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Distribution of coat protein and nucleic acid of *Plum pox virus* (PPV) in seedlings of peach rootstock GF305 and apricot cv. Real Fino

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Summary. In this work, the in-plant distribution of *Plum pox virus* (PPV) in seedlings of peach rootstock GF305 and apricot cv. Real Fino was studied. Symptom severity and the optical density with the ELISA-DASI test were determined in the leaves. Occurrence of the virus (coat protein and nucleic acid) in the stems and petioles was analyzed by tissue printing of sections. The in-plant distribution of PPV was very irregular in both species, even in severely infected plants. It was also more erratic in seedlings showing an irregular distribution of symptoms, mainly in apricot. A correlation between occurrence of symptoms and localization of coat protein and nucleic acid was also observed. The irregular distribution of the virus, as regards both coat protein and nucleic acid, has negative implications for PPV resistance evaluation, since it renders virus detection and secure inoculation more difficult.

Key words: *Prunus persica*, *Prunus armeniaca*, *Plum pox virus*, sharka, tissue printing.

Introduction

Plum pox virus (PPV, Potyvirus, Potiviridae), sharka disease, is characterized by an irregular in-plant distribution throughout its infected natural host plants, particularly *Prunus* species (Albrechtova, 1986; Audergon *et al.*, 1989). This irregular distribution in the plant has negative implications for virus detection, since it means that plants which are really infected may appear healthy on tests (Marenaud and Yürektürk, 1974; Desvignes, 1976), so that particular sampling techniques are required (Morvan and Castelain, 1976).

Diseases and viruses in plants have traditionally been detected by visual symptom observation and by ELISA (Marenaud and Yürektürk, 1974; Desvignes, 1976; Morvan and Castelain, 1976; Albrechtova, 1986; Audergon *et al.*, 1989). More recently, other molecular techniques such as dot-blot hybridization and the PCR have been developed. These molecular techniques are reported to be more sensitive (Varveri *et al.*, 1987; Wetzel *et al.*, 1991; Adams *et al.*, 1999), but they are also more laborious and expensive.

Plum pox virus in tissues are localized by immuno-printing in nitrocellulose membranes using polyclonal (Albrechtova, 1986; Dicenta and Audergon, 1995; Dicenta *et al.*, 2000) or monoclonal antibodies (Cambra *et al.,* 1994a). These techniques normally localize structural coat protein (Cambra *et al.*, 1994a; Dicenta and Audergon, 1995; Knapp *et al.*, 1995; Dicenta *et al.*, 2000), and sometimes non-structural proteins like inclusion proteins (Adamolle and Quiot, 1993). Localization of nucleic acid for other viruses has been attempted using

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radioactive probes (Hsu and Lawson, 1991; Tet-Fatt and Yang-Sun, 1992) and, more recently, digoxigenin-labelled probes (Más and Pallás, 1995; Pallás *et al.*, 1997), but, so far, to our knowledge, not for *Plum pox virus*.

In this work, the in-plant distribution of the coat protein and the nucleic acid of *Plum pox virus* was studied in the leaves, petioles and stems of seedlings of peach rootstock GF305 and apricot cv. Real Fino.

Materials and methods

Plant material

Seedlings of peach rootstock GF305, characterized by their susceptibility to fruit viruses including *Plum pox virus* (Bernhard *et al.*, 1969) and usually used as a rootstock in PPV resistance tests on *Prunus* both *in vivo* (Gabova, 1994; Martínez-Gómez and Dicenta 2000a) and *in vitro* (Martínez-Gómez and Dicenta, 2000b), were used. Also used were seedlings of apricot cv. Real Fino, reported to be susceptible to *Plum pox virus* by Martínez-Gómez and Dicenta (2000a) and previously used as a rootstock in PPV resistance tests (Martínez-Gómez and Dicenta, 1999).

Both GF305 and Real Fino seedlings were grown in 2-litre pots in an insect-proof greenhouse. Three-month-old seedlings were inoculated by grafting a chip from a herbaceous GF305 individual showing strong sharka symptoms. Two months after inoculation, seedlings were subjected to an artificial rest period in a cold chamber at 7°C, in darkness for six weeks. They were then transferred to the greenhouse and were inspected for sharka symptoms two months later. Two plants per cultivar, one with regular and the other with irregular distribution of symptoms (Fig. 1 and 2), were chosen for study.

PPV Isolate

The PPV isolate was RB3.30, a Dideron-Type isolate collected from 'Red Beauty' plum trees at the Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain, and considered representative of the Spanish *Plum pox virus* population (Asensio, 1996).

Symptom evaluation

Symptoms in the leaves were scored on a scale from 0 (no symptoms) to 5 (maximum severity), as usual in resistance tests in apricot (Martínez-Gómez and Dicenta, 1999, 2000a).

ELISA-DASI

To ascertain the presence or absence of *Plum pox virus*, an ELISA-DASI (Double Antibody Sandwich Indirect) assay was applied to the leaves using the 5B monoclonal antibody against the coat protein of PPV in accordance with the protocol of Cambra *et al.* (1994b). Micro-plates (Wells®, Wells, Madrid, Spain) were incubated for 2 h at 37°C with polyclonal rabbit antibodies (1.42 µg/ml in 1%, w:v) (Real-Durviz, Valencia, Spain) and Carbonate buffer (0.159% Na_2CO_3, 0.293% $NaHCO_3$, pH 9.6). Virus extractions were carried out using 1 g of leaf in 5 ml of extraction buffer [2 g Dieca (Sigma, Madrid, Spain) and 20 g PVP-10 (Sigma) in 1000 ml PBS (0.08% NaCl, 0.002% KH_2PO_4, 0.3% Na_2HPO_4 $12H_2O$, 0.02% KCl, pH 7.4)]. Samples were incubated for 16 h at 5°C. After washing 3x5 min with PBS-Tween-20 (0.5 ml/l Tween-20), micro-plates were incubated for 2 h at 37°C in 1% (w:v) Bovine Serum Albumin (BSA, Roche Diagnostics, Mannheim, Germany)-PBS with the specific monoclonal antibodies (0.1 mg/ml) (Real-Durviz). After washing 3 times with PBS-Tween-20, samples were incubated in 1% (w:v) BSA-PBS with an alkaline phosphatase-labelled second antibody (0.1 µg/ml) (Real-Durviz) at 37°C for 2h. Then the micro-plates were washed again and developed with a p-nitrophenol-phosphate colorimetric substrate (Sigma), recording the optical densities (OD) at 405 nm after 60 min. In accordance with Sutula *et al.* (1986), samples with OD double that of the healthy control were considered ELISA-positive.

Coat protein and nucleic acid localization

Two sections in each petiole, 5 mm apart, and ten sections in each stem 5 mm apart (five above and five below the petiole junction) were analyzed. In each section two different cuts about 1 mm apart were made and printed onto nitrocellulose (BA85, Schleicher & Schuell, Munich, Germany) and nylon (Roche Diagnostics,) membranes for localization of PPV coat protein and nucleic acid respectively.

The nitrocellulose membranes were subjected to the protocol described by Cambra *et al.* (1994a), with minor changes. The prints were blocked with 3% (w:v) BSA-PBS. After washing with PBS-Tween-20 for 3x5 min the membranes were incubated in 0.5% (w:v) BSA-PBS for 2 h at 37°C with the same monoclonal antibody as that used in the ELISA-DASI (0.3 µg/ml). After washing 3 times

with PBS-Tween-20, membranes were incubated in 0.5% (w:v) BSA-PBS with an alkaline phosphatase-labeled second antibody (0.3 µg/ml) at 37°C for 2 h. Then they were washed again for 3x5 min.

For nucleic acid localization, the first step was the synthesis of the pBppv1 probe developed by Varveri *et al*. (1987), which was labelled with digoxigenin using PBScrip plasmids and T_7 RNA polymerase as described in Pallás *et al*. (1997). Nylon membranes were subjected to the modified protocol described by Más and Pallás (1995) for *Apple chlorotic leaf spot virus*. The prints were fixed with UV light for 3 min. The membranes were prehybridized, at 68°C, for 2 h, in a hybridization solution [50% deionized formamide, 5xSSC, 2% (w:v) blocking reagent (Roche Diagnostics), 0.1% (w:v) N-lauroylsarcosine, 0.01% (w:v) SDS, pH 7.0]. Hybridization of the digoxigenin-labelled probe to the membranes was carried out for 16 h at 68°C. Membranes were washed for 2x5 min at room temperature after hybridization in washing buffer I [2xSSC, 0.1% (w:v) SDS, pH 7.5], and for 2x15 min at 68°C in washing buffer II [0.1xSSC, 0.1% (w:v) SDS, pH 7.5].

Both the nitrocellulose and the nylon membranes were developed with BCIP/NBT, reagent (Roche Diagnostics) colorimetric substrate, which reacts with alkaline phosphatase in the coat protein localization, and with specific antibodies of digoxigenin conjugated to alkaline phosphatase in nucleic acid localization. The specific dark blue color developed after about 15 min. Lastly, the membranes were dried and the presence or absence of reactions was determined by light microscopy (x40–100 magnification).

Results

Symptom evaluation and ELISA-DASI tests

Figure 1 and 2 summarize symptom severity and OD of the ELISA-DASI test on the leaves of GF305 and Real Fino seedlings. GF305 seedlings showed greater symptom severity, with a mean value of 2.2 and a maximum of 4, than Real Fino apricot seedlings (1.3 and 2).

Similarly, ODs were higher for GF305 (between 0.70 and 2.61 in the positive ELISA-DASI samples, average 1.28), than for Real Fino apricot (between 0.46 and 1.25, average 0.82).

For GF305, there was good correlation between expression of symptoms and ELISA-DASI in the leaves, with generally a higher OD in the leaves with stronger symptoms. In Real Fino, this correlation was observed in the seedling with a regular distribution of symptoms. However, in the seedling with an irregular distribution of symptoms the correlation between manifestation of symptoms and OD was lower. In this seedling, one leaf, without symptoms, was ELISA-DASI positive, while another, with weak symptoms (severity score 1), was negative.

Coat protein and nucleic acid localization

Figure 1 and 2 and Table 1 summarize the presence or absence of both components of *Plum pox virus*, coat protein and nucleic acid, in the stem and petiole tissues of GF305 and Real Fino seedlings.

An irregular distribution of both components of PPV was observed in the stem and petiole sections GF305 and Real Fino seedlings even when seedlings were severely infected and with symptoms regularly distributed throughout. In general, there

Table 1. Percentage of sections in which PPV coat protein, nucleic acid (RNA) or both simultaneously were localized in the petioles and stems of peach rootstock GF305 and apricot cv. Real Fino seedlings with regular and irregular distribution of symptoms.

Rootstock/ cultivar	Distribution of symptoms	Petiole			Stem		
		Coat +	RNA +	Coat + and RNA +	Coat +	RNA +	Coat + and RNA +
GF305	Regular	33	83	33	40	53	37
	Irregular	50	67	50	27	40	10
Real Fino	Regular	100	67	67	53	67	37
	Irregular	33	33	33	27	30	20

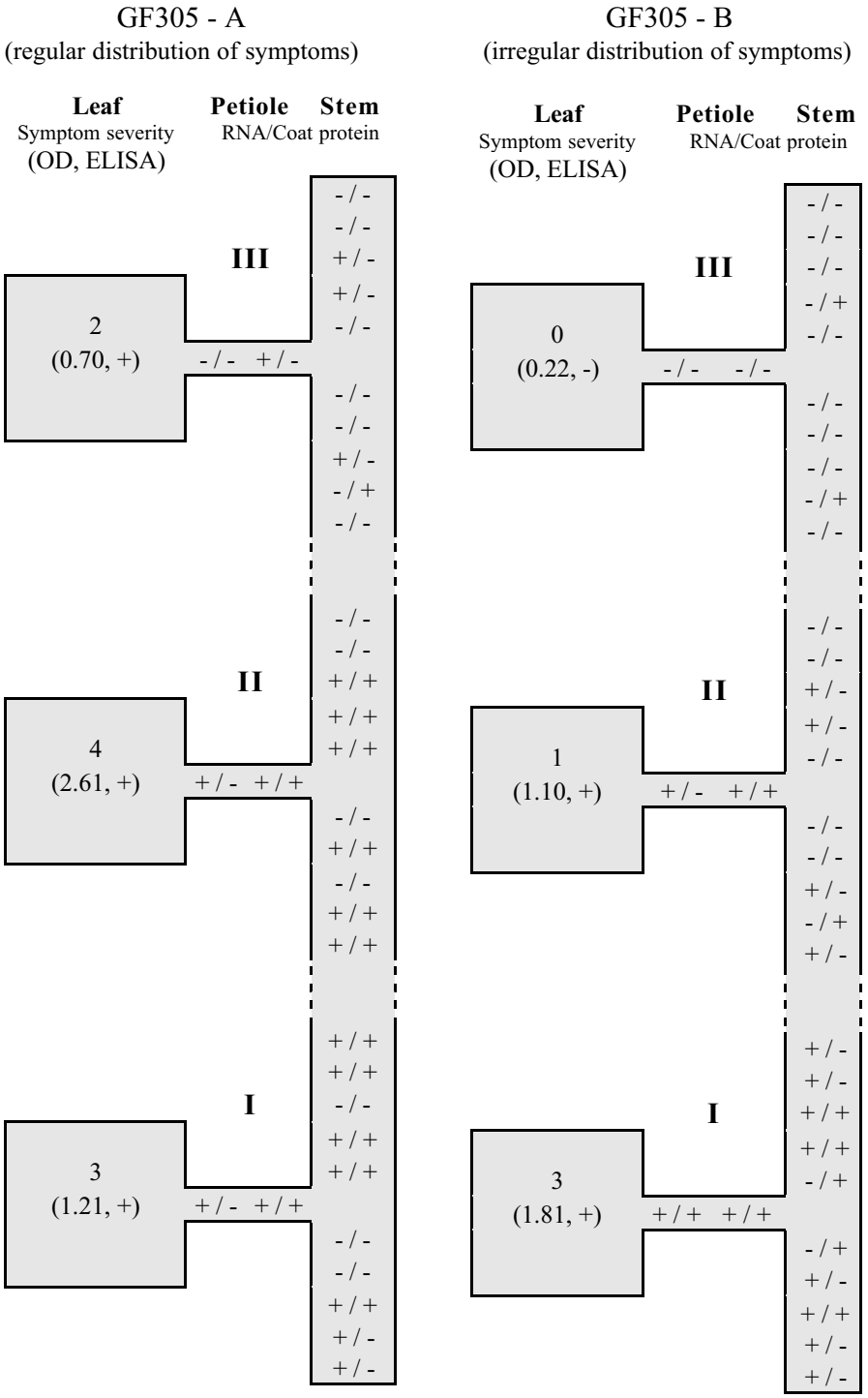

Fig. 1. Schematic representation of stems, petioles and leaves in 3 portions of the seedling (I, II, and III) showing leaf symptom severity (scale from 0 to 5) and ELISA-DASI test (optical densities at 405 nm) in the leaves, in relation to nucleic acid and coat protein localization in petiole and stem sections of peach rootstock GF305 inoculated with PPV. Seedling A with regular distribution of symptoms, and seedling B with irregular distribution of symptoms are compared. OD of ELISA-DASI in control seedlings = 0.25.

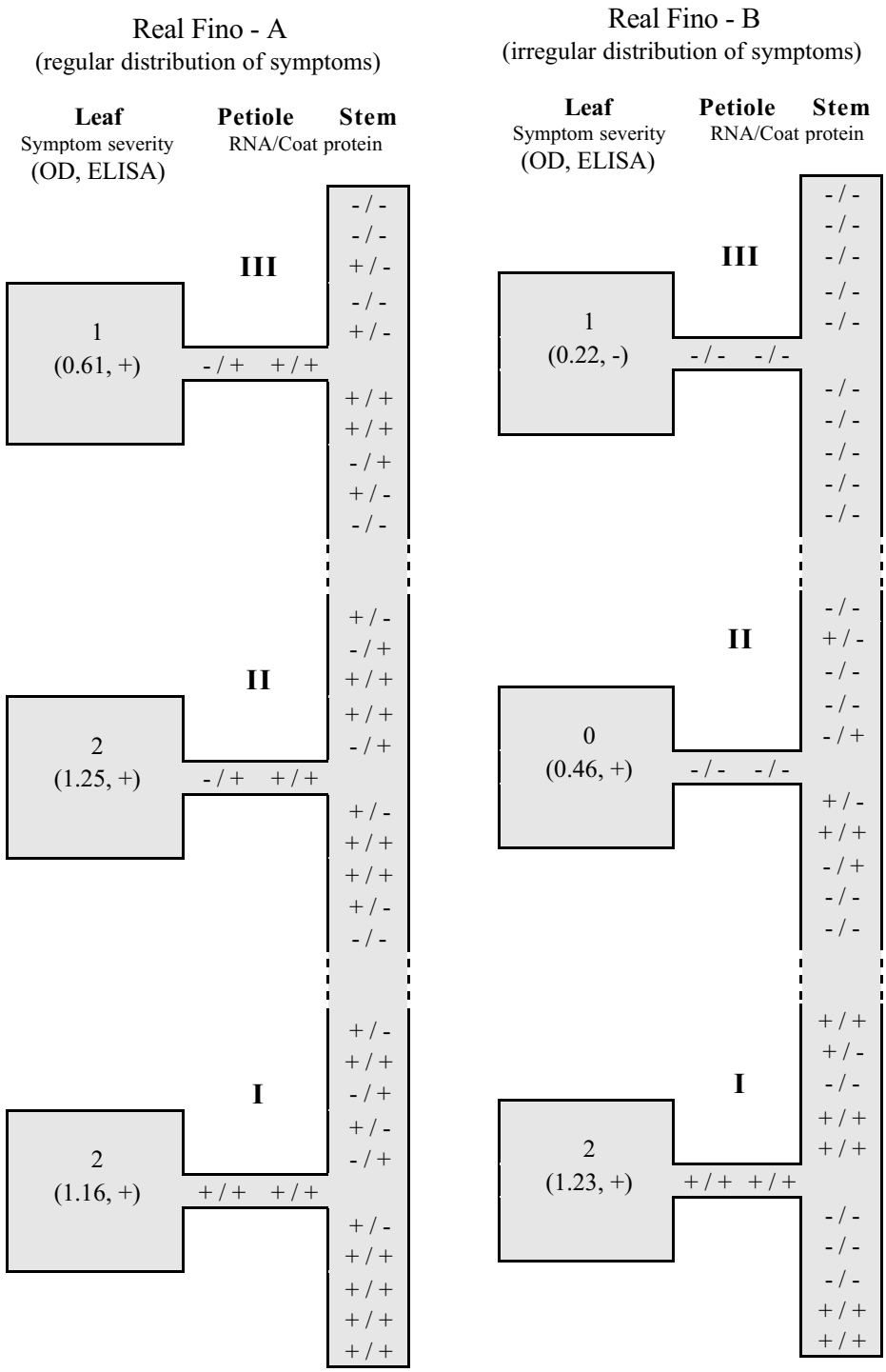

Fig. 2. Schematic representation of stems, petioles and leaves in 3 portions of the seedling (I, II, and III) showing leaf symptom severity (scale from 0 to 5) and ELISA-DASI test (optical densities at 405 nm) in the leaves, in relation to nucleic acid and coat protein localization in petiole and stem sections of apricot cv. Real Fino inoculated with PPV. Seedling A with regular distribution of symptoms, and seedling B with irregular distribution of symptoms are compared. OD of ELISA-DASI in control seedlings = 0.20.

was correlation between symptom severity, the OD of ELISA-DASI in the leaves, and the occurrence of coat protein and nucleic acid in the stems and petioles of GF305 and Real Fino seedlings. The distribution of coat protein and nucleic acid between sections was more regular in seedlings with greater symptoms severity and a higher OD in the leaves (Fig. 1 and 2).

An irregular PPV distribution was more evident in seedlings with an irregular distribution of symptoms. In GF305 seedlings with a regular distribution of symptoms, the virus (coat protein or nucleic acid) was observed in 21% of the petiole and stem sections of the upper third of the plant (part III), where symptoms were weaker, and in 63% of sections in the other two-thirds (parts I and II), where symptoms were stronger. GF305 seedlings with an irregular distribution of symptoms showed PPV in 8% of sections in the upper third of the plant, with no symptoms, but in 52% of sections in the other two parts with symptoms (Fig. 1).

In Real Fino, differences in PPV in-plant distribution between seedlings with regular and irregular distribution of symptoms were greater. In seedlings with a regular distribution of symptoms, the virus (coat protein or nucleic acid) was detected in 46% of petiole and stem sections in the upper third of the plant (weaker symptoms), and in 73% of sections in the other two parts (stronger symptoms). By contrast, seedling with an irregular distribution of symptoms showed no PPV in the upper third of the plant but PPV was detected in 43% of sections in the other two parts (Fig. 2).

Nucleic acid was localized in a greater percentage of seedling sections than was coat protein. In addition, PPV (coat protein and nucleic acid) occurred more often in petiole sections than in stem sections, for both GF305 and Real Fino (Table 1).

As regards correlation in the localization of coat protein and nucleic acid, both PPV components were localized in 42% of GF305 and 50% of Real Fino petioles, and in 23% of GF305 and 28% of Real Fino stems (Table 1).

Discussion

The greater symptom severity and OD with ELISA-DASI on GF305 peach demonstrated that there was a greater accumulation of PPV in this cultivar than in Real Fino apricot.

This greater susceptibility of GF305 compared with apricot has been fully reported by authors using various PPV resistance tests *in vivo* (Martínez-Gómez and Dicenta, 2000a) and *in vitro* (Martínez-Gómez and Dicenta, 2000b). Lower OD values in apricot cultivars than in GF305 were also reported in other PPV detection tests (Cambra *et al.*, 1994b) and in PPV resistance tests (Martínez-Gómez and Dicenta, 2000a). Various studies on coat protein localization likewise reported higher levels of PPV in GF305 tissues than in apricot (Dicenta and Audergon, 1995; Knapp *et al.*, 1995).

These results confirm the value of the GF305 rootstock to test for PPV resistance in *Prunus*, as already reported in other studies (Gabova, 1994; Martínez-Gómez and Dicenta 2000a; 2000b), and show that it is more suitable than Real Fino, used as a rootstock in some tests (Martínez-Gómez and Dicenta, 1999).

The low correlation between symptom expression and OD in the Real Fino seedling with irregular distribution of symptoms shows the importance of using an appropriate sampling procedure when testing for PPV in this species.

The localization techniques showed an irregular distribution of this PPV (coat protein and nucleic acid) in the petioles and stems of both GF305 and Real Fino. They also confirmed the low effectiveness of printing techniques in detecting PPV, as reported by Cambra *et al.* (1994a). An irregular PPV in-plant distribution was also reported in coat protein localization tests on apricot (Dicenta and Audergon, 1995; Knapp *et al.*, 1995; Dicenta *et al.*, 2000).

The severely infected Real Fino seedling showed a more regular PPV distribution, but in lower concentrations than GF305. However, less severely infected Real Fino seedlings with an irregular manifestation of symptoms had a much more irregular distribution of PPV than equivalent GF305 seedlings. Consistent with these results, Desvignes (1988) reported that PPV distribution was more regular in GF305 seedlings than in a number of apricot cultivars. An irregular virus in-plant distribution was also observed in both GF305 and Real Fino seedlings inoculated with *Chlorotic leaf spot virus* and *Prunus necrotic ring spot virus* (Cambra *et al.*, 1986).

In general, nucleic acid was detected more frequently than coat protein in seedling sections. This may indicate that dot-blot hybridization is a more

sensitive localization technique than serological localization (coat protein), as stated by Sánchez-Navarro *et al.* (1996) on *Carnation mottle virus*. The greater frequency of PPV coat protein and nucleic acid in petioles than in stem sections of both GF305 and Real Fino is also reported by other studies on coat protein localization in apricot cultivars (Dicenta and Audergon, 1995; Knapp *et al.*, 1995).

The lack of correlation between the protein and the nucleic acid localization data was due to the erratic in-plant distribution of the virus, perceptible even though the analyzed sections used for the localization of these components were very close together (1 mm apart). This gives an idea of the extremely irregular distribution of PPV in tissues even over minute distances.

This irregular distribution of PPV represents an additional obstacle to determining the resistance of *Prunus* species, which can only be overcome by exhaustive sampling methods including both symptom observation and virus detection of the plants to be tested.

An irregular in-plant distribution of PPV also affects artificial inoculation by chip grafting. Successful inoculation depends on the virus occurring in the bark chip used as the inoculum source. The use of bigger chips close to more severely infected leaves presumably increases the likelihood of the chip containing PPV for purposes of inoculation. As regards selection of an inoculum source, PPV distribution was more uniform in severely infected Real Fino seedlings with good symptom expression, than in GF305. Nevertheless, the use of GF305 as inoculum is more interesting, according to the results obtained here, because PPV concentrations were higher in that rootstock than in Real Fino.

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