Journal of Plant Pathology (2012), **94** (1, Supplement), S1.91-S1.96 *Edizioni ETS Pisa, 2012*

EVALUATION OF *XANTHOMONAS ARBORICOLA* pv. *PRUNI* INOCULATION TECHNIQUES TO SCREEN FOR BACTERIAL SPOT RESISTANCE IN PEACH AND APRICOT

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SUMMARY

Screening for resistance to bacterial spot of apricot (*Prunus armeniaca*) and other stone fruit species such as peach (*P. persica*) and plum (*P. domestica*), caused by the quarantine bacterium *Xanthomonas arboricola* pv. *pruni* requires an inoculation method which is rapid, efficient and which permits to differentiate levels of resistance to this disease. Four inoculation techniques were compared under greenhouse conditions: (i) infiltration in the leaves using a needleless syringe; (ii) injuries caused by a needle; (iii) injuries caused by scissors; (iv) dipping shoots of the plant into the inoculum. All inoculation techniques induced symptoms: infiltration was very efficient and quickly caused severe necrosis and leaf drop; the use of needle was also very efficient but the symptoms were very weak, while the scissors technique was not as efficient as the first two approaches and the symptoms were also very weak. Finally dipping peach shoots into the inoculum was the least efficient in symptom induction, but this technique permitted to obtain more quantitative data suitable for evaluating the level of resistance of a cultivar than all the other techniques (e.g. number of spots per leaf, or damaged surface, calculated disease incidence and resistance index). For this reason, the inoculation technique by immersion was applied to six apricot cultivars to be screened for resistance against bacterial spot. Plants were assessed 15, 28, 38 and 64 days post inoculation for spot number, leaf surface damaged, disease incidence and severity of symptoms. Cv. Orangered was the most resistant, showing low levels of damage to the leaves (10% +/- 3.54) and only a few symptomatic leaves per plant (17.39 +/- 0.56). Cv. Goldbar was the most susceptible with 76.74 +/- 19.39% of symptomatic leaves and high levels of damage per leaf (85 +/- 11.34%). Four other cvs., Bergarouge, Bergeron, Goldrich and Kioto showed intermediate levels of susceptibility to the bacterium. Results of resistance testing were comparable to those observed under field conditions as reported in the literature. This indicates that shoot immersion into the inoculum is an efficient and reliable technique to screen for resistance against bacterial spot of stone fruits under greenhouse conditions.

Key words: bacterial spot, *Prunus*, inoculation techniques, screening for resistance.

Xanthomonas arboricola pv. *pruni* (*Xap*), the causal agent of bacterial spot of stone fruits, is a quarantine pathogen for the European Union (EU) and the European Plant Protection Organisation (EPPO). It mainly causes necrotic lesions on leaves and fruits of peach, plum, apricot and nectarine, but in more severe cases, cankers, defoliations and death of the trees can be observed (du Plessis, 1988). This bacterium has been reported from all continents, and is still expanding its range (EPPO, 2006). In Europe, bacterial spot has been identified first in northern Italy in 1934 (Petri, 1934) where it is now considered as endemic (Battilani, 1999). The disease continues to spread in Europe and outbreaks have occurred more recently in France, Bulgaria, Romania and Ukraine (EPPO, 2006). In Switzerland the disease was detected in 2005 on apricot trees (Pothier *et al.*, 2010), and it is now considered as locally established.

Due to lack of efficient chemical or biological control methods, host resistance might be the most promising solution to combat the disease. Different levels of tolerance against *Xap* have been detected in peach, nectarine, apricot and plum based on field observations (Simeone, 1985; Topp *et al.*, 1989; Layne and Hunter, 2003; Garcin *et al.*, 2005; Garcin and Bresson, 2011). The availability of an artificial inoculation technique allowing evaluations in a standardized quantitative manner of the degree of susceptibility or resistance (tolerance) of different cultivars under greenhouse conditions may foster the selection or identification of resistant or tolerant cultivars. Few techniques for leaf inoculation have been described, mostly on peach (*Prunus persica*) and to a less extent on plum (*P. domestica*) and apricot (*P. armeniaca*). For practical aspects, detached leaf assays have often been used for pathogenicity assays. For example, Boudon *et al.* (2005) immersed detached

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peach leaves in a bacterial suspension and applied a moderate vacuum pressure whereas Randhawa and Civerolo (1985) as well as Hammerschlag (1988) used a syringe without a needle to infiltrate the bacterial suspension into peach leaves. These techniques permitted to observe necrotic lesions 2 weeks after inoculation (Boudon *et al*., 2005) and to obtain differences between clones and cultivars 3 weeks after inoculation (Hammerschlag, 1988). On-tree experiments have been reported for peach and plum, i.e. Miles and co-workers (1977) used a wind-rain machine to expose trees in the field to a concentration of 2×10^6 CFU ml^{-1} and Civerolo (1974) sprayed a selected circular area of peach leaves and obtained lesions 2 days post inoculation (dpi) with a concentration of 10^8 CFU ml^{-1}. A system using pressure with a vacuum pump was used by Scortichini *et al.* (1996) on peach, apricot, Japanese plum and sweet cherry. Depending on the plant species and cultivars tested, the time for obtaining symptoms with this technique ranged from 20 to 25 days. Other systems using an artist's airbrush (Zehr and Shepard, 1996; Shepard *et al*., 1999) or a spray gun connected to a compressed air supply (du Plessis, 1988) permitted to obtain symptoms 4 weeks after inoculation on peach, plum, and apricot (du Plessis, 1988) or 3 dpi in peach at 30°C (Zehr and Shepard, 1996). Du Plessis (1987) used a needle to puncture a droplet directly into the vascular system via the petiole, main vein or secondary vein of plum shoots to study canker development. Finally, the immersion of an actively growing shoot of plum trees in the inoculum has successfully been used in the open field to detect differences in susceptibility between cultivars, and therefore to determine a selection index (Topp and Sherman, 1995).

The objectives of this study were to evaluate different inoculation techniques under greenhouse condition, identify the one that would allow a quantitative evaluation of the resistance level of a genotype to *Xap* and to test the technique on a set of apricot cultivars for which field resistance data were available.

Two-year-old saplings of the rootstock GF305 (hereafter referred as "peach plants") were grown in 5 litre pots containing a mixture of peat and loam and were pruned before short term storage at 4°C in a cold chamber. Six different apricot cultivars were chosen for their range of resistance or susceptibility mainly based on field observations made by Garcin and Bresson (2005), with two to eight replicates per cultivar. These plants were grafted on the plum rootstock Saint-Julien 655-2, potted in 2 litre pots and kept in a cold chamber at 2°C for long term storage. Peach plants were obtained from the Pépinières de Saxon (Switzerland), and apricot scions were from the Agroscope Changins-Wädenswil nursery at Conthey (Switzerland). All peach plants were grown under greenhouse conditions at 23°C, 60% relative humidity under natural conditions of light for one month and apricots for two weeks before being transferred to a quarantine greenhouse. No fertilization was applied. Four strains of *Xap* were used, two of which were collected in Valais, (Switzerland) in 2005 (XA1.29) and in 2007 (XA1.51), respectively. The other two strains were the pathotype strain NCPPB 416 isolated from *P. salicina* in New Zealand in 1953 and the sequenced strain CFBP 5530 (Pothier *et al*., 2011) isolated from *P. persica* in Italy in 1989. For long-term storage, the strains were kept in 40% glycerol at -80°C. A loopful of a pure culture of each bacterial strain was separately grown on peptone yeast extract glycerol agar (NYGA) (Turner *et al*., 1984) at 28°C for 36 h. Cultures were then suspended in 1% sterile KCl and adjusted using a Genesys 10 UV spectrophotometer (Spectronic Unicam, USA) to an optical density of 0.5 at 600 nm corresponding to 1×10^8 CFU ml^{-1}, as estimated by counting on YPGA plates. After adjusting the concentration of the different strains separately, equal volumes of the bacterial suspensions were mixed just before inoculation. Four different inoculation techniques were tested on two peach plants per technique, presenting 5 to 10 actively growing shoots. The same techniques were repeated with sterile KCl (resuspension buffer of *X. arboricola* pv. *pruni* strain mix) on different plants to serve as negative control for comparison. The first technique consisted in dipping scissors into the inoculum before cutting 4 to 10 leaves per branch in the middle of the lamina. With the second technique 8 wounds per leaf were made on 4 to 8 leaves per branch with a needle, and droplet of inoculum of *ca*. 5 µl was placed on each wound. With the third technique the inoculum was infiltrated with a 1 ml syringe without needle in 8 sites of the abaxial side of 4 to 8 leaves per shoot. Infiltration was stopped when a clear water-soaked spot appeared around the inoculation point. Finally, the fourth inoculation technique consisted in the immersion of 4 to 5 actively growing shoots with 3 to 6 leaves in the bacterial suspension and gentle agitation for about 5 seconds until the leaf surfaces were fully wetted on both sides. Following inoculations, conditions were set at 85% relative humidity and 23°C during daytime and 18°C during the night. No extra light nor fertilization were applied.

The following data were recorded from inoculated peach plants, according to the inoculation technique used: (i) total number of leaves with symptoms (scissors); (ii) number of necrotic spots per leaf developing from the 6 to 8 inoculation points (needle and syringe); (iii) number of symptomatic leaves per shoot (dipping). All data were then expressed as percentage. Assessments were made 1, 2 and 3 weeks post inoculation. For screening apricot cultivars, four assessments were made 15, 28, 38 and 64 days post inoculation, taking into account four different traits: (i) number of spots per leaf on four leaves per plant presenting most symptoms (ref-

erence leaves); (ii) percentage of surface damaged by the disease on those four leaves; (iii) disease incidence determined by counting the number of leaves per plant showing symptoms and by dividing by the total number of leaves per branch; and (iv) resistance index calculated with the following formula:

$$RI = \frac{[(n_1 \times 1) + (n_2 \times 2) + (n_3 \times 3) + (n_4 \times 4)]}{N}$$

where n_1 is the number of leaves presenting 0 to 25% damage, n_2 26 to 50%, n_3 51 to 75%, n_4 76 to 100% and N is the total number of leaves per plant. Student's *t*-tests were calculated for each assessment using software JMP (SAS Institute, USA). The area under disease progress curve (AUDPC) was calculated for disease incidence and resistance index with Microsoft Excel (Microsoft Corporation, USA) using the following formula:

$$AUDPC = \sum_{i=1}^{n-1} \left[\frac{(t_{i+1} - t_i)(y_i + y_{i+1})}{2} \right]$$

where *t* is the time in days of each measurement, *y* is the variable at each measurement and *n* is the number of measurements.

Incidence and severity of bacterial spot on inoculated peach plants varied greatly depending on the inoculation technique used. The first symptoms could be observed between one and two weeks post inoculation (wpi). Water-soaked spots caused by infiltration became necrotic after already 1 wpi, resulting rapidly in high infection percentages (100%, Table 1). Symptoms were severe and many leaves dropped from 1 to 2 wpi, so that no quantitative results, e.g. size of the spots, could be scored three weeks after inoculation. Yellow halos were present on all leaves around the water-soaked spot caused by the syringe 2 wpi.

In average, 23% of the wounds caused per leaf by infected needles became necrotic 2 wpi, and 3 wpi typical necrotic spots were observed at all inoculation points (Table 1). As for the infiltration technique, yellow halos around the inoculation point were present on all leaves 2 wpi. Also in this case no quantitative data could be collected 3 wpi, because the spots remained too small. The technique using scissors did not permit to distinguish more than 4 spots per leaf developing closed to the wound area on the 50 leaves, even at 3 wpi, because the spots were so small that the damaged surface could not be quantified. Nevertheless, at 3 wpi disease incidence reached 48%. Finally, dipping the shoots into the inoculum permitted to obtain much more quantitative information pertaining to: (i) number of symptomatic leaves per branch; (ii) number of spots per leaf; (iii) percentage of damaged leaf area caused by the disease on the leaves. Dipped shoots presented in average about 11% and 38% of symptomatic leaves 2 and 3 wpi, respectively (Table 1). This method was consequently

Table 1. Disease responses to *Xanthomonas arboricola* pv. *pruni* in peach obtained after inoculation by wounding with a needle, by infiltration with a syringe, by cutting the leaf with infected scissors and by dipping the plants into the inoculum. Standard deviations, when relevant, are indicated in brackets.

Days Post Inoculation	Needle[1] (*n* = 50)	Infiltration[2] (*n* = 50)	Scissors[3] (*n* = 50)	Immersion[3] (*n* = 30)
7	0	100	0	0
14	23 (18.78)	100	16	11.29 (8.53)
21	100	100	48	38.19 (12.43)

[1] percentage of necrotic spots per leaf that have developed from the inoculation point
[2] percentage of necrosis per leaf obtained from the inoculation point
[3] disease incidence (%)

Table 2. Mean and standard deviation (std. dev.) of each trait measured from the apricot varieties 64 days post inoculation. Means sharing the same letter were not significantly different using a *t*-test ($p < 0.05$).

Cultivar	Nb. of replicates	Spot number[1]		Surface damaged (%)[1]		Disease incidence (%)		Resistance index	
		mean	std. dev.	mean	std. dev.	mean	std. dev.	mean	std. dev.
Bergarouge	5	17.70b	10.15	31.50b	14.53	65.27b	15.43	0.86b	0.28
Bergeron	3	8.00ab	4.77	45.83b	25.04	49.56b	26.17	1.16b	0.67
Goldbar	8	31.63bc	18.38	85.00c	11.34	76.74bc	19.39	3.04c	1.31
Goldrich	2	12.00b	6.36	20.00ab	13.60	66.67b	20.40	2.50bc	1.06
Kioto	6	12.92b	9.03	37.08b	20.76	65.65b	21.84	1.13b	0.47
Orangered	2	3.50a	2.83	10.00a	3.54	17.39a	0.56	0.17a	0.01

[1] Calculated with data from the four most damaged leaves per plant

chosen for a further test to assess the susceptibility to *Xap* of different apricot cultivars. Negative controls of all inoculation techniques did not develop *Xap* symptoms.

All apricot cultivars exhibited symptoms during the experiments, and a great variation of resistance levels to bacterial spot was observed (Table 2). The first symptoms appeared 1 wpi on cv. Goldbar, whereas all other varieties remained symptomless (data not shown). 'Goldbar' reached rapidly (28 dpi) 70% of symptomatic leaves and up to 76% of infected leaves at the end of the time-course experiment (64 dpi). The disease developed less rapidly in all other cultivars, and at the end of the experiment, cvs Bergarouge, Goldrich and Kioto had about 65% of their leaves showing symptoms, and cv. Bergeron 50%. Only cv. Orangered had less than 20% symptomatic leaves during the whole experiment (Fig. 1A).

Regarding the four leaves per plant presenting the strongest reaction (reference leaves), only cv. Orangered had significantly less leaf surface damaged and cv. Goldbar had significantly more than the other varieties, but when the average spot number was taken into account, cv. Orangered was the only variety with significantly less spots than the other 5 (Table 2). Although cvs Kioto, Bergarouge and Bergeron had a relatively high disease incidence 64 dpi, the resistance index (Table 2 and Fig. 1B) shows that their leaves remained moderately damaged, opposite to cvs Goldrich and Goldbar, for which a high disease incidence was accompanied by a heavier damages on the leaves. 'Orangered' was the most resistant cultivar in our experiment, taking both disease incidence and resistance index into consideration. Regarding the AUDPC of the disease incidence, no significant difference could be observed between cvs Bergeron, Kioto, Goldrich and Bergarouge. Only cv. Orangered was significantly more resistant [AUDPC of 2309 +/-737 standard deviation (std dev)] than the other cultivars, which ranged from AUDPCs of 4583 (+/-2186 std dev) for cv. Bergeron to 7524 (+/-1655 std dev) for cv. Bergarouge, to 10526 (+/-2746 std dev) for cv. Goldbar, which was significantly more susceptible (Fig. 2). When considering the AUDPC of the resistance index, however, cv. Goldrich was significantly more susceptible (AUDPC of 196 +/-31 std dev) than the cvs Bergarouge, Kioto and Bergeron, which ranged from AUDPCs of 92 (+/-47 std dev) for cv. Bergeron to 102 (+/-33 std dev) for cv. Kioto (Fig. 2). 'Orangered' was again significantly more resistant (AUDPC of 23, +/-7 std dev) and cv. Goldbar was significantly more susceptible (AUDPC of 299, +/-140 std dev).

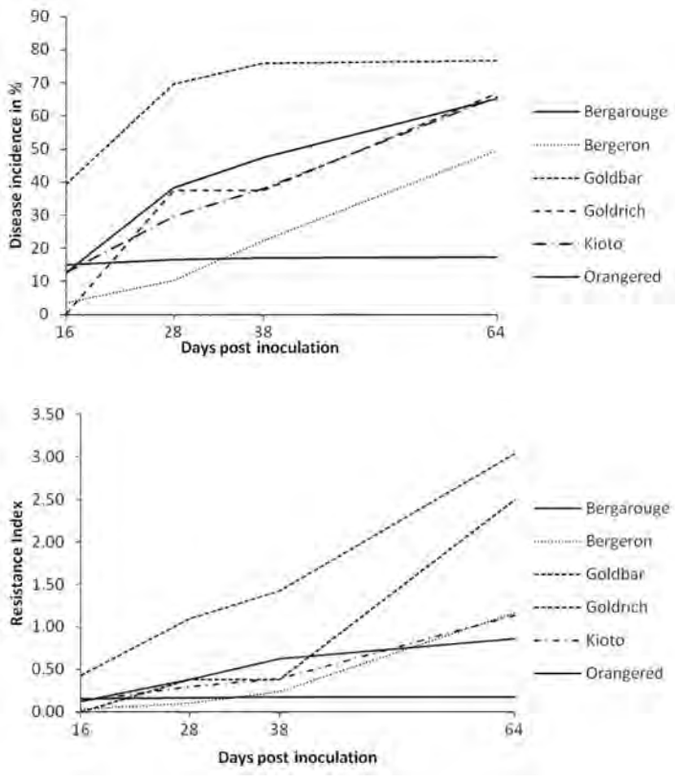

Fig. 1. A. and B. Disease incidence (a) and resistance index (b) for six apricot cultivars 15, 28, 38 and 64 days post inoculation.

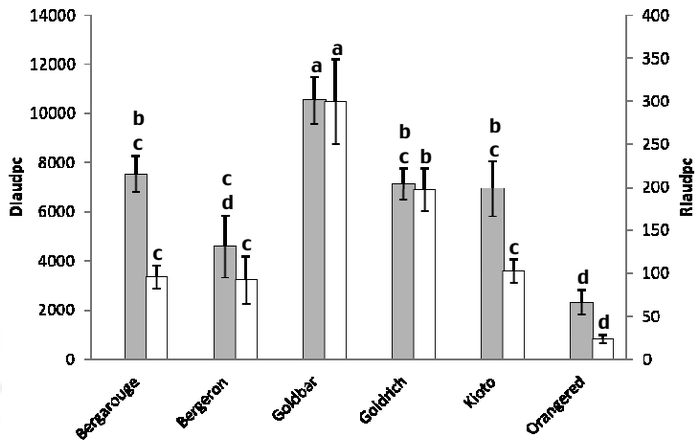

Fig. 2. AUDPC of the disease incidence (DIaudpc, in grey) and AUDPC of the resistance index (RIaudpc, in white) for the six apricot cultivars. Means (+standard error) sharing a letter were not significantly different using a *t*-test ($p < 0.05$).

It was possible to induce *Xap* symptoms with all four inoculation techniques tested. However, this study shows that quantitative expression of symptoms caused by *X. arboricola* pv. *pruni* may vary greatly in peach depending on the inoculation technique used. The high inoculum pressure induced by infiltration resulted rapidly and consistently in a high level of damage but the symptoms were so severe that often the inoculated leaves fell. Wounds caused by scissors and needles re-

sulted into symptom development although the reactions were not sufficiently quantitative. In both the latter cases, small necrotic points were visible after three weeks but neither their exact number nor the surface of the leaf damaged could be estimated. Inoculation by dipping the shoots into inoculum resulted in a larger array of symptoms and therefore in more variables useful to measure or count. This proved to be the method that offered the best reproducibility without approaching the values zero or 100%, which would not differentiate various levels of susceptibility/resistance of the host. This last technique can be considered as a more natural and less invasive method of inoculation than infiltration via a syringe (Kvitko *et al*., 2009). For these reasons we selected this method to assess the levels of resistance to *Xap* of several apricot cultivars and to compare them with field resistance evaluations.

Garcin and Bresson (2005, 2011) have assessed resistance to bacterial spot of different apricot cultivars in naturally contaminated orchards observing important differences in susceptibility that were partly dependent on the yearly climates. The six cultivars that we used in this study were also evaluated by Garcin and Bresson (2011) for disease incidence over 5-7 years in the field. Their study classified cvs Goldbar and Goldrich as very susceptible, cvs Bergeron and Kioto as intermediate, and cvs Bergarouge and Orangered as the least susceptibles, which is in accordance with our findings.

Measurements on the four reference leaves for spot number and severity has proved not to be precise enough for an appropriate comparison between the different varieties. 'Goldrich' varied for disease reaction depending on the publication, i.e. Garcin and Bresson (2011) classified it as susceptible, whereas Simeone and Scortichini (2005) found it to be the most tolerant among 33 cultivars tested. In our study, cv. Goldrich had a medium disease incidence, but the resistance index showed that symptomatic leaves were more damaged than those of cvs Kioto, Bergarouge and Bergeron, so that in our experiment cv. Goldrich was retained as rather susceptible. Finally, cv. Bergarouge had a high disease incidence score but most of the leaves were almost undamaged, showing a potential of tolerance to the disease. In all cases, with scores between 17 and 70% of symptomatic leaves per cultivar, our artificial inoculations on young plants in a greenhouse led to a much higher disease incidence than that reported (2 and 38%) from field trials by Garcin and Bresson (2011). We anticipate that this could probably be due to the fact that working with small plants in greenhouse permits to score also small lesions, which under field conditions may remain unnoticed or masked by lesions due to other biotic or abiotic factors.

In this study, a dip inoculation technique was used to identify apricot cultivars showing a high resistance level to *Xap.* The results obtained largely overlap with field observations reported in the literature (Zaccardelli, 1998). Although this technique may require, depending on the country, a quarantine greenhouse and other containment facilities, we came in three months time to very similar conclusions to those based on field trials under natural conditions over a period of several years. Our technique is consequently time-efficient and permits to consider the total amount of leaves per plant as well as the progression of the disease on the same leaves without the risk of confusion with other damages provoked by other pathogens or abiotic factors.

ACKNOWLEDGEMENTS

Financial support was provided by the Swiss State Secretariat for Education and Research (SBF C08.0124 and SBF C07.0139). We thank Rolf Blapp, Jürgen Krauss and Verena Knorst for plant grafting and greenhouse support.

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