SUMMARY

Three major *Plum pox virus* (PPV) strains have been identified as prevalent in most of the central- and east-European countries, i.e. PPV-M, PPV-D and PPV-Rec. PPV-Rec, which arose from homologous recombination between PPV-M and PPV-D, has been recognised only recently. Although important studies on its genetic variability have been done in the last years, the biological and epidemiological data are scarce. It is generally thought that recombination events may provide some selective advantage to the recombinant isolate over its parent variants to become established in nature. Therefore, the behaviour of different PPV-Rec isolates in mixed infection with either PPV-M or PPV-D was studied by simultaneous co-inoculation of *Nicotiana benthamiana* plants. A stable balanced inter-strain mixed infection could be detected in about 40% of individual plants. No clear-cut strain-specific behaviour of PPV isolates in term of competitiveness in mixed infection could be observed. This further confirmed the existence of biological variability within single PPV strains.

Keywords: *Nicotiana benthamiana*, PPV-Rec, strain.

*Plum pox virus* (PPV, genus *Potyvirus*) is the causal agent of Sharka, the most detrimental disease of stonefruit trees worldwide. The vast majority of the currently identified PPV isolates is assigned to three major strains, i.e. PPV-M, PPV-D and PPV-Rec (Garcia and Cambra, 2007; Glasa and Candresse, 2008). The PPV-Rec strain consists of an ensemble of closely related isolates derived from a single homologous recombination event between PPV-M and PPV-D, with the recombination cross-over situated in the 3´terminal part of the NIb gene. PPV-Rec isolates are widespread in several central and east European countries and have been occasionally detected in Turkey, Pakistan and Canada (Glasa et al., 2004a; Kollerová et al., 2006; Candresse et al., 2007; Thompson et al., 2009).

Recombination is considered to play an important role in RNA virus evolution and can provide additional sources of variation with unpredictable effects on virus pathogenicity (Worobey and Holmes, 1999). Despite the wide distribution of PPV-Rec isolates, limited data are available on their biological properties. It is generally assumed that recombination can provide some selective advantage to the recombinant isolate over parent variants to become established in nature (Lai, 1992; Aaziz and Tepfer, 1999).

*Nicotiana benthamiana* Domin. is an experimental host plant commonly used for PPV maintenance and multiplication. This herbaceous species enables a relatively fast evaluation of biological experiments. All PPV strains infect *N. benthamiana* systemically and reach high concentration in its tissues (Kollerová et al., 2008).

The objective of this study was to evaluate the competitiveness of PPV-Rec isolates in experimental mixed infections of *N. benthamiana* with isolates of PPV-D and PPV-M, all of which were from a laboratory collection. Initially, the isolates were obtained from different Prunus spp. (Table 1), transmitted from the original hosts by mechanical inoculation to *N. benthamiana* and long term stored in lyophilised infected tissues. PPV-D was obtained from the viral collection of INRA Montpellier, France. The isolates had been characterised previously (Teycheney et al., 1989; Glasa et al., 1997, 2004a; Kollerová et al., 2008).

Inoculum sources were obtained by mechanical inoculation of *N. benthamiana* plants. Leaves systemically infected with the respective PPV isolate were harvested at 10-14 days post inoculation (dpi), sliced, mixed and weighted in order to prepare equal inoculum lots, which were stored at -80°C in order to use similar inoculum for each experiment. The relative virus concentration in the inocula from different isolates was compared by DAS-ELISA as described (Glasa et al., 2003) using the polyclonal antiserum AS196 produced in the laboratory of INRA Montpellier, France (Dallot et al., 2001). All samples were analysed together on a single plate (Sarstedt, USA), so that OD values could be compared.

Viral inoculum was prepared by homogenizing leaves from systemically infected *N. benthamiana* leaves 1:10
(w/v) in 0.1 M phosphate buffer, pH 7.2. A homogeneous lot of *N. benthamiana* at the 3-4 leaf stage was double-inoculated with either a PPV-Rec and a PPV-M isolate, or a PPV-Rec and a PPV-D isolate in the ratio 1:1, to ensure the same initial conditions for each isolate. The first fully developed basal leaf of *N. benthamiana* was inoculated by rubbing the inoculum on the leaf surface previously dusted with carborundum. To avoid a hierarchical effect because of the alternate leaf arrangement, both inocula were placed separately on the opposite halves of the same leaf (Fig. 1). Usually, 15 plants were co-inoculated for each combination. In parallel, five plants, single-inoculated with the respective isolate, were used as controls. Plants were monitored daily by visual inspection. Systemically infected apical leaves were harvested 21 and 40 dpi and the presence of virus isolate was specifically tested by immunocapture-reverse transcription PCR (IC-RT-PCR).

Plant extracts for immunocapture were prepared by grinding infected *N. benthamiana* leaves (1:20, w/v) in phosphate buffered saline (PBS) containing 0.05% (v/v) Tween-20 and 2% (w/v) polyvinylpyrrolidone. Clarified sap (100 µl) was incubated overnight at 4°C in tubes precoated with 1 µg/ml of anti-PPV IgG AS196. Tubes were then washed twice with sterile PBS-T and used for cDNA synthesis.

For specific detection of PPV isolates belonging to three different strains, a two-step RT-PCR protocol was applied. First, cDNA was synthesised using AMV reverse transcriptase and pdN6 primers (Promega, USA) as described (Glasa et al., 2002), then aliquots of the RT reaction were used in independent PCR reactions with strain-specific primer sets according to Šubr et al. (2004a), using GoTaq® DNA polymerase (Promega, USA).

PPV-Rec has been recognised only recently (Glasa et al., 2004a), thus information on its biology and epidemiology is still limited. The requirements to be met for evaluating the competitiveness of PPV-Rec isolates in mixed infection with PPV-D or PPV-M isolates were a successful virus multiplication in the double-inoculated experimental host and a persistence of infection. The host chosen for this study was *N. benthamiana* which,

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### Table 1. PPV isolates used in this study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain</th>
<th>Original host</th>
<th>Country of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BULG</td>
<td>Rec</td>
<td>Plum</td>
<td>Bulgaria</td>
<td>Glasa et al., 2004</td>
</tr>
<tr>
<td>BOR-3</td>
<td>Rec</td>
<td>Apricot</td>
<td>Slovakia</td>
<td>Glasa et al., 1997</td>
</tr>
<tr>
<td>Horomerice</td>
<td>Rec</td>
<td>Plum</td>
<td>Czech Republic</td>
<td>Glasa et al., 2004</td>
</tr>
<tr>
<td>CAH-2</td>
<td>M</td>
<td>Apricot</td>
<td>Slovakia</td>
<td>Glasa et al., 2004</td>
</tr>
<tr>
<td>Kr-4</td>
<td>M</td>
<td>Apricot</td>
<td>Slovakia</td>
<td>Glasa et al., 2004</td>
</tr>
<tr>
<td>VAR-2</td>
<td>M</td>
<td>Peach</td>
<td>Slovakia</td>
<td>Glasa et al., 2004</td>
</tr>
<tr>
<td>BOJ-3</td>
<td>D</td>
<td>Plum</td>
<td>Slovakia</td>
<td>Glasa et al., 1997</td>
</tr>
<tr>
<td>BOR-1</td>
<td>D</td>
<td>Plum</td>
<td>Slovakia</td>
<td>Glasa et al., 1997</td>
</tr>
<tr>
<td>Dideron</td>
<td>D</td>
<td>Apricot</td>
<td>France</td>
<td>Teycheney et al., 1989</td>
</tr>
</tbody>
</table>

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Fig. 1. Inoculation of *N. benthamiana* plants and assessment of infection.
although it is not a natural PPV host, thus it does not reflect the conditions underlying PPV installation in the natural host and the possible selective advantages deriving to different PPV sources by factors such as aphid transmission rate and *Prunus* host adaptability, still represents an interesting model for studying interactions between viral isolates. In fact, notwithstanding natural host preferences, all PPV strains infect readily *N. benthamiana*, although they may differ in the speed of systemic invasion (Kollerová et al., 2008).

A possible "host" effect is frequently observed under natural field conditions. For example, PPV-Rec isolates are frequently detected in plums but rarely in peach. Likewise, under experimental conditions, PPV-Rec isolates are less effectively aphid-transmitted to peach and their infection to GF305 is mostly symptomless (Glasa et al., 2002, 2004a, 2004b). On the contrary, PPV-M isolates are known to cause fast epidemics in peach (Dallot et al., 1998), and in experimental conditions they are more virulent than PPV-D to *Prunus salicina* (Capote et al., 2005). Our previous investigations with strain-mixed infections have also shown that peach GF305 seedlings are better suited for infection by PPV-M than PPV-D and that the more effective replication and faster systemic spread of PPV-M caused replacing of PPV-D in GF305 after superinfection, but not vice versa. Several combinations of PPV isolates gave similar results (Šulbr et al., 2004b, 2006).

All the isolates used in this study were readily transmitted to *N. benthamiana* by mechanical inoculation (100% infection). The relative concentration of virus in different inocula compared by DAS-ELISA did not reveal significant differences among isolates providing evidence that the IgG used for immunocapture reacted broadly with all the tested isolates. Under field conditions, the viral population is forced through a severe bottleneck during aphid transmission that can lead to an important reduction in polymorphism and thus would limit its adaptation rate (Ali et al., 2006). The mechanical inoculation used in our experiments, on the other hand, allowed us to infect the opposite halves of the same leaf provided in equal opportunity for infection of each of tested *PPV* isolates.

Table 2. Persistence of PPV isolates in *N. benthamiana* after double-inoculation with different combinations of PPV-Rec and PPV-M.

<table>
<thead>
<tr>
<th>Plant no</th>
<th>Rec&lt;sub&gt;Horomerice&lt;/sub&gt;/M&lt;sub&gt;CAH-2&lt;/sub&gt;</th>
<th>Rec&lt;sub&gt;BULG&lt;/sub&gt;/M&lt;sub&gt;CAH-2&lt;/sub&gt;</th>
<th>Rec&lt;sub&gt;BOR-3&lt;/sub&gt;/MKr-4</th>
<th>Rec&lt;sub&gt;BOR-3&lt;/sub&gt;/MVAR-2</th>
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</table>

n.t. = not tested (plant death not due to virus infection)
Isolate-specific behaviour of PPV Journal of Plant Pathology (2010), 92(1), 267-271

At 40 dpi, a roughly equal proportion of double- and single-infected plants were detected in the case of PPV-Rec/PPV-M combinations (26 vs 31 plants), whereas for PPV-Rec/PPV-D, this proportion was 25 vs 43 plants. After co-inoculation of BOR-3 and VAR-2 (PPV-Rec/PPV-M), both isolates were detected in all plants (Table 2). However, the PPV-D isolate BOJ-3 was never detected after co-inoculation with PPV-Rec isolates BOR-3 or BULG (Table 3), although BOJ-3 was readily transmitted to N. benthamiana in single inoculations.

Results showed that two distinct PPV isolates could co-infect a N. benthamiana plant up to 40 dpi. If a double infection became established and was detected 21 dpi, both isolates persisted up to 40 dpi, without displacing one another. Moreover, the coexistence of BOR-3/BOR-1 and BOR-3/VAR-2 was certified up to 110 dpi (data not shown). Only in two cases (plant No. 10 from RecHoromerice/MCAH-2 and plant No. 3 from Rec BOR-3/MKr-4 combination), the assay performed 40 dpi disclosed the emergence of the second isolate which had not been detected 21 dpi. This is likely due to the low concentration of the second isolate at the time of the first detection assay.

Dietrich and Maiss (2003) showed the existence of a spatial separation of PPV population in mixed infections, for only a few cells were double-infected. When only one isolate is repeatedly detected in a double-infected host, this may be consequent to the likely displacement of the second isolate in the early stage of infection due to the more rapid invasion of healthy tissues by the first isolate, although virulence and ability to persist in mixed infection are obviously not in direct connection with the speed of systemic movement (Šubr et al., 2006).

Occurrence of mixed PPV infections has been reported in nature and the coexistence of different viral isolates in Prunus hosts has been experimentally proven (Candresse et al., 1998; Myrta et al., 1998; Szemes et al., 2001; Capote et al., 2006; Kollerová et al., 2006; Kajic et al., 2008). As shown in our experiment, a stable balanced inter-strain mixed infection was established in about 40% of inoculated N. benthamiana plants. Such persisting mixed infections may not be equally common in Prunus trees or, if yes, they are detectable with difficulty because of the uneven virus distribution within the tree. Moreover, complete displacing of one PPV population by another in double-inoculated Prunus trees may take place. Based on the present results, we cannot draw definite conclusions on strain-specific behaviour of PPV in N. benthamiana in single infections (e.g. degree of aphid transmissibility, symptom expression on woody indicators) within PPV-Rec.

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>Rec+D/Expt</th>
<th>Rec+D/Expt</th>
<th>Rec+D/Expt</th>
<th>Rec+D/Expt</th>
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n.t. = not tested (plant death not due to virus infection)
ACKNOWLEDGEMENTS

We thank Dr G. Labonne (INRA Montpellier, France) for the generous gift of the Dideron isolate and the AS196 PPV antiserum. This work was supported by the grants APVV-51-0402-07 from Slovak Research and Development Agency and 2/0027/09 from the Scientific Grant Agency of Ministry of Education and Slovak Academy of Sciences.

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Received July 21, 2009
Accepted November 16, 2009

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