization of several closteroviruses, the taxonomic relationship of this once heterogeneous group of viruses is more defined (Fauquet et al., 2005; Karasev, 2000). GLRaVs belong to the family *Closteroviridae* (Martelli et al., 2002) which is composed of three genera: (a) *Closterovirus*, e.g., GLRaV– 2, (b) *Ampelovirus*, e.g., GLRaV-1, -3 and -5 and tentative members of GLRaV – 4 and – 9 (Alkowni et al., 2004), and (c) *Crinivirus*.

Grapevine leafroll-associated virus 1 (GLRaV-1) is one of the most important types (Martelli et al., 1997). Particles of GLRaV-1 are filamentous and contain a coat protein (CP) with an *M*r of 39000 (Gugerli et al., 1984). A replicative form double-stranded RNA (dsRNA) species of 19 kb and several smaller dsRNAs are consistently isolated from GLRaV-1-infected tissues (Habili and Rezaian, 1995). These smaller dsRNA species arise from infection with mixed viruses or may be ubgenomic molecules. One of the smaller dsRNA species extracted from GLRaV-1-infected tissues hybridizes to a DNA probe made from the 19 kb viral genome (Habili and Rezaian, 1995; Habili et al., 1997). Subgenomic RNA species are considered to be part of gene expression strategies utilized by closteroviruses (Agranovsky, 1996) and have been found in *Beet yellows virus* (BYV) (Agranovsky et al., 1994), *Lettuce infectious yellows virus* (LIYV) (Klaassen et al., 1995) and *Citrus tristeza virus* (CTV) (Hilf et al., 1995).

Partial purification of some of these putative closteroviruses has allowed development of serological methods (ELISA) for their detection (Demke and Adams, 1992; Hu et al., 1990; Rowhani, 1992; Rowhani et al., 1997). Serological detection of GLRaV, however, requires the availability of multiple antibody preparations capable of detecting all of the known types of this virus (Forsline et al., 1996). Other molecular tests, such as reverse transcriptase polymerase chain reaction (RTPCR), have also been developed for their detection (Rowhani et al.,

2000). Rather than relying on antibody reactions, RTPCR specifically tests for molecular sequences that are unique to a particular pathogen.

Traditionally, detection of leafroll in grapevines is achieved by biological indexing, which requires graft-inoculation of sensitive cultivars with candidate material and observation of symptom development over an 18-month period (Goheen, 1970). Using serological techniques, rapid detection of certain GLRaVs has become possible. The detection of GLRaVs is based on bioassays, ELISA and PCR, which have a limited reliability and sensitivity. Bioassays are used widely, but are time-consuming, unreliable and require glasshouse infrastructure. The low concentration of viruses associated with GLRaVs, and their uneven distribution in infected tissues, together with the variation in seasonal titre, make detection by ELISA methods difficult to use and unreliable due to their low sensitivity.

This study was designed to determine the extent to which results of the direct tissue blotting assay (DTBA) and ELISA agree with each other and with visual diagnosis for detecting GLRaV-1 in grapevine tissues.

MATERIAL AND METHODS

Plant tissue

Plants with visible leafroll symptoms were collected from the different location (Kragujevačko, Podgorsko, Trsteničko i Župsko) in Serbia. Leaves from these plants were simultaneously tested for viruses with direct tissue blotting assay (DTBA) and with enzyme-linked immunosorbent assay (ELISA).

DTBA Methods

Tissue blotting technique was described by Lin et al., 1990 and Couceiro et al., 2006. Leaves were tightly rolled and cut with a razor blade. Cut surfaces were pressed firmly but gently onto membrane of nitrocellulose with 0.45 μm pore size (AppliChem, Darmstad). The membranes were blocked in nonfat dried milk at 1% in extraction buffer for grapevine (0.14 M NaCl, 0.01 M Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 3.1 mM NaN_3 and 0.05% Tween-20, adjusted to pH 7.4), during one hour at room temperature or overnight at 4-6°C. Membranes were then incubated for 2-2.5 h at room temperature or overnight at 4-6°C in monoclonal antibody (1:1000 dilution) conjugated to alkaline phosphatase (Sediag, Dijon, France). After incubation the membranes were washed carefully for at least five minutes, three times, in a saline buffer (0.085% NaCl,

0.05% Tween 20 in distilled water). The membranes were than dipped directly in alkaline phosphatase streptavidin conujgated antibody solution. After incubation the membranes were washed three times for five minutes in a saline buffer. The membranes were than covered with BCIP-NBT(5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium) ready to use liquid substrate (Sigma B-1911). The purple colour was developed after 30 minutes of incubation, therefore the reaction was not stopped (with distilled water) until membrane starts getting dark and the positive control is clear.

ELISA Methods

Tisuue extracts were placed into microplates coated with a specific polyclonal and monoclonal antibodies against GLRaV-1. Each plate contained wells with a known GLRaV-1 infected control, wells with sample buffer only, wells with a known healthy control and wells for each unknown sample. ELISA procedures followed methods described by Clark and Adams, 1977. The plant tissue extract was prepared by powdering about 200 mg of grape leaf tissue with a mortar and pestle and ground in 2ml of grape extraction buffer ((0.2 M Tris–HCl, 0.14 M NaCl,2% PVP, 0.5 ml l)1 Tween 20, pH 8.2).). The samples were centrifuged (16,000g for 10 min) in a bench top centrifuge and 100 µl of the supernatant transferred in duplicate to a microtitre plate to which the primary antibody had been bound (1:500 dilution in coating buffer [15 mM Na2CO3, 35 mM NaHCO3, pH 9.6]) at 37˚C. A positive control and negative control (provided by the manufacturer) and extraction buffer alone were included. The plate was incubated for 16 h at 4˚C, washed five times with PBST (140 mM NaCl, 1.5 mM KH2PO4, 8.09 mM Na2HPO4, 2.7 mM KCl, 0.5 ml Tween 20, pH 7.4), and 100µl conjugated antibody (1:500 dilution in conjugate buffer [PBST, 2% PVP, 2 g BSA]) added. The plate was incubated for 2 h at 37˚C, washed, and 100 µl freshly prepared 4-nitrophenyl phosphate (0.1 g ml) 1 substrate buffer [100 mM Na2CO3, 1 mM MgCl2, pH 9.8] added. The plate was incubated at 37˚C for 1 h and the absorbance at 405 nm was read using a plate reader The reading was repeated after a further 1 h incubation. According to manufacturer's recommendations, the sample was considered infected when absorbance readings with a spectrophotometer UNISCAN set at 405 nm was 0.3-1.2 OD, and uninfected if A405 was 0.050–0.065 OD.

RESULTS

Grapevine samples with characteristic symptoms for virus infected plants were collected from the different location (Kragujevačko, Podgorsko, Trsteničko i Župsko) in Serbia. The presence of GLRaV-1 was correlated with leaf symptoms including vein banding, leaf deformation, line pattern and mosaic in vines collected from the field. Samples showing typical dispersed mosaic or complete leaf discoloration, typical leaf rolling and interveinal discoloration on vine leaf.

Fig. 1. – Different grapevine samples with characteristic symptoms
Sl. 1. – Uzorci vinove loze sa karakterističnim simptomima

In a further study ELISA methods was used for virus detection, sixteen samples were tested on different leafroll viruses with various combinations of antibodies in ELISA (Table 1). All tested samples were positive on Grapevine leafroll virus type 1 and were negative on the other types of GLRaV. The ELISA readings

(specific data not shown) were clean and clear with a minimum absorbance at 405 nm of over 0.550 from infected vines and an average of 0.022 from healthy controls.

Table 1 – ELISA detection of grapevine leafroll viruses in collected samples

Tabela 1 – Određivanje prisutva virusa uvijenosti ELISA testom u uzorcima lišća vinove loze

Viticultural regions Vinogradarski regioni	Samples Uzorci	Viruses – Virusi				
		GLRaV 1	GLRaV 2	GLRaV 3	GLRaV 5	GLRaV 7
Župsko	1	+	-	-	-	-
	2	+	-	-	-	-
	3	+	-	-	-	-
	4	+	-	-	-	-
Trsteničko	5	+	-	-	-	-
	6	+	-	-	-	-
	7	+	-	-	-	-
	8	+	-	-	-	-
Podgorsko	9	+	-	-	-	-
	10	+	-	-	-	-
	11	+	-	-	-	-
	12	+	-	-	-	-
Kragujevačko	13	+	-	-	-	-
	14	+	-	-	-	-
	15	+	-	-	-	-
	16	+	-	-	-	-

We were particularly interested in developing a DTBA Methods system for detection of GLRaV-1 in grapevine. To evaluate the effectiveness of DTBA Methods, comparative studies were carried out with ELISA. The experiments were repeated at least three times with similar results. Grapevine samples collected from the field were tested on Grapevine leafroll viruses types 1, 2. 3, 5

and 7, and samples were positive only on GLRaV-1. This results were confirmed results that we were got with ELISA tests.

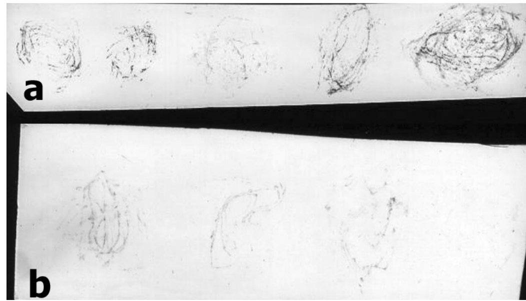

Fig. 2. – DTBA revealed that the examined grapevine samples were virus-infected (a), Healthy grapevine leaf samples (b)

Sl. 2. – DTBA pokazuje da su uzorci vinove loze pozitivni na prisustvo virusa (a), Zdravi uzorci lišća vinove loze (b)

DISCUSSION

Grapevine viruses can cause severe losses by substantially reducing yield, affecting fruit quality and shortening the lifespan of infected plants in the vineyard. Using different methods we were tried to find faster and more sensitive way in detecting grapevine lefroll viruses.

Extensive efforts have been carried out in identifying GLRaV-1 infected vines from collected plants both by DTBA Methods and by ELISA. To confirm the performance of DTBA and to test its utility for the detection of GLRaV-1 from infected field plants, comparative ELISA and DTBA detection assays were carried out using grapevine samples with typical symptoms. Vineyards inspected regularly showed considerably fewer symptoms. Serological tests revealed that this type of infection was attributed by GLRaV-1. The samples were collected from naturally infected grapevine plants in few vineyards in Serbia.

Two kinds of samples were used: samples collected in plants bearing symptoms and samples from healthy control plants. First, we tested the presence of GLRaV-1 by DAS ELISA using a commercial polyclonal antiserum that reacted specially with GLRaV-1. The samples were first tested by ELISA for the presence of different GLRaV viruses (GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-5 and GLRaV-7) and only GLRaV-1 was detected. All samples which were positive by

ELISA were also positive by DTBA. This demonstrated its very high sensitivity for GLRaV-1 diagnosis.

Grapevine leafroll 1 is a phloem limited virus and turn purple during DTBA, making results unambiguous an easy to interpret. DTBA analysis is especially useful for non-destructively sampling large numbers of plants for GLRaV. DTBA tissue blots detect virus antigens only at the surface of the cut, thus results are inconsistent for tissues with scattered phloem concentrations. The ELISA tests used homogenized tissue extracts which potentially represent all phloem cells and any associated antigens, thereby giving ELISA an advantage over DTBA for testing leaves. The fact that tissue blots detect virus antigens present on a cut surface at any chosen location makes DTBA useful for monitoring GLRaV movement in a plant. DTBA cannot quantify virus titer, but can be used for quick detection GLRaV in a plant. DTBA also could be useful in detection of other grapevine viruses. DTBA has also been shown to be particularly valuable in situations where there is an uneven distribution of viral antigens (Hsu and al., 1991).

Grapevine leafroll agents other than GLRaV-1 are present in Serbian vineyards (Kuzmanović et al., 2003, Starović et al., 2006, Starović et al., 2007, Paunović et al., 2007) and DTBA can be used for their detection in further investigation.

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PRIMENA DTBA I ELISA METODE U DOKAZIVANJU VIRUSA UVIJENOSTI LISTA VINOVE LOZE – 1

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REZIME

Sindrom uvijanja lista vinove loze je oboljenje koje prouzrokuje grupa virusa koja se naziva *Virusi vezani za uvijanje lista vinove loze* (GLRaV-1–9). Više različitih metoda je razvijeno za detekciju ove grupe virusa, kao što su indeksiranje, serološke i molekularno biološke metode. Zbog relativno male koncentracije virusa u zaraženoj biljci potrebno je pronaći metodu koja ima najveću osetljivost pri njegovoj detekciji. U ovom radu u cilju detektovanja virusa GLRaV, ispitivane biljke su pored vizuelnog pregleda obrađivane i pomoću ELISA i DTBA metoda. Poređenjem ELISA i DTBA metode na simptomatičnim biljkama utvrđeno je da se ove dve metode sa jednakom tačnošću mogu koristiti u otkrivanju GLRaV-1 u listu vinove loze.

Ključne reči: vinova loza, vizuelni test, ELISA test, «tissue blot» test

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