Detection of the Phytosanitary Status of Stone Fruit Cultivars under *in vitro* Conditions in Hungary

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Abstract

Prunus species are prone to infections by a range of pathogens (LAIMER 2002). Stone fruit plantations are mainly affected by *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *European Stone Fruit Yellows phytoplasma* (ESFY). The production of elite plants of pathogen-free stone fruit cultivars includes the application of effective methods for the detection and elimination of the major stone fruit tree viruses and phytoplasmas. The method of micropropagation offers a quick possibility for breeders to introduce new cultivars into the production. Virus testing by molecular techniques of *in vitro* plants requires only tiny samples and reduces the danger of false negatives. First results of the virus testing originated from *in vitro* peach cultivars are presented.

Keywords: peach; *in vitro*; virus testing; PPV; PNRSV; PDV; ESFY; ELISA; PCR

INTRODUCTION

Micropropagation is a widespread method for propagation of stone fruit rootstocks and cultivars. According to the EU requests for the healthy planting material the phytosanitary control of the *in vitro* plants has to be put into the practice in case of the most common stone fruit viruses like PPV, PDV and PNRSV as well as ESFY phytoplasma. ESFY has recently been encountered for the first time in Austria and Hungary (RICHTER 1998; SÜLE 1999). Continuous control of the planting material produced under *in vitro* conditions the spread of the mentioned casual agents could be reduced.

MATERIALS AND METHODS

Plant material: *In vitro* cultures of six peach cultivars Fantasia, Redhaven, Suncrest, Cresthaven, Biscoe and Frederica maintained in the cultivar collection of the Research Institute for Fruitgrowing and Ornamentals Budapest were established during May–June 2000, when shoots were in the stage of active growth and infection pressure in the field was still permissive.

Micropropagation: 5–10 cm long, actively growing shoot tips were surface sterilised and established on modified MS medium. Cultures were kept in a culture room at 22°C, 2000 Lux light intensity and 16/8 hrs photoperiod and subcultured following routine procedures of micropropagation. Descendants from every individual shoot tips were handled as separate clones. The health status of the individual shoot tips was determined at the beginning of the multiplication phase to decide further measures to be taken.

DAS-ELISA: The assay principle based on the classical DAS-ELISA technique (CLARK & ADAMS 1977) was followed with small modifications of the extraction protocol. Diagnostic kits for PPV (broad range), PNRV and PDV were purchased from Loewe Biochemica GmbH. All other chemicals were from

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Sigma-Aldrich Ltd. Nunc Maxisorp ELISA plates were used for all tests. Entire *in vitro* plants were prepared as samples by roller press homogenisation with a final dilution 1:20 in sample buffer, additionally containing 0.2% (w/v) bovine serum albumin (BSA).

We preferred 16 hours incubation of samples at 4°C for PPV and PNRV tests, because we obtained clearer differences between positive and negative samples. Enzyme-substrate reactions were stopped with 3M NaOH after one hour incubation at 28°C. Optical densities were measured at 405 nm with Metertech Mini ELISA reader.

Polymerase chain reaction (PCR)

Plum pox virus (PPV) detection: Immunocapture-RT-PCR method was used for confirmation of negative results of PPV ELISA tests. The protocol developed by LAIMER *et al*. (2001) was used with some modifications. The sap of *in vitro* plants was incubated at 4°C overnight in ELISA plates coated with PPV specific antibody (Loewe). After washing RT-PCR mix was pipetted into the wells of the ELISA plates. After reverse transcription (38°C, 45 min) the samples were transferred into PCR tubes and the amplification was performed in a PDR-91 thermal cycler. PCR products were separated on 2% agarose gels and photographed.

European stone fruit yellows (ESFY) phytoplasma detection: The extraction method described by KOBAYASHI *et al*. (1998) was used for the DNA isolation from *in vitro* plant samples. Nested PCR reaction was carried out in a PDR-91 thermal cycler. The primers (PA2F/R and NPA2F/R), the thermal cycling programs and the composition of the master mix were used as described by HEINRICH *et al*. (2001). PCR products were separated on 1.5% agarose gels and photographed.

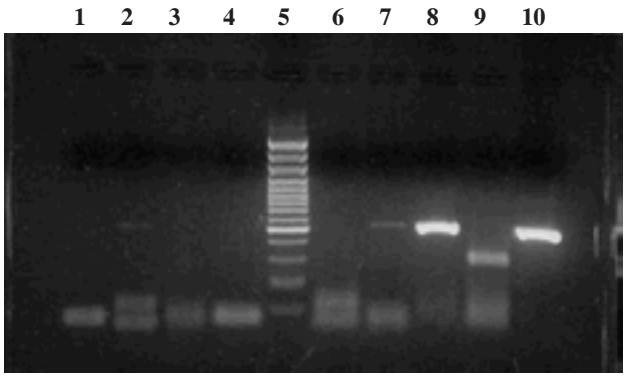
RESULTS

It was found, that the success of the *in vitro* establishment and the survival rate of the *in vitro* cultures like the multiplication rate of the peach cultivars depend not only the growth habit of the cultivar, but also on the level of virus infection. 50 actively growing shoot-tips were surface sterilised from all of the cultivars. The survival rate of the partly infected Frederica cultivar (Table 1) is much higher than the survival rate of highly or double infected cultivars, like Fantasia. Similar effects of virus infection could be detected in case of the propagation rate. Significant difference were found between the propagation rate of the virus infected and virus free shoot-tips of the same cultivar (data not shown).

Seventeen clones of the peach cultivars were tested for PPV, PDV and PNRV by ELISA. All of the cultivars were found infected with PPV (Table 2). Partly infection was found in case of Frederica cultivar, but one of the shoots was found to contain also PNRV. So far, no PDV infection could be detected in the examined cultivars. In case of Fantasia both viruses, PPV and PNRV were detected, even in the same shoot

Table 1. Survival rate and multiplication rate of the established peach cultivars

Cultivar	No. established plants	Survival rate (%)	Multiplication rate
Biscoe	50	10	2.2
Frederica	50	16	3.6 (–) 2.3
Fantasia	50	2	1.3
Suncrest	50	4	3.2 (–) 1.8
Cresthaven	50	2	1.2
Redhaven	50	2	1.6



1. Orangered –
2. Szegedi Mammut +
3. "5" –
4. Biscoe 2 –
5. Marker
6. Biscoe 4 –
7. Biscoe 5 +
8. ESFY positive Marianna +
9. DNA of ESFY negative control plant –
10. DNA of ESFY positive control plant +

Figure 1. ESFY detection by nested PCR. Products of amplification were separated in 1.5% agarose gel

Table 2. Pathogen infection of individual shoot tips tested by ELISA for PPV, PDV and PNRSV as well as by PCR for PPV and ESFY (n.t. not tested)

Clones	ELISA						PCR	
	PPV (OD_{405})		PDV (OD_{405})		PNRV (OD_{405})		PPV	ESFY
Biscoe 2	2.0 +	+	0.097	–	0.030	–	+	–
Biscoe 3	2.0 +	+	0.140	–	0.013	–	n.t.	–
Biscoe 4	2.0 +	+	0.222	–	0.119	–	n.t.	–
Biscoe 5	2.0 +	+	0.147	–	0.182	–	n.t.	+
Frederica 1	0.019	–	0.087	–	0.023	–	–	–
Frederica 2	0.340	–	0.279	–	0.308	+	–	–
Frederica 3	0.191	–	0.079	–	–0.092	–	–	–
Frederica 4	0.034	–	0.062	–	0.011	–	–	–
Frederica 5	0.083	–	0.073	–	0.001	–	–	–
Frederica 6	0.153	–	0.126	–	0.015	–	–	–
Frederica 7	2.0 +	+	0.089	–	0.056	–	+	–
Frederica 8	2.0 +	+	0.136	–	0.154	–	n.t.	–
Fantasia 2	2.0 +	+	0.100	–	1.663	+	n.t.	–
Suncrest 1	0.081	–	0.093	–	0.003	–	–	–
Suncrest 2	1.687	+	0.092	–	0.005	–	n.t.	–
Cresthaven	2.0 +	+	0.188	–	0.065	–	n.t.	–
Redhaven 2	2.0+	+	0.054	–	0.059	–	n.t.	–
Negative control	–0.050	–	0.011	–	0.036	–	n.t.	n.t.
Positive control	2.0 +	+	2.0 +	+	2.0 +	+	n.t.	n.t.
Negative N. benth	–0.050	–	0.138	–	–0.046	–	n.t.	n.t.

tip. This fact could be an explanation for the poor *in vitro* growth of this cultivar.

Seven clones were found negative in the ELISA examination for PPV infection. These results were controlled by IC-RT-PCR, representing a more sensitive method. However, all the PPV negative results were confirmed (Table 2).

We tested by nested PCR every clone for the presence of ESFY phytoplasmas. The concentration of this pathogen is very low in *in vitro* plants. For this reason we used this very sensitive method. Only one clone of the examined cultivars was found infected by ESFY. This material will be further propagated and used for elimination trials.

DISCUSSION

The results of the experiments carried out so far show that differences could be detected in the level of virus infection between the different shoot tips, even when they originated from the same tree. Considerating the growing number of *in vitro* propagated stone fruit rootstocks and cultivars as well as the EU regulations requiring healthy planting material, it appears reasonable to carry out a continuous health control of the *in vitro* established material at the very beginning of the propagation phase. The use of the very sensitive methods for virus and phytoplasma detection could allow to gain time in the virus elimination procedure. This represents a good possibility to reduce the impact of virus or phytoplasma infections in European planting material.

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