EFFICIENCY OF INOCULATION OF PEACH GF305 SEEDLINGS WITH *PLUM POX VIRUS* BY DIFFERENT METHODS

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Summary. – Peach GF305 is frequently used as rootstock in experiments to evaluate the resistance of different species of *Prunus* to *Plum pox virus* (PPV) because of its extreme susceptibility. However, transmission of PPV in *Prunus* species is sometimes problematic due to its low concentration or uneven distribution in these species. To determine the most effective way of transmitting the virus, different infection methods (by aphids, grafting, mechanical infection and injection) were tested using Dideron PPV isolates. The most effective method was the grafting of herbaceous material with inoculum derived from similar herbaceous material. Infection by aphids was more laborious and less effective than grafting, showing many disadvantages. Neither mechanical infection nor injection transmitted the virus.

Key words: sharka; Plum pox virus; GF305 peach; Prunus; aphids; grafting; mechanical infection; inoculation

Introduction

Peach GF305 was selected in the sixties by INRA (Grande Ferrade Research Station, Bordeaux, France). As rootstock it is little used despite its good vigour and the fact that the scion comes early in bearing (Lichou and Audubert, 1992). It is very susceptible to a large number of woody plant viruses including PPV (sharka disease) (Bernhard *et al.*, 1969), and for this reason it is frequently used in quarantine systems to detect fruit tree viruses such as PPV (Waterworth, 1994). For the same reason it is used as a susceptible rootstock in experiments to evaluate the PPV resistance of different *Prunus* species, and also as material for preserving and multiplying isolates or as inoculum source (Audergon and Morvan, 1990; Gabova, 1994; Martínez-Gómez and Dicenta, 1999, 2000a, 2000b).

In resistance evaluation, correct inoculation of peach GF305 with PPV is essential to prove the presence of the virus in the plants to be evaluated. Some authors (Materrazzi *et al.*, 1991; Martínez-Gómez and Dicenta, 1999) have mentioned difficulties in inoculating the peach GF305 plants. Possible reasons included the low concentration and irregular distribution of the virus in *Prunus*, both in the plant and in the tissues (Albrechtova, 1986; Dicenta and Audergon, 1995a,b), the negative effect of high temperature or the influence of vegetative state of the plants on virus multiplication (Hubert *et al.*, 1988).

In natural conditions in the field, PPV is transmitted by aphids (Kunze and Krozal, 1971; Labonne and Lauriot, 1991; Avinent *et al.*, 1994) and although this method was used in the evaluation of resistance, the obtained results were not conclusive (Gabova, 1994). In evaluation trials in general, the main method used is grafting of a chip or a chip-bud from a diseased peach GF305 (Audergon and Morvan, 1990; Martínez-Gómez and Dicenta, 1999), although more efficient and easy methods would be appreciated when a large number of plants have to be inoculated. In this sense, injection of sap (Boyé and

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Abbreviations: DASI-ELISA = double-antibody indirect sandwich enzyme-linked immunosorbent assay; PPV = *Plum pox virus*

Desvignes, 1986; Desvignes, 1997) and mechanical inoculation (Materrazzi *et al.*, 1991) have been tested with a smaller success.

The aim of the present study was to compare the effectiveness of different inoculation methods applicable to peach GF305 seedlings in order to find the most suitable one for routine PPV resistance evaluation assays.

Materials and Methods

Plant material. Peach GF305 seedlings were obtained from seeds of the trees growing in the experimental field station of the Servicio de Investigaciones Agrarias (SIA), Zaragoza, Spain or from INRA-UGAFL, Avignon, France. Seedlings of *Nicotiana benthamiana*, which can accumulate large amounts of PPV (Kamenova, 1990), were used as control. Seedlings of the susceptible apricot Real Fino were used as inoculum source in grafting inoculation assays.

PPV isolates. Two PPV isolates, both of the Dideron type, namely Laverune (in assays of PPV transmission by aphids in the INRA, Avignon, France) and RB3.30 (in other assays in the CEBAS-CSIC, Murcia, Spain) were used.

Infection by aphids was carried out using *Myzus persicae* (Sulzer), which were bred in laboratory on pepper plants. The aphids were first kept without feeding for 1 hr and then were placed onto young leaves of three-month-old peach GF305 showing strong PPV symptoms for 2 mins (the acquisition phase). Later, these viruliferous aphids were placed onto three-month-old peach GF305 to be inoculated for 2 hrs (the transmission phase). In each treatment, 1, 3, 5, 7, and 10 aphids were placed onto each plant. 100 seedlings in the first treatment and 20 seedlings in the rest of treatments were assayed. Afterwards aphids were killed by a treatment with Peropal® (Azociclotim 25%) before putting them back into an insect-proof greenhouse.

Infection by chip, chip-bud and approach grafting. Three months after sowing peach GF305 stones in pots, when the stems measured about 5 mm in diameter, the seedlings were inoculated by grafting a piece of bark (chip) or bark with a bud (chip-bud) from diseased GF305 plants showing strong sharka symptoms. In the case of approach grafting the stems of diseased and healthy GF305 plants were brought into contact after their barks had been stripped. Twenty plants were inoculated in each treatment.

Mechanical infection. Two months after sowing peach GF305 stones in pots, the stems of about 3 mm in diameter were mechanically infected with inoculum from leaves of infected peach GF305 or *N. benthamiana*. Four leaves per seedling were infected by using carborundum as abrasive and rubbing on each leaf 30 ml of an extract of the diseased tissue in the inoculation buffer (0.05 mol/l phosphate and 0.05% bentonite, pH 7) in a ratio of 1:2. Twenty plants were inoculated in each treatment.

Infection by injection. Stems of two-month-old peach GF305 were injected by a syringe in four different sites with an extract (1:2) from infected leaves of peach GF305 or *N. benthamiana* in the inoculation buffer supplemented with 2% polyvinylpyrrolidone following the protocol of Desvignes (1997). Twenty plants were inoculated in each treatment.

Optimization of infection by chip grafting. Other experiments were carried out to determine the effect of different factors on the efficacy of infection by chip grafting. The following factors were studied: The woody (four-months-old and about the stem with 8 mm in diameter) or herbaceous (two-months-old and about 3 mm in diameter) state of rootstock to be inoculated; woody or herbaceous nature of the chip-material used as inoculum source; species used as inoculum source (GF305 peach or Real Fino apricot) and number of chips used in inoculation (1, 2 or 3). By combining all these factors, 18 different inoculation treatments by chip grafting were carried out, using ten plants per treatment.

Optimization of inoculation by injection. Different types of peach GF305 tissues, namely primary roots from 1-week-old seedlings, hypocotyls from 1-week-old seedlings and stems from 1-month-old seedlings were injected with inoculum from a woody and herbaceous sources as described above. *N. benthamiana* plants were also used as control. Ten plants per treatment were used.

In all experiments, two months after inoculation, the peach GF305 plants were transferred to an artificial lethargy in a cold chamber at 7°C in darkness for 6 weeks. They were then transferred to an insect-proof greenhouse and the leaves of inoculated plants were inspected for symptoms 2 months later. A DASI-ELISA using PPV-specific monoclonal antibodies (Cambra *et al.*, 1994) was also applied to check the presence of the virus. Only in the virus inoculation by aphids, the DASI-ELISA was applied only sporadically to verify the presence or absence of the virus. The presence of symptoms and the DASI-ELISA positive for the virus were used as proof that the inoculation was effective. A statistical comparison of the ratio of the number of plants with symptoms to the total number of plants used was performed by the X^2 test (Mead *et al.*, 1993).

Results and Discussion

As expected, all the plants (GF305 and *N. benthamiana*) showing symptoms of sharka were always positive in DASI-ELISA and *vice versa,* demonstrating these two species as suitable sharka indicators.

Comparison of virus inoculation efficiency by aphids, grafting, mechanical inoculation and injection

The obtained results (Table 1) showed a low rate of transmission PPV to peach GF305 seedlings by aphids, namely 40% in the best condition (10 aphids per plant). These results are lower than those described in herbaceous plants (about 90%) by Morvan and Chastelliere (1980).

The use of aphid inoculation seems suitable in assays of natural transmission (Avinent *et al.,* 1994), but in the case of inoculation of a large number of plants in the resistance evaluation, its low effectiveness is an important handicap. Also, with this type of inoculation we introduce another factor, the interaction plant-vector-virus described by Audergon *et al.* (1995) which may affect the evaluation.

Table 1. Number of peach GF305 plants showing sharka symptoms after inoculation with PPV Laverune isolate of Dideron type by aphids

No. of aphids applied	No. of plants inoculated	No. of plants with symptoms	Percentage of plants with symptoms[1]
1	100	3	3 (b)
3	20	2	10 (b)
5	20	3	15 (a,b)
7	20	4	20 (a,b)
10	20	8	40 (a)

[1]The percentage values followed by the same letters in parentheses shows no significant difference by the X^2 test (P = 0.01).

According to these results certain number of aphids is requested to reach a good effectiveness of the method. Labonne and Lauriot (1991) reported 50,000–200,000 aphids in a tree during the fly phase of the aphids, with a rate of viruliferous aphids of 1/1,000. On the basis of these and our results, 30–50 aphids per plant could be requested to assess the inoculation. On the other hand, the inoculation by aphids shows many disadvantages because of laborious management of aphids. Also, there is an important risk in taking viruliferous aphids in the greenhouse and contaminate the rest of the trials.

Grafting was a more efficient method of virus inoculation (Table 2). Although there were no significant differences between the grafting methods used, the approach method was most effective (100% success rate), followed by chip (80%) and chip-bud grafting (70%). These results are comparable with those of Dosba *et al.* (1987) who obtained a 25%–100% success rate by chip grafting inoculation.

Table 2. Percentage of peach GF305 and *N. benthamiana* plants showing symptoms of sharka and reacting as PPV-positive in DASI-ELISA after inoculation with PPV RB3.30 isolate of Dideron type by different methods

Species	Inoculation method		Percentage of plants with symptoms and PPV-positive in DASI-ELISA[1]
Peach GF305	Grafting	Chip-bud	70 (a)
		Chip	80 (a)
		Approach	100 (a)
	Mechanical	Herbaceous inoculum	0 (c)
		Woody inoculum	0 (c)
	Injection	Herbaceous inoculum	0 (c)
		Woody inoculum	0 (c)
N. benthamiana	Mechanical	Herbaceous inoculum	90 (a)
		Woody inoculum	60 (a)
	Injection	Herbaceous inoculum	30 (b)

[1]The numbers followed by the same letters in parentheses show no significant differences by the X^2 test (P = 0.01).
Twenty repetitions per treatment.

The only reference available on PPV inoculation using the approach grafting concerned pea (*Pisum sativum*) and peach GF305 and Dideron type PPV isolates with the success rate of only 20% (Kerlan *et al.*, 1978). Such a low rate was probably due to the graft incompatibility of the species used.

Among the three graft methods, chips (bark) are a readily available source of virus inoculum. On the other hand, chip-buds are not, but it is possible to confirm the presence or absence of the virus when the buds sprout. Virus inoculum is even less available when the approach grafting is used, even though it seems to guarantee the infection.

The mechanical and injection inoculations were effective only in *N. benthamiana* plants; the inoculation by injection was less effective (30%) than the mechanical inoculation (75%) (Table 2). No peach GF305 plant was successfully infected using either inoculation.

The results of mechanical inoculation correspond to those of Bernhard *et al.* (1969) and Morvan and Chastelliere (1980) who considered mechanical inoculation with PPV suitable for herbaceous but not for woody plants. These results are in accord with those obtained by Materrazzi *et al.* (1991) who mentioned a great influence of physiological stage of peach GF305 on the efficiency of transmission; thus this method is not suitable for selection testing.

As regards inoculation by injection, Boyé and Desvignes (1986) and Desvignes (1997) succeeded to infect 10%–50% of peach GF305 plants using a Dideron type of PPV isolate, while they obtained a higher success rate (up to 100%) with a Marcus type of PPV isolate. Other inoculation methods such as placing papers moistened with extracts from diseased tissues below the bark gave negative results (Boyé and Desvignes, 1986).

Optimization of virus inoculation by chip grafting

These experiments showed the importance of the vegetative state of rootstocks on the effectiveness of inoculation; 83% of herbaceous but only 11% of woody rootstocks was successfully infected (Table 3). This low success rate in woody rootstocks might due to the increased lignin content of the cell wall; the latter may harden during lignification (Grisebach, 1977) and so may provide a barrier against pathogens including viruses (Vance *et al.*, 1980).

Within woody rootstocks, there was little difference between the herbaceous or woody state of the inoculum used (10 and 12% success rates, respectively) (Table 3). The woody inoculum was not used for young rootstocks because this grafting is not viable. Hardly any difference was observed between the success rates obtained using chips from diseased apricot stems (35.5%) and those from diseased peach GF305 stems (34.5%) (Table 3).

Finally, although inoculating with more chips increased the success rate of virus inoculation (30%, 35%, and 40%

Table 3. Percentage of peach GF305 plants showing symptoms of sharka and reacting as PPV-positive in DASI-ELISA after inoculation with PPV RB3.30 isolate of Dideron type by different methods of chip grafting

State of rootstock	State of inoculum	Type of inoculum	No. of chips grafted	Percentage of plants with symptoms and PPV-positive in DASI-ELISA[1]
Woody	Woody	GF305	1	0 (d)
			2	10 (c)
			3	30 (b)
		Real Fino	1	10 (c)
			2	10 (c)
			3	10 (c)
	Herbaceous	GF305	1	10 (c)
			2	0 (d)
			3	10 (c)
		Real Fino	1	10 (c)
			2	10 (c)
			3	20 (b, c)
Herbaceous	Herbaceous	GF305	1	80 (a)
			2	100 (a)
			3	70 (a)
		Real Fino	1	70 (a)
			2	80 (a)
			3	100 (a)

[1]The numbers followed by the same letters in parentheses show no significant differences by the X^2 test (P = 0.01).
Ten repetitions per treatment.

with 1, 2, and 3 chips, respectively), the improvement was not proportional to the extra effort required. For the best inoculation procedure (herbaceous rootstock – herbaceous chip), the success rates were 75%, 90%, and 85% with 1, 2, and 3 chips, respectively (Table 3).

Optimization of inoculation by injection

The results obtained in the experiments on optimization of the inoculation by injection (Table 4) showed that this type of inoculation with PPV isolate of Dideron type was effective only in plants of *N. benthamiana*. The injection in different types of peach GF305 tissues (primary roots from 1-week-old seedlings, hypocotyls from 1-week-old seedlings, and stems from 1-month-old seedlings) gave negative results.

Contrary to our results, the virus inoculation by injection was found successful by Boyé and Desvignes (1986), who obtained a 50% success rate; however, neither the number of plants nor the isolates assayed were specified. Using the same method, Desvignes (1997) managed to infect 10% of plants using a PPV isolate of Dideron type, while the success rate rose to 100% when a PPV isolate of Marcus type was used.

In conclusion, the most successful virus inoculation method for peach GF305 with PPV isolates of Dideron type is chip grafting of herbaceous peach GF305 with an inoculum also from herbaceous peach GF305 showing clear sharka symptoms. For a guaranteed virus inoculation the more time consuming approach grafting is recommended. The virus inoculation by aphids is not recommended for resistance evaluation purposes because of its low effectiveness and laborious performance.

Table 4. Percentage of peach GF305 and *N. benthamiana* plants showing symptoms of sharka and reacting as PPV-positive in DASI-ELISA after injection with PPV RB3.30 isolate of Dideron type by different methods

Species	Inoculum source	Type of injection in seedlings	Percentage of plants with symptoms and PPV-positive in DASI-ELISA[1]
GF305 Peach	Woody	Primary root	0 (b)
		Hypocotyl	0 (b)
		Stem	0 (b)
	Herbaceous	Primary root	0 (b)
		Hypocotyl	0 (b)
		Stem	0 (b)
N. benthamiana	Herbaceous	Stem	40 (a)

[1]The numbers followed by the same letter show no significant differences by the X^2 test (P = 0.01).
Ten repetitions per treatment.

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