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Sanitary selection of sour cherry cv. Marasca (*Prunus cerasus* cv. Marasca) in Croatian largest plantation "Vlačine"

Zdravstvena selekcija višnje Maraske (*Prunus cerasus* cv. Marasca) u najvećoj Hrvatskoj plantaži "Vlačine"

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ABSTRACT

Sour cherry cv. Marasca is considered a native Croatian variety with the best fruit quality in the Northern Dalmatia region. The current production is not sufficient to cover the demand, therefore new orchards should be established with pathogen-tested planting material. To select virus- and bacteria-free mother trees, a survey was conducted in the largest Croatian Marasca plantation "Vlačine". ELISA on 205 trees (51 elite, 103 average, 51 below average) confirmed the presence of prunus necrotic ringspot virus (PNRSV) on 101 trees (49.3%); petunia asteroid mosaic virus (PeAMV) and prune dwarf virus (PDV) on 10 trees each (4.9%); raspberry ringspot virus (RpRSV) on 7 trees (3.4%); cherry leafroll virus (CLRV), arabis mosaic virus (ArMV) and plum pox virus (PPV) on 3 trees each (1.5%); and apple mosaic virus (ApMV) on 1 tree (0.5%). For PNRSV, significant ELISA detection sensitivity was found, with twigs in dormant period as more reliable virus source compared to leaves during the growing season. In addition, six trees were positive on little cherry virus 2 (LChV-2) by RT-PCR. Along with very frequent latent infections, following symptoms were observed during pre-harvest period: yellow spots on leaves (ApMV); yellowing, stunted growth, leafless twigs with leaves only at the tip (PDV), bark splitting (PeAMV), reduced leaf size (RpRSV), chlorotic/necrotic ring spots (PNRSV), and uneven fruit ripening (LChV-2). Eleven trees were free of all viruses and bacteria defined by EPPO cherry certification scheme, including PPV. The selected virus- and bacteria-free elite trees represent a valuable genetic source for propagation and further clonal selection.

Keywords: ELISA, RT-PCR, symptoms, viruses, bacteria

SAŽETAK

Višnja Maraska se smatra hrvatskom autohtonom sortom s najboljom kvalitetom plodova na području sjeverne Dalmacije. Trenutna proizvodnja ne zadovoljava potrebe tržišta stoga bi trebalo podići nove voćnjake korištenjem sadnog materijala testiranog na patogene. S ciljem pronalaska matičnih stabala bez virusa i bakterija istraživanje njihove prisutnosti provedeno je u najvećem hrvatskom nasadu višnje Maraske „Vlačine". Rezultati testiranja serološkom metodom (ELISA) na 205 stabala (51 elitno, 103 prosječna, 51 ispodprosječno) potvrdili su prisutnost virusa nekrotične prstenaste

pjegavosti trešnje (PNRSV) u 101 stablu (49.3%); virusa zvjezdastog mozaika petunije (PeAMV) i kržljavosti šljive (PDV) u 10 stabala svaki (4.9%); virusa prstenaste pjegavosti maline (RpRSV) u sedam stabala (3.4%); virusa uvijenosti lista trešnje (CLRV), virusa mozaika gušarke (ArMV) i virusa šarke šljive (PPV) u tri stabla svaki (1.5%); virusa mozaika jabuke (ApMV) u jednom stablu (0.5%). Značajna razlika u osjetiljvosti testa zabilježena je kod PNRSV-a prilikom čega su se grančice u mirovanju vegetacije pokazale kao pouzdaniji uzorak u usporedbi sa listovima prikupljenima tijekom vegetacije. Dodatno, šest stabala se metodom RT-PCR pokazalo pozitivno na virus sitnih plodova trešnje 2 (LChV-2). Pored vrlo čestih latentnih infekcija, u periodu pred berbu zabilježeni su simptomi: žuta pjegavost listova (ApMV); žućenje, zaostajanje u rastu, gole grane s listovima samo na vršnom dijelu (PDV); pucanje kore (PeAMV), smanjeni listovi (RpRSV), klorotični/nekrotični prstenovi (PNRSV) i neujednačeno dozrijevanje (LChV-2). Slobodnim od svih virusa i bakterija definiranih EPPO certifikacijskom shemom za trešnju, uključujući i virus šarke šljive, pokazalo se 11 stabala. Izdvojena elitna stabla bez virusa i bakterija predstavljaju vrijedan genetski materijal za razmnožavanje i daljnju klonsku selekciju.

Ključne riječi: ELISA, RT-PCR, symptoms, viruses, bacteria

INTRODUCTION

Fruit production has a special place in the agricultural production of the Republic of Croatia. Among fruit species grown, sour cherry cultivar Marasca (*Prunus cerasus* cv. Marasca) has a special place and importance.

The peak of Marasca production was reached in the 1970s, when the number of trees exceeded 1.000.000 and a total production of 12.150 tons. Since then, the number of trees and the amount of fruit produced have decreased, and intensive production has remained only in area of Ravni Kotari. The production has remained relatively stable, mainly because of the company Maraska, which regularly buys all the available amounts of fruit and uses the fruit from its largest plantation called "Vlačine" (Medin, 1997). The total production in the Republic of Croatia is currently about 1,500 tons, most of which is in Zadar County. It is estimated that the food industry, together with export and retail, can currently process between 6,000 and 10,000 tons of fruit, for which about 500-1000 ha of new orchards should be established. To achieve this, it is necessary to modernize production. This includes the use of virus- and bacteria-free planting material for the new plantations and control of their spread in the existing plantations.

The first studies related to virus diseases of sweet and sour cherry in the territory of former Yugoslavia were conducted in the 1960s. Symptoms of ring spot disease commonly found in cherry seedlings in nurseries, peach ring spot virus in nurseries on seedlings and on some local sweet and sour cherry cultivars and die-back of old cherry plants with suspected presence of two viruses - prunus necrotic ringspot virus and arabis mosaic virus were mentioned (Šarić and Panjan, 1964). The first confirmation of viruses in sour cherry Marasca date back in the late 1970s (Šarić and Velagić, 1980), with the occurrence of prunus necrotic ringspot virus (PNRSV), prune dwarf virus (PDV), arabis mosaic virus (ArMV), strawberry latent ringspot virus (SLRV), cucumber mosaic virus (CMV) and soybean mosaic virus (SMV). Since 1988, the Institute for Plant Protection, now within Croatian Agency for Agriculture and Food, has monitored the presence of plum pox virus (PPV) in nurseries and has confirmed the presence of this virus on several occasions (Kajić et al., 2012). Recently, there is no written data on the incidence of the other 15 viruses listed in the recommendations of the European and Mediterranean Plant Protection Organization (EPPO). According to the experience from the countries of the region, especially Serbia (Mandić et al., 2007), frequent occurrence of the viruses should be expected in the trees which are currently used as a source of buds for the production of planting material.

The most common bacterial diseases of stone fruit trees are bacterial leaf spot disease caused by *Xanthomonas arboricola* pv. *pruni*, crown gall caused by *Agrobacterium tumefaciens* and bacterial canker caused by *Pseudomonas syringae* pathovars *syringae* and *morsprunorum*. While *X. arboricola* pv. *pruni* mainly infects peach and plum, bacterial canker caused by *Pseudomonas syringae* pv. *morsprunorum* is one of the most serious diseases affecting sour cherry

trees (Bultreys and Kaluzna, 2010).

The aim of this study was to investigate the presence of viruses and bacteria, defined by the EPPO standard PM 4/29(1) - Cherry Certification Scheme (EPPO, 2000), in the plantation "Vlačine", the largest Croatian plantation of sour cherry Marasca. The result of this investigation should fill the information gap on the presence of sour cherry viruses and bacteria in Croatia, and provide additional data on their frequency, associated symptoms and sampling time. In addition, virus and bacteria free trees with good agronomic traits will be selected as potential mother plants (i.e. source of buds for planting material) and could be used as a source material for clonal and sanitary selection programs in the future.

MATERIALS AND METHODS

Plant material

The study was conducted in the largest plantation of cv. Marasca named "Vlačine", located in Zemunik Donji (Northern Dalmatia). With the aim of obtaining an objective assessment of the health status in 190 ha plantation, 205 trees were selected according to the visual assessment of yield, fruit size and coloration, and symptoms of viral and bacterial diseases. Based on the above criteria, the trees were classified into three categories: elite/excellent (E-51), average (A-103) and below average (BA -51 trees with reduced growth and partial dieback). In July 2014, just before harvest, leaves from different parts of the canopy (east, west, north, south) were used as a potential antigen source for serological testing (ELISA). In the absence of data on the optimal timing and procedure for sampling, each tree was resampled in November (dormant period) by collecting four twigs per tree using the same sampling scheme as for leaves. Samples were labeled, placed in plastic bags, and stored at -20 °C until testing.

Serological tests

Collected leaves/twigs were tested for the presence of 11 viruses: apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), arabis mosaic virus (ArMV), petunia asteroid mosaic virus (PAMV), cherry leaf roll virus (CLRV), prune dwarf virus (PDV), prunus necrotic ringspot virus (PNRSV), raspberry ringspot virus (RpRSV), strawberry latent ringspot virus (SLRSV), tomato black ring virus (TBRV) and plum pox virus (PPV). ELISA was performed using commercial kits from LOEWE® Biochemica GmbH (Germany) according to the manufacturer's instructions. Petioles and main veins/cortical shavings were taken separately from each leaf/twig in the sample and mixed in an average sample of 0.2 g for each tissue type and pulverized in a mortar with a pestle and liquid nitrogen. All the other steps were performed according to the manufacturer's instructions. Spectrophotometric measurements were performed using BIOTEK EL800 spectrophotometer (BioTek, USA) at a wavelength of 405 nm. The samples with absorbance greater than three times the average value of the negative controls were considered positive.

Molecular tests - viruses

Between ELISA-negative elite trees 17 of them with the best agronomic traits were additionally tested for four viruses by reverse transcription polymerase chain reaction (RT -PCR): little cherry virus 1 and 2 (LChV-1 and LChV-2), cherry mottle leaf virus (CMLV) and cherry green ring mottle virus (CGRMV). Total RNA was isolated from cortical scrapings of twigs using RNeasy Plant Mini Kit (QIAGEN, Valencia, USA). The integrity and quality of RNA was checked using NanoPhotometer P330 spectrophotometer (Implen, Germany). Detection of CMLV was performed as described by Ma et al. (2014), while for CGRMV, LChV-1 and LChV-2, initial denaturation of total RNA was performed on 75°C for 10 min and RNA was stored on ice until use for one-step RT -PCR. For the preparation of 50 µl reaction mixture One-Step RT -PCR Kit (Qiagen, Valencia, USA) was used according to the manufacturer's recommendations with the addition of 1X Q-solution, 0.6 µM of each primer and 5 ng of total RNA. Reaction conditions were as follows: Reverse transcription at 52°C for 30 min, initial activation step 95°C for 15 min, 35 cycles: 94°C for 30 sec, 60°C - LChV-1 57°C - LChV-2, 56°C – CMLV, 55°C - CGRMV for

45 sec, 72°C for 1 min; final extension 72°C for 7 min. Primers CMLV-5F/CMLV-3R (Ma et al., 2014), CGRMV1/CGRMV2 (Li & Moch, 2005) LChV-1 F1/R1 (Osman et al., 2012) were used, while for LChV-2 primers LCHV2LO2/LCHV2UP2 (Rott and Jelkmann, 2001) and LC26L/LC26R (Eastwell and Bernardy, 2001) were used. In addition, RT -PCR was used as a confirmatory test for PNRSV in cases where ELISA-results from leaves differed from those of twigs. For this purpose, five trees were selected from each tree category (elite, average, below average), total RNA was extracted as described previously, and universal primers JC-10 and JC-12 were used in RT -PCR as described by Hammond et al. (1999), with an annealing temperature of 50˚C and other conditions as described above. After gel electrophoresis in 1.5% 1xTBE agarose gel, the products were visualized on the UV transilluminator (Bio-Rad, USA).

Molecular tests - bacteria

Marasca trees that were found to be negative for all viruses tested were again visually inspected after flowering for the presence of bacterial diseases caused by *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *morsprunorum* and *Xanthomonas arboricola* pv. *pruni*. No symptoms reminiscent of *A. tumefaciens* or *X. arboricola* pv. *pruni* were observed. Only mild leaf spots, possibly indicative of *Pseudomonas* sp. infection, were observed on eight trees which leaves were re-sampled and used for further testing. Portions of the leaves were macerated in PBS buffer for 20 minutes. Extracts were plated out on King's medium B, incubated at 27 °C and examined after 48 hours (Schaad et al. 2001). Suspect colonies were used for DNA extraction using the DNeasy Plant mini kit (Qiagen, Germany). Primers D21 and D22, which amplify internal transcribed spacers (ITS1), were then used according to Manceau and Horvais (1997).

Symptom observation

Possible symptoms of viral and bacterial infections were tracked/observed during the sampling periods in 2014. In 2015, after the health status of individual trees was known through laboratory tests, inspections of trees were carried out and any changes observed were compared with the results of serological and molecular tests.

RESULTS

Serological tests

ELISA-results confirmed a relatively high incidence of PNRSV (101 trees, 49.3%) with much less frequent PAMV and PDV (10 trees each, 4.9%), RpRSV (7 trees, 3.4%), CLRV, ArMV and PPV (3 trees each, 1.5%) and ApMV (1 tree, 0.5%). The presence of other viruses included in the study was not confirmed in any tree. In the elite, average and below average categories, 32 (62.7%), 47 (45%) and 25 (49%) trees were found to be free of ELISA-tested viruses, giving an overall infection rate of 49.3%. In virus-infected trees, single infections predominated (91.7%), while mixed infections with PDV+PNRSV and PDV+PeAMV+PNRSV+RpRSV were detected in three plants each (1.5%). Comparing the results from leaves collected in June with those from twigs collected during dormancy, cortical scrapings taken from dormant twigs were found to be a more reliable source, yielding more positive samples. However, when a particular virus was detected in leaves, it was also confirmed from twigs, but not vice versa, i.e. much more positive results were obtained with twigs. A detailed overview of ELISA-results is presented in Table 1.

Molecular tests - viruses

The results of RT-PCR performed on 17 elite trees using specific primers for LChV-1, LChV-2, CMLV, and CGRMV confirmed the presence of LChV-2 in six trees (35%). Differences in detection efficiency were observed with different primer pairs: four positive results were recorded with primer pair LCHV2LO2/LCHV2UP2 and another two positive results with the second primer pair used - LC26L/LC26R (Figure 1). None of the trees tested positive with both primers. After serological and molecular tests, out of the 205 trees tested, 11 trees were found to be free from all 15 viruses tested.

Table 1. ELISA-results for 11 viruses performed on 205 Marasca trees (51 elite and below average each, 103 average). Results are shown only for viruses found in survey. ApMV – apple mosaic virus, ArMV – arabis mosaic virus, CLRV – cherry leaf roll virus, PDV – prune dwarf virus, PAMV – petunia asteroid mosaic virus, PNRSV – prunus necrotic ringspot virus, RpRSV – raspberry ringspot virus, PPV – plum pox virus

Tree category	Plant material	ApMV	ArMV	CLRV	PDV	PAMV	PNRSV	RpRSV	PPV
Elite (51)	Leaves	0	0	1	0	0	9	0	0
	Twigs	0	1	3	1	1	29	1	1
% of infection		0	1.9	5.9	1.9	1.9	56.9	1.9	1.9
Average (103)	Leaves	0	0	0	3	0	18	1	1
	Twigs	0	0	0	5	6	50	3	2
% of infection		0	0	0	4.9	5.8	48.5	5.9	1.9
Below average (51)	Leaves	0	0	0	2	0	10	1	0
	Twigs	1	0	0	4	3	22	3	0
% of infection		1.9	0	0	7.8	5.9	43.1	5.9	0

Molecular tests – bacteria

Molecular tests did not confirm the presence of *Pseudomonas* sp. in none of the eight trees tested.

In sensitivity comparison tests for PNRSV, all 15 samples/trees yielded PCR products of the expected size of 641 base pairs, using either leaf or twig tissue as the potential virus source.

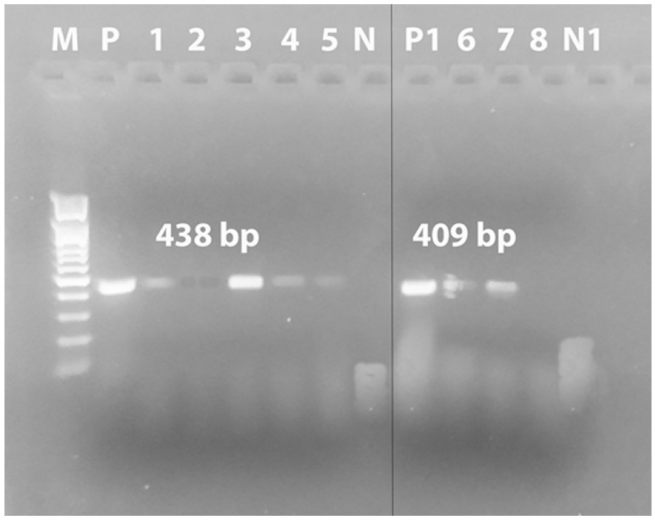

Figure 1. RT-PCR results for LChV-2 conducted using primer pair LCHV2LO2/LCHV2UP2 with expected product size of 438 bp (left) and primer pair LC26L/LC26R with expected product size of 409 bp (right). M – marker; P and P1 – positive controls for primers LCHV2LO2/LCHV2UP2 and LC26L/LC26R, respectively, N and N1 – corresponding negative controls, 1-8 – different samples. Positive results were obtained in samples 1, 3, 4, 5, 6 and 7. Not all tested samples are shown on gel

Symptom observation

The symptoms observed in Marasca cherry trees when infected with certain viruses and their descriptions can be found in Figures 2 to 7. However, it should be noted that not all infected trees showed signs of virus infection - in some trees the infection was probably latent or the symptoms were not apparent at the time of visual inspection.

DISCUSSION

ELISA-results showed relatively high infection of Marasca trees with PNRSV, especially in trees selected as elite (56.9%), with decreasing percentage in average trees (48.5%) and below average tree category (43.1%). Similar results were reported by Mandić et al. (2007) from Serbia, where PNRSV was found as the most frequent virus (35.7%) in sour cherry collections. The determined total virus infection rate of 49.3% is higher than that determined in Serbia (Mandić et al., 2007), but similar to the virus infection rate of 41% in commercial stone fruit orchards in Bosnia and Herzegovina (Matić et al., 2008).

Considering the percentage of virus-free plants in different categories (elite - 62.7%, average - 45% and below average - 49%), visual inspection was only partially successful in selecting virus-free plants, and many infections in "Vlačine" orchard were asymptomatic.

Figure 2. Yellow spots on leaves of Marasca cherry trees infected with apple mosaic virus (ApMV)

Figure 3. Yellowing and stunted growth of Marasca cherry tree infected with prune dwarf virus (PDV) – comparison of tree sizes of infected and virus-free plant (left); leaf growth only at the tips of branches/shoots (right)

Figure 4. Bark splitting of branches of Marasca tree infected with petunia asteriod mosaic virus (PeAMV)

Figure 5. Leaf size from uninfected (bottom) and infected (top) Marasca cherry tree infected with raspberry ringspot virus (RpRSV). Leaves of approximately the same age were taken from adjacent trees

Figure 6. Chlorotic and necrotic spots on Marasca tree infected with prunus necrotic ringspot virus (PNRSV)

Figure 7. Uneven fruit ripening on Marasca tree infected with little cherry virus 2 (LChV-2)

Alternatively, the absence of visible symptoms can be addressed to virus-host interactions, especially with detected numerous variants of PNRSV, some of which do not induce symptoms in *Prunus* sp. (Howell And Mink, 1988), or even environmental conditions, especially high temperatures, present at the time of visual inspection.

For detection of PNRSV Salem et al. (2003) reported spring as the best season and summer as less reliable for ELISA. This is consistent with the results of our study, as leaves collected in summer were found to be 63% less effective for virus detection compared to phloem tissue from twigs collected during the dormant period. Therefore, as reported by several authors (Sanchez-Navarro et al., 1998; Mourty et al., 2000) and the results of this study, if sampling for ELISA cannot be done in appropriate period, molecular methods such as RT-PCR should be chosen as more reliable and appropriate detection methods, especially in the case of sanitary selection where false negative results can have long-term negative consequences.

Little cherry disease, caused by little cherry virus 1 (LChV-1) and little cherry virus 2 (LChV-2), is an economically important viral disease of sweet and sour cherry with worldwide distribution (Martelli et al., 2012). LChV-2 confirmed in six Marasca trees with uneven fruit ripening is consistent with symptoms previously described by other authors (Jelkmann and Eastwell, 2011). As previously reported (Vončina et al., 2016), at least two LChV-2 variants are present in Croatia, suggesting significant sequence variability. Both causal agents of little cherry disease, LChV-1 and LChV-2, are characterized by high genome diversity (Candresse et al., 2013; Jelkmann et al., 2008; Tahzima et al., 2019), so new knowledge about genome diversity could help in the development of new detection methods capable of detecting a wide range of different strains.

The symptoms of different viral diseases described in this study are consistent with those reported from other countries and authors: yellow line pattern, bright yellow spots and rings due to ApMV (Hadidi et al., 2011), yellowing, reduced growth and leaf drop due to PDV (Nemeth, 1986; Hadidi et al., 2011), bark splitting due to PeAMV (Koenig and Kunze, 1982), chlorotic and necrotic spots due to PNRSV (Hadidi et al., 2011) and problems with uneven fruit ripening on trees infected with viruses causing little cherry disease as reported by Eastwell (1997) and Milbrath and Reynolds (1964). The absence of virus symptoms on many trees infected with different viruses or virus combinations could be due to virus-host interactions, virus strains, environmental conditions (especially temperatures above 30-35 °C), confirming that visual inspection and symptomatology have no diagnostic significance in case of most viruses included in the study.

None of the symptoms of the diseases caused by *A. tumefaciens* or *X. arboricola* pv. *pruni* were detected by visual inspection in the "Vlačine" plantation. The presence of causal agent of bacterial spot and canker, *Pseudomonas* sp., was not confirmed with molecular tests in this investigation.

Since the predominant transmission pathway for most of the viruses confirmed in the study is through planting material, special attention should be paid to the quality of planting material in nurseries. According to the Croatian legislation on cherry planting material, until 2017 only monitoring of nurseries for PPV was mandatory. Since 2017, the "Regulation on the marketing of propagating material and seedlings for fruit production" has been in force (Official Gazette, 2017). Sour cherry planting material should be free from 15 viruses and bacteria from the genus *Pseudomonas*, which were included in this study. Current Regulation on marketing of fruit propagating material is clearly dividing CAC category and pathogen-tested categories (certified, basic and pre-basic). The requirements for mother plants are different and pathogen-tested material production is controlled by competent delegated body. In the last decade, producers of various stone fruits in Croatia have become aware of the harmful effect that various viral and bacterial diseases can have, especially in fruit trees, where the expected lifetime of plantations, quality and quantity of yields can be significantly reduced due to viral and bacterial diseases. Hopefully, new standards and legislative adopted from 2017 will have a positive impact on the quality of planting material.

CONCLUSIONS

This study fills the information gap about sour cherry viruses in Croatia that have occurred in the last 40 years. Compared to viral diseases, bacterial diseases were shown to be less prevalent and important in "Vlačine" plantation. *Prunus* necrotic ringspot virus proved to be the most dominant virus found in sour cherry cv. Marasca (confirmed in almost half of the examined trees), while other viruses occurred sporadically at a rate of 0.5 to 4.9%. Variations in virus titre between growing and dormant periods were noted, with twigs in dormant period providing a reliable source of PNRSV for ELISA detection. Observed symptoms and plant vigour were found to be less reliable sources of information on virus infection. A prerequisite for successful fruit production is the use of clean and health-tested planting material. In this study, 11 trees that were free of 15 tested viruses and economically important bacteria were selected as potential candidates for the pre-basic planting material. This is an important prerequisite for the production of planting material according to the standards of the European and Mediterranean Plant Protection Organization (EPPO).

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