SHORT COMMUNICATION

COMPETITIVENESS OF DIFFERENT *PLUM POX VIRUS* ISOLATES IN EXPERIMENTAL MIXED INFECTION REVEALS RATHER ISOLATE- THAN STRAIN-SPECIFIC BEHAVIOUR

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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 stone-fruit 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).

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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 tisues. 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

Table 1. PPV isolates used in this study.

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

(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 4ºC 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 synthetised 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,

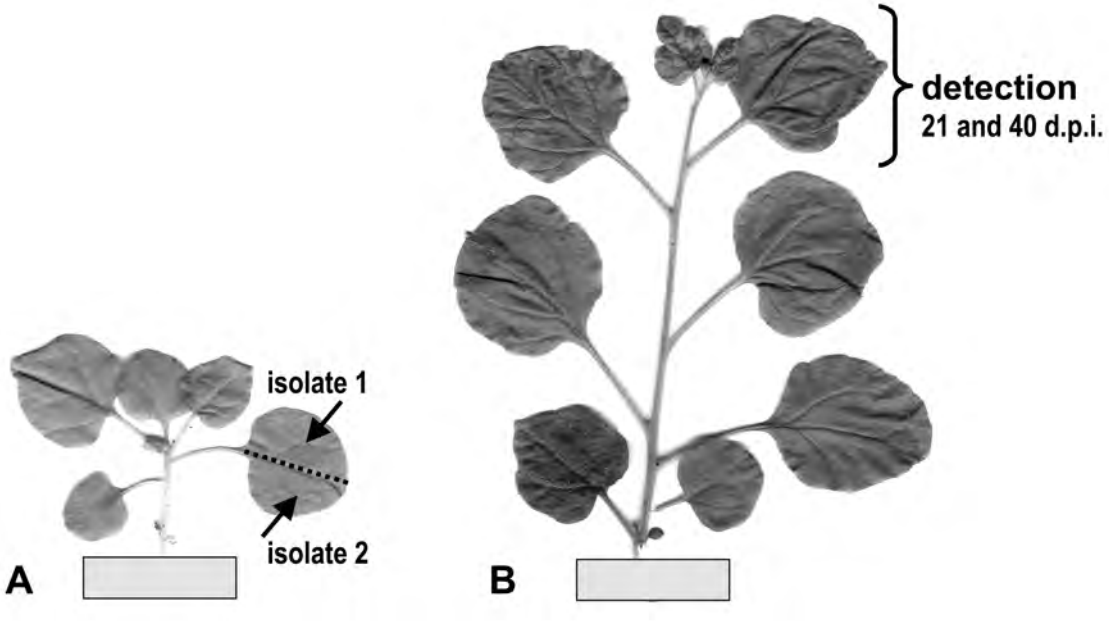

Fig. 1. Inoculation of *N. benthamiana* plants and assessment of infection.

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

	$Rec^{Horomerice}/M^{CAH-2}$		Rec^{BULG}/M^{CAH-2}		Rec^{BOR-3}/M^{Kr-4}		Rec^{BOR-3}/M^{VAR-2}	
Plant no.	21 dpi	40 dpi	21 dpi	40 dpi	21 dpi	40 dpi	21 dpi	40 dpi
1	Rec	Rec	M	M	M	M	Rec+M	Rec+M
2	Rec+M	Rec+M	Rec	Rec	M	M	Rec+M	Rec+M
3	Rec	Rec	Rec+M	Rec+M	M	Rec+M	Rec+M	Rec+M
4	Rec	Rec	Rec	Rec	M	M	Rec+M	n.t.
5	Rec	Rec	Rec	Rec	M	M	Rec+M	Rec+M
6	Rec	Rec	Rec+M	Rec+M	Rec+M	Rec+M	Rec+M	Rec+M
7	Rec+M	Rec+M	Rec+M	Rec+M	M	M	Rec+M	Rec+M
8	Rec	Rec	Rec	Rec	M	M	Rec+M	Rec+M
9	Rec+M	Rec+M	Rec	Rec	M	M	Rec+M	Rec+M
10	Rec	Rec+M	Rec+M	Rec+M	M	M	Rec+M	Rec+M
11	Rec	Rec	Rec	Rec	M	M	n.t.	n.t.
12	Rec	Rec	Rec+M	Rec+M	M	M	Rec+M	Rec+M
13	Rec	Rec	Rec	Rec	M	M	Rec+M	Rec+M
14	n.t.	n.t.	Rec	Rec	M	M	Rec+M	Rec+M
15	Rec+M	Rec+M	Rec+M	Rec+M	M	M	Rec+M	Rec+M
Rec	10	9	8	8	0	0	0	0
M	0	0	1	1	14	13	0	0
Rec+M	4	5	6	6	1	2	14	13

n.t. = not tested (plant death not due to virus 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 (Šubr *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, ensured massive virus transfer to the plants and eliminated such a bias. Furthermore, placing separate inocula on the opposite halves of the same leaf provided an equal opportunity for infection for each of tested PPV isolates.

Overall, four combinations of PPV-Rec vs PPV-M (Horomerice/CAH-2, BULG/CAH-2, BOR-3/Kr-4, BOR-3/VAR-2) and five combinations of PPV-Rec vs. PPV-D (BOR-3/BOR-1, BOR-3/Dideron, BOR-3/BOJ-3, BULG/BOJ-3, Horomerice/Dideron) were analysed. Regardless of the isolate combination tested, no aggravation of symptoms nor growth reduction were observed in double-infected in comparison with single-infected plants.

As shown in Table 2 and 3, a relatively frequent occurrence of double infections was found in the experi-

Table 3. Persistence of PPV isolates in *N. benthamiana* after double-inoculation with different combinations of PPV-Rec and PPV-D.

	Rec^{BOR-3}/D^{BOR-1}		$Rec^{BOR-3}/D^{Dideron}$		Rec^{BOR-3}/D^{BOJ-3}		Rec^{BULG}/D^{BOJ-3}		$Rec^{Horomerice}/D^{Dideron}$	
Plant no.	21 dpi	40 dpi	21 dpi	40 dpi	21 dpi	40 dpi	21 dpi	40 dpi	21 dpi	40 dpi
1	D	D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	Rec+D	Rec+D
2	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
3	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
4	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	n.t.	D	D
5	D	D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
6	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
7	Rec+D	n.t.	D	D	Rec	Rec	Rec	Rec	D	D
8	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
9	Rec+D	Rec+D	Rec	Rec	Rec	Rec	Rec	Rec	D	D
10	Rec+D	Rec+D	Rec+D	Rec+D	Rec	Rec	Rec	Rec	D	D
11	Rec+D	Rec+D	Rec+D	Rec+D			Rec	Rec	D	D
12	Rec+D	Rec+D	Rec+D	Rec+D			Rec	Rec	D	D
13	Rec+D	Rec+D	Rec+D	Rec+D			Rec	Rec	D	D
14	Rec+D	Rec+D	Rec	Rec			Rec	Rec	D	D
15	Rec+D	Rec+D	Rec+D	Rec+D			Rec	Rec	D	D
Rec	0	0	2	2	10	10	15	14	0	0
D	2	2	1	1	0	0	0	0	14	14
Rec+D	13	12	12	12	0	0	0	0	1	1

n.t. = not tested (plant death not due to virus infection)

ments. 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 $Rec^{Horomerice}/M^{CAH-2}$ and plant No. 3 from Rec^{BOR-3}/M^{Kr-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 (Capote *et al*., 2006; Šubr *et al*., 2006).

Based on the present results, we cannot draw ultimate conclusions on a clear-cut strain-specific behaviour of PPV isolates in term of competitiveness in mixed infection in *N. benthamiana*. This is, however, in line with previous observation (Glasa *et al*., 2004a, 2004b) that registered intra-strain biological variability (e.g. degree of aphid transmissibility, symptom expression on woody indicators) within PPV-Rec.

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