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APPLICATION OF QPCR FOR PLUM POX VIRUS DETECTION DURING CRYOTHERAPY

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Abstract: For the purpose of removing viruses from infected plant material, cryotherapy is a novel application of the plant cryopreservation technique. The use of various cryotherapy procedures and treatments necessitates the examination of cryo-treated material for the presence of the targeted pathogen in order to gauge the effectiveness of the therapy. In our study, we evaluated the efficiency of reverse transcription-polymerase chain reaction (RT-PCR) and qPCR (quantitative PCR) methods for the detection of plum pox virus (PPV) in cryo-treated material of plums 'Belošljiva' and 'Crvena Ranka'. A qRCR assay showed higher sensitivity in comparison to conventional RT-PCR.

Keywords: plum pox virus, qPCR, cryotherapy, efficiency

Introduction

A safety measure against the unintentional loss of plant germplasm collections, the latest biotechnology-based conservation strategies, including various *in vitro* procedures, complement *ex situ* conservation techniques. Cryopreservation, or the storage of plant material at extremely low temperatures (-196°C), has emerged as a crucial method for the long-term preservation of plant germplasm. Recently, it has been shown that cryopreservation can be employed for other uses than germplasm conservation – for eradicating viruses. Cryotherapy is a cutting-edge method of removing pathogens from infected plant material. Once the methodology for the specified genotype has been established, the technique requires only the most basic tools found in a tissue culture laboratory. The main difficulty in applying cryotherapy is the vastly varied responses to the treatment between genotypes of the same species. For a dozen species, a number of procedures using various cryotherapy techniques have been documented.

The most devastating viral disease in stone fruits, including plum, is plum pox virus (PPV) that is present in Serbia since 1930ies. Other known viruses infecting stone fruits were very rarely detected in plums in the country. In the

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past decade, research on PPV in Serbia has brought new knowledge on PPV distribution, genetic diversity, and epidemiology (Jevremović, 2013). According to the estimates, about 70% of the plum trees are infected with three major PPV strains (PPV-M, -D, and -Rec). PPV represents the main threat to the production and existence of plum genotypes in an open field. Cryotherapy, as method for PPV eradication from stone fruit species is an old idea, but not much investigated and utilized in the practice. An important segment in this process is the analysis of the regenerated cryopreserved material. Highly sensitive assay is required to detect PPV, even in a very low concentration due to the suppression with the applied cryotherapy method.

In this paper we presented the results of the application of quantitative Polymerase Chain Reaction (qPCR, Real-time PCR) for PPV detection in cryopreserved plums.

Materials and methods

The initial material for this experiment consisted of two PPV-infected autochthonous plums 'Belošljiva' and 'Crvena Ranka'. Aseptic cultures of these two plum cultivars were established on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) as described in detail by Jevremović et al. (2022). Prior cryotherapy, apical shoot tips (1 mm large) and axillary buds were dissected from the shoots and analyzed by qPCR to confirm the presence of PPV. Cryotherapy was performed on plum shoot tips (aprox. 1.5 mm long) from 4-week-old plantlets growing on MS media using aluminum cryo-plates according to D- and V-cryo-plate methods (Jevremović et al., 2022).

A total of 111 pool samples of *in vitro* shoots (average 10 plants per sample, in total about 1100 plants) of the plums 'Belošljiva' and 'Crvena Ranka' were tested (65 samples of 'Belošljiva' and 46 samples of 'Crvena Ranka'). *In vitro* shoots regenerated from control and cryopreserved explants were continuously tested during multiplication for the presence of PPV.

At the first stage (the first two subcultures after regrowth), all samples were tested with conventional RT-PCR analysis. To evaluate these results and obtain the highest efficiency of detection, all samples were further tested using qPCR.

Total nucleic acids (TNA) were isolated from 0.2 g fresh *in vitro* shoots with 2% CTAB buffer according to the protocol of Li et al. (2008). Extracted RNA was used for two-step reverse-transcription (RT) analysis. Reverse transcription (RT) reactions were performed in two steps with Maxima reverse transcriptase (ThermoScientific, USA). Obtained cDNA was subjected to PCR reaction with

P1/P2 universal primers (Wetzel et al., 1991). RT and PCR reactions were performed in TPersonal thermal cycler (Biometra, Germany). PCR products were analysed by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized by Gel Doc EZ System (Biorad Laboratories, USA). The presence of an expected fragment of 243 bp was considered as a positive reaction.

Samples were further tested using primers and TaqMan probes (Olmos et al., 2005). The reaction mixture (20 µl) consisted of: 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 6.25 U 1 × MultiScribe RT (Applied Biosystems, USA), 10U RNase Inhibitor Mix (Applied Biosystems, USA), 1 µM P241 primer, 0.5 µM each of P316D and P316M primers, 200 nM TaqMan PPV-DM probe and 5 µl RNA template. The reaction was performed with the following thermocycling conditions: 15 min at 48°C 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C. Data acquisition and analysis were conducted in a StepOnePlus™ Real-Time PCR System (Applied Bio-systems, USA) and StepOne™ v2.3 Software package (Life technologies, USA).

Results and discussion

Using RT-PCR, the plum pox virus was detected in 67 out of 111 samples tested (60.36%). An expected 243 bp fragment was obtained in 39 samples of plum 'Belošljiva' and 28 of plum 'Crvena Ranka' (Figures 1 and 2). In certain positive samples (Figure 1, lines 8, 9, 11, 12, 14-18; Figure 2, line 18), expected fragments were clear, and strong suggesting a relatively high virus concentration in the samples. In some samples, bands on the gel were very faint and barely visible (Figure 2, lines 5, 12, 14-17).

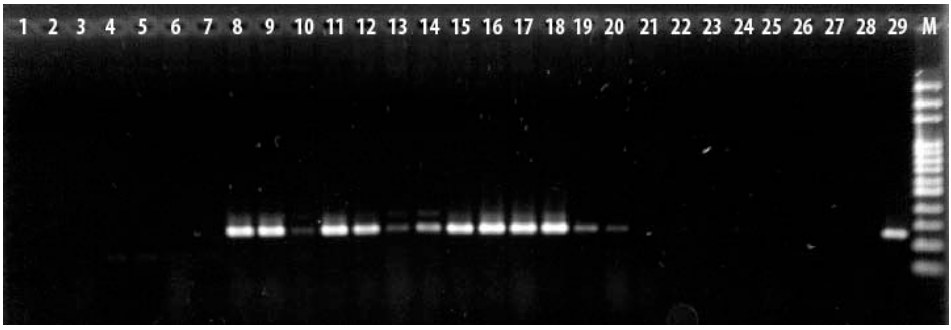

Figure 1. RT-PCR detection of plum pox virus in *in vitro* plants after cryotherapy (lines 1-28: analysed samples, line 29: positive control; line M: 100 bp DNA Ladder (Solis BioDyne, Estonia)

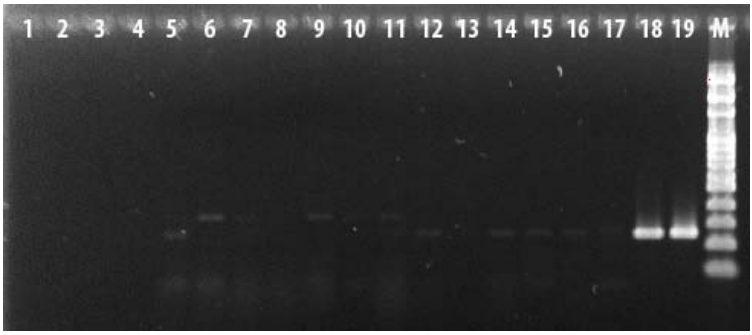

Figure 2. RT-PCR detection of plum pox virus in *in vitro* plants after cryotherapy (lines 1-18: analysed samples, line 19: positive control; line M: 100 bp DNA Ladder (Solis BioDyne, Estonia)

Using a qPCR assay, PPV was confirmed in 82 tested samples (73.87%). Conventional RT-PCR failed to detect PPV in 15 samples that were positive with qPCR (Figures 3 and 4). In control (non-cryo-treated) plants, PPV was detected with both assays.

Quantitative PCR proved to be a more reliable method for the analysis of *in vitro* plants after cryotherapy. Due to the low virus concentration in regenerated plants, a highly sensitive method is needed to evaluate the health status of the material and the success of cryotherapy. Cryotherapy has a significant impact on virus concentration in treated plants and can eradicate PPV in certain treatments and genotypes (Jevremović et al., 2022).

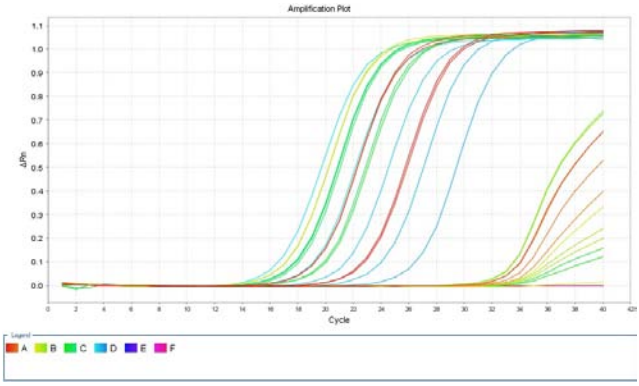

Figure 3. qPCR detection of plum pox virus in analyzed samples

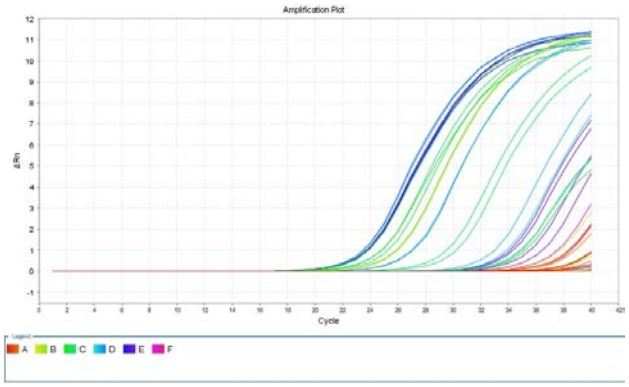

Figure 4. qPCR detection of plum pox virus in analyzed samples

In published studies on plum pox virus eradication with different methods (hemotherapy, thermotherapy, cryotherapy, and their combinations) ELISA (Enzyme-Linked ImmuneSorbent Assay) and RT-PCR methods were performed (Brison et al., 1997; Manganaris et al. 2003; Paunović et al., 2007; Polak and Hauptmanova 2009). Since its discovery, PCR has become the gold standard for the molecular detection of a large number of plant pathogens. Real-time PCR provide a more rapid, sensitive and reliable diagnosis of PPV (Olmos et al., 2006). For the first time, two molecular methods were evaluated in our study to gain access to the health status of cryopreserved plum material. Based on our results, qPCR should be used in the analysis of the plant material on the presence of PPV during cryotherapy.

Conclusion

The findings of our investigation supported the excellent specificity and sensitivity of the qPCR technique for the detection of PPV in *in vitro* shoots of the plum cultivars 'Belošljiva' and 'Crvena Ranka'. Higher sensitivity is one of the characteristics of qPCR in contrast to PCR.

Acknowledgement

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