Diagnostics[1]
Diagnostic

Xanthomonas arboricola pv. *pruni*

Specific scope

This standard describes a diagnostic protocol for *Xanthomonas arboricola* pv. *pruni*.

Specific approval and amendment

Approved in 2005-09.

Introduction

Bacterial spot caused by *Xanthomonas arboricola* pv. *pruni* was described for the first time in the USA (Michigan) in 1903 on Japanese plum. The disease is now reported from almost all continents where stone fruits are grown. *X. a. pruni* attacks only *Prunus* species and particularly the fruit crops. It is best known as a pathogen of plum, nectarine and peach (Stefani *et al*., 1989), but it has been reported on apricot (Scortichini & Simeone, 1997), almond (Young, 1977) and cherry as well. Species of the Sino-Japanese group (*P. japonica* and *P. salicina*) are generally more susceptible than European plums (Bazzi *et al*., 1990; Bazzi & Mazzucchi, 1980, 1984; Topp *et al*., 1989). Other hosts are Japanese apricot (*P. mume*), Chinese wild peach (*P. davidiana*), *P. buergeriana*, *P. crassipes* and *P. donarium*. Ornamental species of *Prunus* are also attacked.

Identity

Name: *Xanthomonas arboricola* pv. *pruni* (Smith, 1903) Vauterin, Hoste, Kersters & Swing 1995.
Synonyms: *Xanthomonas campestris* pv. *pruni* (Smith, 1903) Dye, *Xanthomonas pruni* (Smith) Dowson.
Taxonomic position: *Bacteria, Gracilicutes*.
EPPO code: XANTPR.
Phytosanitary categorization: EPPO A2 list no. 62, EU Annex designation II/A2.

[1]The figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

Detection

Disease symptoms

Symptoms of bacterial spot can be observed on leaves, fruits, twigs and branches (EPPO, 1997).

Symptoms on leaves

On peach leaves, infection is first apparent on the lower surface as small, pale-green to yellow, circular or irregular areas with a light-tan centre. These spots soon become evident on the upper surface as they enlarge, becoming angular and darkening to deep-purple, brown or black. The immediately surrounding tissue may become yellow. The diseased areas drop out, usually after darkening in colour, but they may drop out prior to the colour change, giving a shot-hole appearance to the leaf. Often, a dark ring of diseased tissue is left with the formation of the shot hole. Spots are usually concentrated towards the leaf tip, because the bacteria accumulate in this region in droplets of rain or dew. Bacterial ooze may be associated with the spots. Severely infected leaves turn yellow and drop off. Atypical symptoms reported for peach include a grey leaf spot on the upper surface, and a case in which bacteria infiltrated a large area, giving the leaf a greenish-yellow, translucent appearance. A severe defoliation can occur, leaving a carpet of yellow chlorotic leaves under the trees of susceptible cultivars.

On plum leaves, initial symptoms are angular water-soaked spots, rapidly turning reddish-brown, then dark brown and necrotic, whereas chlorosis is minimal and less apparent than on peach leaves. The necrotic spots frequently perforate, so that a shot-hole effect can be pronounced. On almonds, apricots and cherries, leaf symptoms are similar to those on peach, but rarely of importance.

Symptoms of bacterial spot on leaves can sometimes be confused with injuries caused by fungi or copper preparations. However, copper lesions are larger (2–6 mm in diameter) and often round in shape.

Symptoms on fruits

On peach fruits, small circular brown spots appear on the surface. They become sunken, the margins are frequently water-soaked, and there are often light-green haloes which impart a mottled appearance to the fruit. As a result of natural enlargement of the fruit, pitting and cracking occur in the vicinity of the spots. These cracks are often very small and difficult to see, but where heavy infection has occurred on young fruits they can be extensive, severely damaging the fruit surface. Gum flow, particularly after rain, may occur from bacterial wounds; this may easily be confused with insect damage. Similar symptoms may appear on apricots and almonds.

On plum fruits, symptoms may be quite different; large, sunken black lesions are common on some cultivars, while, on others, only small pit-like lesions occur. On cherries, early fruit infection results in distorted fruit, and bacteria may be found from the epidermis to the stone.

As a general rule, symptoms on fruits appear 3–5 weeks after petal fall and develop until the skin colour changes, when ripening process begins and some physiochemical parameters change. Symptoms often occur after hail damage.

Symptoms on twigs

On peach twigs, spring cankers occur on the top portion of overwintering twigs and on water sprouts before green shoots are produced; initially small, water-soaked, slightly darkened, superficial blisters, they extend 1–10 cm parallel to the long axis of the twig and may even girdle it. In this case the tip of the twig may die, while the tissue immediately below the dead area, in which the bacteria are present, is characteristically dark; this is the so-called 'black tip' injury. Twig infections later in the season result in summer cankers, which appear as water-soaked, dark-purplish spots surrounding lenticels. These later dry out and become limited, dark, sunken, circular to elliptical lesions with a water-soaked margin.

On plum and apricot twigs and branches, cankers are perennial, in contrast to peach, and continue developing in twigs 2 and 3 years old. The inner bark is penetrated, resulting in deep-seated cankers which deform and kill twigs. For more information, see Dunegan (1932), Anderson (1956), Hayward & Waterston (1965), Moffett (1973), McIver (1973), Gasperini *et al*. (1984), Du Plessis (1988), Goodman & Hattingh (1988), Shepard (1994) and Ritchie (1995).

Detection on symptomatic plant material

Isolation

In all *Prunus* species, bacteria can be isolated from symptomatic leaves showing water-soaked angular spots, or from immature fruits, or from twigs and branches with cankers. Isolation from ripening fruits is troublesome and, as ripening process continues, isolation of viable cells of the pathogen is no longer possible.

A few small pieces of tissue (1–2 mm) are taken from the margin of the lesion and crushed in a mortar or comminuted in a sterile Petri dish, adding a few drops of sterile water or sterile PBS buffer. After crushing, a further 2–3 mL of sterile water or sterile PBS buffer is added and the suspension is left to macerate 1–2 min (longer maceration would cause oxidation of the sample, resulting in loss of bacterial cell viability). The dilution-plate method should be used to streak 10–30 µL of the suspension onto YDC (yeast extract-dextrose-calcium carbonate agar) or YPGA (yeast-peptone-glucose agar) (Appendix I). The YDC or YPGA agar plates are incubated at 27 ± 2°C for 2-3 days. *X. a. pruni* colonies are convex, smooth, mucoid and glistening: colour is bright, creamy yellow, with a tendency to darken a little, turning yellow-orange with age. Typical colonies should be re-streaked onto Nutrient Agar plates to obtain pure cultures for further identification. Final confirmation requires a pathogenicity test on leaves of peach or plum seedlings.

Other bacterial species (e.g. *Pantoea agglomerans*) may show similar yellow colonies on the above media. *Pseudomonas syringae*, causing bacterial canker of stone fruits, does not produce bright yellow colonies on YDC or YPGA, but produces fluorescent pigment on King's B medium whereas *X. a. pruni* does not.

Detection on symptomless plant material

X. a. pruni may survive as an epiphyte on *Prunus* hosts in orchards or nurseries, associated with buds and leaf scars. From these, it can enter the host before full healing of the leaf scar, or through stomata, during the following growing season. Symptomless plant material may be analysed with the methods used for testing and certification of nursery propagation material prior to marketing and used in routine tests (Zaccardelli *et al*., 1995). These have been validated on peach and plum but not on cherry, almond or apricot, although satisfactory results are likely to be achieved on those species also.

Sample size

Samples of nursery material should consist of 100 dormant scion chips, while samples from orchards in winter should consist of 100 1-year twigs. One scion chip for each tree or twig should be cut and the 100 pieces collected in a Stomacher bag. If single large trees (used to obtain scion chips for grafting) are to be tested, 30 twigs from each tree should be cut and 100 chips taken from them.

Extraction

30 mL of 0.05 M sterile K-phosphate buffer, pH = 7.0 is added to the Stomacher bag and crushed for 3 min at room temperature. The suspension is filtered through sterile gauze into a 50 mL centrifuge tube and spun for 5 min at 480 *g*. The supernatant is poured into a new tube and centrifuged again at 12 000 *g* for

10 min. The supernatant is discarded and the pellet resuspended with 1 mL of phosphate buffer to obtain the final concentrate. A portion of the final concentrate (0.5 mL) is added to 50–70 µL of sterile glycerol and kept at –20°C. The rest is used for direct isolation and IFAS.

Direct isolation

30 µL of the final concentrate and its 10- and 100-fold dilutions are plated on SP (sucrose-peptone agar) (Appendix I) and incubated for 3 days at 27°C. Colonies resembling *X. a. pruni* are selected and purified on YDC or YPGA agar plates.

Immunofluorescence (IF)

The final concentrate and its 10-fold and 100-fold dilutions are used for indirect immunofluorescent colony staining (IF). The standard protocol described in EU (1998) is followed, using a polyclonal antibody with a titre of the crude antiserum not less than 1: 2000. The IF test should be performed on freshly prepared sample extracts: if IF needs to be performed on extracts with glycerol added (kept at –80°C), remove glycerol by adding 1 mL of 10 mM PBS, pH 7.2 (NaCl 8 g; KCl 0.2 g; Na_2HPO_4. $12H_2O$ 2.9 g; KH_2PO_4 0.2 g; distilled water to 1 L), centrifuge for 15 min at 7000 *g*, discard supernatant and resuspend in 1 mL PBS.

Suspect colonies are purified and identified according their morphology on agar plates and further tested for their ability to elicit a Hypersensitivity Reaction (HR) on bean pods (Klement, 1963) or on tomato leaves cv. 'Roma' (Lelliott & Stead, 1987), or else by IFAS and/or protein profiling. A reliable PCR protocol is not yet available, either for detection or for identification of pure cultures.

Identification

The presence of *X. a. pruni* is suspected when the colony morphology on the bacterial culture media and the biochemical tests are those typical of the pathovar. Pure cultures can be identified by Protein Profiling (SDS-PAGE), Fatty Acids Methyl-Ester profile analysis (FAME) or with REP-PCR. Final confirmation requires a pathogenicity test on leaves of peach seedlings cv. 'sunhigh' or other susceptible peach or plum cultivar (Randhawa & Civerolo, 1985; Ritchie *et al*., 1993).

Description of pathogen

Gram negative rod, motile by one flagellum, measuring 0.2–0.4 × 0.8–1.0 µm, strict aerobe with an optimum growth temperature range of 24–29°C. Virulence to peach, plum, apricot, almond and cherry may be remarkably different among strains and populations. Cross infections between different host species are common, but not always possible.

Biochemical tests

According to Fahy & Persley (1983) and Schaad (1988): Gram reaction (negative); presence of oxidase (lack); glucose metabolism (oxidative); aesculin hydrolysis (positive); gelatine liquefaction (positive); protein digestion (positive); starch hydrolysis (negative); urease production (negative); potato soft rot (slimy yellow growth); growth at 35°C in yeast broth (positive); growth in 2% NaCl (positive); growth in 5% NaCl (negative).

Protein profiling

Colonies of the putative *X. a. pruni* are grown on GYCA agar medium for 48 h at 28°C and then subcultured on the same agar medium for additional 48 h at the same temperature. Protein extraction, purification, protein gel-electrophoresis (SDS–PAGE) and interpretation of the electrophoresis results are described in Vauterin *et al*. (1991). A positive protein profiling analysis is achieved when the protein profile of the presumptive culture is identical to that of the positive control (Kersters, 1990).

Fatty acid profiling (FAME)

Colonies of the putative *X. a. pruni* are grown on trypticase soy agar for 48 h at 28°C and an appropriate FAME procedure is applied. A positive FAME test is achieved when the profile of the presumptive culture is similar to that of the positive control (Sasser, 1990).

Pathogenicity tests

Detached leaf bioassay

Following the method of Randhawa & Civerolo (1985), young fully expanded leaves (3–6th leaf from the top) are detached from peach seedlings cv. 'sunhigh', or any other peach cultivar known to be susceptible to *X. a. pruni*, grown in the glasshouse. The leaves are briefly washed under running tap water to remove dirt and disinfected for 40–60 s with 70% ethanol. They are rinsed repeatedly in sterile water and immediately used for inoculation. Bacterial suspension at concentration of 10^7 cfu mL^{-1} is prepared. Leaves or parts of them, abaxial side upward, are placed on several layers of sterile blotter. Inoculum is infiltrated by using a syringe without needle and by applying gentle and steady pressure while holding the open end of the syringe against the leaf until a 2- to 4-mm-diameter area of mesophyll tissue is water-soaked. 8–10 sites on each leaf are inoculated approximately 1 cm apart. The leaves are lightly blotted to remove any excess of inoculum. In the same way, negative controls are prepared using sterile water (instead of bacterial suspension), and a positive control using suspension of a known strain of *X. a. pruni* at 10^7 cfu mL^{-1}. All inoculated leaves (test sample, negative control, positive control) are placed on 0.5% water agar and incubated for two weeks at 25°C under fluorescent lights (60–75 $\mu E \times s^{-1} \times m^{-2}$) timed to a 16 h photoperiod.

For a positive result, after 6–9 days all inoculated sites should exhibit confluent water soaking, becoming dark brown and brittle necrotic spots often surrounded by a greyish

white or purple margin. Bacterial ooze occurs frequently on older lesions.

When *X. a. pruni* is isolated from plum, the pathogenicity test may conveniently be performed on a susceptible plum cultivar, e.g. 'Friar', 'Laroda', 'Frontier', 'Angeleno', 'Black Star', 'Shiro' (Bazzi *et al*., 1990; Simeone, 1990). Differences in aggressiveness have been observed among some strains after inoculation in several host plants (Du Plessis, 1988, Scortichini *et al*., 1996).

Inoculation of plants with X. a. pruni

Plants of susceptible peach or plum cultivars or rootstocks (peach cvs. 'Barrier', 'Catherine', 'Parade', 'Royal Glory' or 'Reach Lady', plum cvs. 'Black Beauty', 'Black Diamond', 'Calita' or 'Mariana') can be inoculated by two protocols. Following Randhawa & Civerolo (1985), young leaves on young shoots are infiltrated using a plastic syringe without a needle, applying gentle and steady pressure while holding the open end of the syringe against the leaf until the mesophyll tissue is water-soaked. Following Du Plessis (1988), plants are maintained at 25–27°C and 95–100% RH for 8 h before inoculation. The first young but fully expanded leaves from the tip of the shoots are spray-inoculated on the abaxial side with a spray gun connected to a compressed air supply. Both protocols use bacterial suspensions of 10^7 cfu mL^{-1}. The plants should be maintained under glasshouse conditions at about 25°C and high humidity. Lesions can be recorded 1–4 weeks after inoculation.

Reference material

ATCC 19312; CFBP 2535; ICMP 51; LMG 852; NCPPB 416.

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM7/– (in preparation).

Further information

Further information on this organism can be obtained from:

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Appendix I Media

YDC agar medium (Stolp & Starr, 1964): yeast extract 10.0 g; dextrose (glucose) 20.0 g; calcium carbonate (light powder) 20.0 g; agar 15.0 g; distilled water to 1.0 L

YPG agar medium (Lelliott & Stead, 1987): yeast extract 5.0 g; bacteriological peptone 5.0 g; glucose 10.0 g; agar 20.0 g; distilled water to 1 L; (pH 6.5-7.0).

SP agar medium (Hayward, 1960): bacteriological peptone 5.0 g; sucrose 10 g; potassium bi-phosphate 0.05 g; magnesium sulphate 0.25 g; agar 15 g; distilled water to 1.0 L; (pH 6.8). Sterilize by autoclaving 15 min at 121°C, cool to about 50°C and then add 10 mL L^{-1} of a 1% cycloheximide (actidione) sterile solution.