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DETECTION AND IDENTIFICATION METHODS AND NEW TESTS AS DEVELOPED AND USED IN THE FRAMEWORK OF COST 873 FOR BACTERIA PATHOGENIC TO STONE FRUITS AND NUTS

Xanthomonas arboricola pv. *pruni*

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SUMMARY

Xanthomonas arboricola pv. *pruni* (*Xap*), the causal agent of bacterial spot disease of stone fruits and almond, is regulated as a quarantine pathogen in the European Union and the European and Mediterranean Plant Protection Organization (EPPO). *Xap* can have an epiphytic phase and/or be latent and, consequently, it can be transmitted by different types of plant material. Effective quarantine measures require specific, sensitive and rapid methods to detect *Xap* in propagative material or new reservoirs. Laborious and time-consuming methods for the diagnosis of *Xap* are recommended in the current EPPO standard protocol. However, new several pathogen-specific PCR and quantitative real-time PCR assays have been developed that enable direct detection of *Xap* in symptomatic and symptomless plant samples. A concise resource of current methods for *Xap* detection and identification, based on assessment and development activities within the framework of COST 873, is presented.

Key words: PCR, real-time PCR, isolation, biochemical tests, FAME, MALDI-TOF MS, pathogenicity tests.

INTRODUCTION

Xanthomonas arboricola pv. *pruni* (*Xap*) is the causal agent of bacterial spot disease of stone fruits. It is considered one of the most important bacterial pathogens affecting stone fruits, almond and *Prunus* spp. worldwide (Young, 1977; Jindal *et al.*, 1989; Civerolo and Hatting, 1993; Akhtar *et al.*, 1995; Ritchie, 1995; Scortichini, 1995; Janse, 2006; Palacio-Bielsa *et al.*, 2010). Some ornamental species such as *Prunus laurocerasus* can also be affected (Anonymous, 2009; Marchi *et al.*, 2011). *Xap* is regulated as a quarantine pathogen in many countries as in phytosanitary legislation of the European Union (Anonymous, 2000 and amendments), or the European and Mediterranean Plant Protection Organization (EPPO) (Anonymous, 2003). An effective prevention against the introduction and spread of *Xap*, needs quarantine measures and specific, sensitive and rapid detection protocols. Currently, in many countries, only visual inspections once a year for typical *Xap* symptoms are required to certify plants in nurseries as free from the disease (López *et al.*, 2010), which is clearly insufficient to prevent dissemination of its causal agent, for which the existence of an epiphytic phase is well established, as well as latent infections, that enable spreading by propagative material (Dhanvantari, 1971; Goodman and Hattingh, 1986; Zaccardelli *et al.*, 1998).

The current EPPO standard protocol for the diagnosis of bacterial spot of stone fruits (Anonymous, 2006a) is based on isolation of the pathogen using general agar media, followed by identification of pure cultures by biochemical tests, protein and fatty acids methyl ester (FAME) profiling, repetitive extragenic palindromic PCR (REP-PCR), serological and pathogenicity tests. The development of several pathogen-specific PCR assays, however, now enables direct detection of *Xap* in symptomatic or symptomless plant samples, and a significant improvement for the identification of putative *Xap* isolates (Weller *et al.*, 2007; Park *et al.*, 2010; Ballard *et al.*, 2011; Palacio-Bielsa *et al.*, 2011; Pothier *et al.*, 2011a, 2011b, 2011c, 2011d).

Comprehensive reviews of currently available information on detection, diagnosis, phytosanitary measures and identification of bacterial diseases of stone fruits, including bacterial spot caused by *Xap*, have been recently published (Janse, 2010; López *et al.*, 2010).

The main objective of this contribution is to provide a concise resource of current methods applied to detect and identify *Xap*, based on assessment and development activities within the framework of COST 873.

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HOST RANGE

Xap has been reported to cause bacterial spot disease in a wide range of *Prunus* species, such as *Prunus armeniaca* (apricot), *P. avium* (sweet cherry), *P. blireiana* (flowering plum), *P. buergeriana*, *P. cerasus* (sour cherry), *P. crassipes*, *P. davidiana* (Chinese wild peach), *P. domestica* (plum), *P. donarium*, *P. dulcis* (almond), *P. laurocerasus*, *P. mahaleb*, *P. mume* (Japanese apricot), *P. persica* (peach), *P. persica* var. *nectarina* (nectarine) and the Sino-Japanese plum group (*P. japonica* and *P. salicina*). It affects different cultivars more or less severily according to their susceptibility (Young, 1977; Bazzi *et al.*, 1990; Simeone, 1990; Ritchie, 1995; Anonymous, 2003). Other *Prunus* spp. as well as *Sorbus japonica* have also been reported as hosts, but only based on inoculation assays (Bradbury, 1986).

According to the EPPO, the presence of *Xap* has been reported in different hosts from some European countries (Anonymous, 2006b, 2006c), namely: France, in peach, apricot and plum (Boudon *et al.*, 2005; André and Rouzet, 2007); Italy, in plum, peach, apricot and *P. laurocerasus* (Stefani *et al.*, 1989; Bazzi *et al.*, 1990; Scortichini and Simeone, 1997; Marchi *et al.*, 2011); Montenegro, in almond (Panic *et al.*, 1998; Arsenijevič and Obradovič, 2008); Hungary, in plum (Németh *et al.*, 2007); the Netherlands, in *P. laurocerasus* (Anonymous, 2009); Spain, in almond (Palacio-Bielsa *et al.*, 2010) and other *Prunus* species such as plum, peach and nectarine (Roselló, 2007; Roselló *et al.*, this issue); Switzerland, in apricot and Japanese plum (*P. salicina*) (Anonymous, 2006b; Pothier *et al.*, 2010). *Xap* has been considered as an emergent pathogen in some of these countries. Several other European countries, from which less information about hosts is available, such as Bulgaria, Republic of Moldova, Romania, Russian Federation, Slovenia and Ukraine, have reported only local outbreaks (Anonymous, 2006c).

DETECTION AND IDENTIFICATION

All the methods described below have been developed and/or practiced in laboratories involved in COST 873, and some other promising new methods are mentioned only with literature references. The described protocol includes isolation, serological and molecular techniques to detect *Xap* from symptomatic or symptomless samples of leaves, buds, fruits and cankers. Identification methods based on different biological principles are also detailed.

GENERAL ISOLATION METHODS

General culture media. Two general media (YPGA and YDC) are recommended in the EPPO standard (Anonymous, 2006a) for the isolation of *Xap* from symptomatic samples and have been also used in COST 873.

YPGA (yeast-peptone-glucose agar) (Ridé, 1969; Lelliot and Stead, 1987). Per 1 litre distilled water: 5 g yeast extract; 5 g bacteriological peptone; 10 g glucose; 20 g agar; pH 7.0. After incubation at 25°C for 3-4 days, colonies are about 3 mm in diameter, round, with entire margins, domed, smooth, mucoid and pale yellow to yellow, although after some days they appear more coloured. Comparison with reference strains is advised.

YDC (yeast extract-dextrose-calcium carbonate agar) (Stolp and Starr, 1964). Per 1 litre distilled water: 10 g yeast extract; 20 g dextrose (glucose); 20 g $CaCO_3$ (light powder); 15 g agar; pH 7.0. The autoclaved medium should be cooled to 50°C in a water bath, and $CaCO_3$ suspended by swirling before pouring the plates. After incubating at 27°C for 2-3 days, *Xap* colonies appear pale yellow to yellow, round, mucoid and domed.

Wilbrink agar (Koike, 1964). Per 1 litre distilled water: 5 g bacteriological peptone; 0.5 g K_2HPO_4; 0.25 g $MgSO_4 \cdot 7H_2O$; 10 g sucrose; 18 g agar; pH 7.0. After incubation at 25°C for 2-3 days, *Xap* colonies appear pale yellow to yellow, round, mucoid and domed.

SP (sucrose peptone agar) (Hayward, 1960). This medium was used by Zaccardelli *et al.* (1995) for isolation from buds and leaf scars on asymptomatic branches, and it is also recommended by the EPPO standard protocol (Anonymous, 2006a). Per 1 litre distilled water: 5 g bacteriological peptone; 10 g sucrose; 0.05 g potassium bi-phosphate; 0.25 g magnesium sulphate; 15 g agar; pH 6.8. After 48 h at 27°C, *Xap* colonies are circular, with a mean diameter of 1.2 mm, cream-coloured, mucoid and domed.

Addition of sterile 250 mg l^{-1} cycloheximide (Sigma-Aldrich, USA) to these media is suggested for avoiding fungal growth.

Semi-selective culture media. *mXCP1 medium.* This medium has been modified from the original XCP medium (McGuire *et al.*, 1986) (H. Koenraadt, personal communication). Per 1 litre of sterile distilled water: 10 g peptone; 20 g soluble potato starch; 10 g KBr; 0.25 g $CaCl_2$ (anhydrous); 20 g agar; pH 7.0. The autoclaved medium should be cooled to 45°C before adding the following filtered sterilized ingredients: 0.4 ml cephalexin (Sigma-Aldrich, USA) of a stock solution (25 mg ml^{-1}); 0.6 ml 5-fluorouracil (Sigma-Aldrich, USA) of a stock solution (5 mg ml^{-1}); 1 ml nystatin (Sigma-Aldrich, USA) from a stock solution (200,000 U ml^{-1}); 0.14 ml tobramycin (Sigma-Aldrich, USA) from a stock solution (0.8 mg ml^{-1}); 1.5 mg crystal violet (Sigma-Aldrich, USA); 10 ml Tween 80 (autoclaved separately). After incubation at 28°C for 3-5 days, *Xap* colonies appear yellow, mucoid and the hydrolysis of Tween 80 can be observed as a clear zone around the colonies.

XPSM (Civerolo *et al.*, 1982). Semi-selective medium

XPSM is advised by Schaad *et al.* (2001), and its use has been also reported by Shepard and Zehr (1994), and Ballard *et al.* (2011) for the isolation of epiphytic *Xap* populations from symptomless plant material. After incubation at 27°C for 6-7 days, *Xap* colonies appear greyish white in colour, convex, shiny and opaque. The efficiency of this medium has not been evaluated in COST 873.

ISOLATION TECHNIQUES

Isolation from symptomatic samples. From leaves, cankers or fruits, superficial disinfection is not usually required. Use 50 µl of macerates, obtained as indicated below, for plating on YPGA (Ridé, 1969; Lelliot and Stead, 1987) or other media. Incubate the plates at 25°C for 3-4 days and check for typical colonies.

The isolation of *Xap* from symptomatic samples is often easy since the number of culturable bacteria is usually high. When environmental or cultural conditions are not favourable for the pathogen, or treatments such as copper have been applied during crop management (Pulawska *et al.*, 1997), the number of *Xap* culturable cells is expected to be very low and isolation plates can be overcrowded with saprophytic bacteria. In such cases, plating 100 µl of the macerates and their decimal serial dilutions (10^{-1} and 10^{-2}) in larger (130 mm diameter) plates is advised.

Leaves. Select untreated leaves with typical young bacterial spots, preferably surrounded by a water-soaked halo. Wash approximately 1 g (fresh weight) of leaves, collected in sterile plastic bags, in 15 ml of sterile distilled water or phosphate buffered saline (PBS) (per 1 litre of distilled water: 8 g NaCl; 0.2 g KH_2PO_4; 1.15 g Na_2HPO_4; 0.2 g KCl; pH 7.2), let them stand for 15 min without shaking and plate 50 µl. Alternatively, cut small pieces of 2-3 spots in 4.5 ml of sterile distilled water or PBS and comminute them in Petri dishes, waiting for 5-15 min before plating.

Cankers. Select the canker sample from margins between diseased and healthy tissue. In spring cankers (black tip injury), the tissue immediately below the necrotic area is recommended. In summer and in perennial cankers, select the water-soaked margin when present. Try to avoid dry necrotic tissues because populations of the pathogen are usually low. To find the active margin of the canker it is necessary to localize it by eliminating the epidermis with a flamed scalpel. Dissect 3-5 small portions (*ca.* 1 cm^2) of the leading edge of the lesion, comminute them in a sterile Petri dish with 4.5 ml of sterile distilled water or PBS and let them stand for 15-30 min before plating.

Fruits. Pathogen isolation from ripening fruits can be troublesome and, in the last stages of the ripening process, its recovery may become impossible (Anonymous, 2006a). Select small pieces of tissue (*ca.* 1 cm^2) from the margin of the fruit lesions and comminute them in a sterile Petri dish, add 4.5 ml of sterile distilled water or PBS and leave them to stand for 1-2 min (longer periods would cause oxidation of the sample, resulting in a loss of bacterial cell viability). Almond nuts can be also washed in sterile plastic bags in 15 ml of sterile distilled water or PBS or alternatively, cut 2-3 small pieces at the edge of the external or internal lesions, comminute them in Petri dishes with 4.5 ml of sterile distilled water or PBS and leave them to stand for 5-15 min before plating.

The same washed or comminuted tissue preparations can also be used for immunofluorescence (IF), ELISA and PCR analyses.

When analysing symptomatic samples, good correlation is expected between isolation, real-time PCR (Palacio-Bielsa *et al.*, 2011) and the optimised conventional PCR from Pagani (2004) (see López *et al.*, this issue).

Isolation from asymptomatic samples. Symptomless leaves, buds of fruits can also be analyzed without superficial sterilization. Use 50 µl of washed or comminuted tissues (obtained as indicated below) for plating on the same media. These same washings or crushes can also be used for IF, ELISA and DNA extraction followed by PCR.

Leaves. Wash approximately 1 g (fresh weight) of mature leaves and proceed as for symptomatic samples.

Buds. Add 10 ml of sterile distilled water or PBS to 20 buds (approximately 0.7 g) in a sterile plastic bag, slightly comminute them and leave them to stand for 5 min. Proceed as indicated above for leaf samples. According to Anonymous (2006a), samples can also consist of 100 dormant scion chips and, if single large trees are to be tested, 30 twigs from each tree should be cut and 100 chips taken from them randomly.

Fruits. According to Shepard and Zehr (1994), 5 to 10 peach or plum fruits can be introduced in an Erlenmeyer flask with sterile distilled water until they are covered and treated for 5 min in an ultrasonic bath. Then, 10^{-1} and 10^{-2} dilutions can be plated on XPSM medium (Civerolo *et al.*, 1982) and incubated at 27°C for 7 days. In all cases, after incubation, select typical colonies and identify them after one purification step on a general medium (Nutrient agar, YPGA) by the techniques described below (biochemical, serological and molecular tests, as well as pathogenicity tests).

SEROLOGICAL DETECTION TECHNIQUES

According to the EPPO standard for *Xap* (Anonymous, 2006a), symptomatic or symptomless plant material can be screened using IF provided that specific commercial antibodies are available. Samples of 100 pieces of tissue containing buds and leaf scars were ana-

lyzed by IF and isolation on SP medium with good results (Zaccardelli *et al.*, 1995). However, as indicated below for identification of pure cultures, specificity problems have been found with the evaluated antibodies in the frame of COST 873.

MOLECULAR DETECTION TECHNIQUES

Plant DNA extraction methods. *Heat treatment.* In most cases, satisfactory real-time PCR results are obtained with heat treatment (96ºC for 10 min and subsequent cooling on ice) of washed leaves samples (Palacio-Bielsa *et al.*, 2011). However, for maximum accuracy, a reliable and simple DNA extraction protocol for plant tissue is indicated below (Llop *et al.*, 1999).

Simple DNA extraction with isopropanol (modified from Llop *et al.,* 1999). Useful for washings and comminuted asymptomatic tissues and for comminuted tissue from lesions of infected leaves, buds, twigs and fruits (see above). Centrifuge 1 ml of the tissue sample extract at 13,000 *g* for 10 min. Discard the supernatant and resuspend the pellet in 500 µl filtered sterilised extraction buffer [200 mM Tris HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS; 2% polyvinilpyrrolidone (PVP)]. Vortex the resuspended pellet and shake for 1h at room temperature (RT). Centrifuge the extract at 5,000 *g* for 5 min and transfer 450 µl of the supernatant to a clean Eppendorf tube. Add 450 µl isopropanol, mix gently and let stand for 30 min at RT. Centrifuge the mixture at 13,000 *g* for 10 min. Discard the supernatant and dry the pellet on air. If a coloured precipitate (brown or green) at the bottom of the tubes is observed, remove it carefully while discarding the supernatant to obtain a cleaner DNA. Resuspend the pellet in 100 µl sterile distilled water and determine DNA concentration and purity by spectrophotometry. Use for PCR or store at -20ºC.

DNA extraction with CTAB (Doyle and Doyle, 1990). This procedure can be used to extract total DNA from lesions of infected plants or from asymptomatic samples, especially when a high amount of inhibitors is suspected. CTAB buffer (2% CTAB hexadecyltrimethylammonium bromide; 1.4 M NaCl; 0.2% 2-mercaptoethanol; 20 mM EDTA; 100 mM Tris-HCl, pH 8.0) is preheated to 60°C in a water bath. Centrifuge 500 µl of the samples at 13,000 *g* for 10 min. Discard the supernatant and add 400 µl CTAB buffer. Vortex the tubes to mix, add 0.4 µl 2-mercaptoetanol and mix by inverting. Incubate at 60°C in a water bath for 20 min. Add 400 µl chloroform-isoamyl alcohol (24:1), mix gently but thoroughly to get a homogeneous mix and centrifuge at 13,000 *g* for 15 min. Transfer the aqueous phase (400 µl) to a clean Eppendorf tube avoiding disturbing or mixing phases. Add 240 µl cold isopropanol and mix gently by inverting. Centrifuge at 13,000 *g* for 5 min and discard supernatant. Add 500 µl wash buffer (10 mM ammonium acetate in 76% EtOH) and incubate for 5 min at RT. Centrifuge at 13,000 *g* for 5 min, discard supernatant carefully and dry the pellet on air. Resuspend the pellet in 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). Use for PCR or store at -20°C.

Negative and positive controls should be included in the described DNA extraction and PCR protocols.

PCR detection protocols. The conventional PCR protocols described by Park *et al.* (2010) and Pothier *et al.* (2011a), as well as the real-time PCR protocols from Ballard *et al*. (2011) and Palacio-Bielsa *et al.* (2011) (see under "Identification by molecular techniques"), can be used for the analysis of symptomatic samples after performing the DNA extraction procedures described above (Fig. 1). The real-time PCR protocol of Palacio-Bielsa *et al.* (2011) is very efficient for symptomatic as well as symptomless samples in most of the hosts. Conventional PCR from Park *et al.* (2010) was not evaluated in the framework of COST 873.

The sensitivity of conventional simplex-PCR and duplex-PCR protocols from Pothier *et al.* (2011a) was evaluated for spiked apricot leaf tissue. In the simplex-PCR assay, a limit of detection of approximately 5 x 10 or 5×10^2 CFU per reaction was obtained when using *Xap*Y17 and *Xarb*Q primers, respectively. Established sensitivity of the duplex-PCR is 5×10^2 CFU per reaction (Pothier *et al.*, 2011a).

Regarding the real-time PCR from Ballard *et al.* (2011), that is a Bio-PCR involving an additional growth step of tissue washes at 28°C for 6 days on XPSM agar medium (according to Shepard and Zehr, 1994), was developed for detection of *Xap* in symptomless peach tissues. Although satisfactory results have been reported by the authors, this method has not been evaluated in the framework of COST 873.

The quantitative real-time PCR protocol from Palacio-Bielsa *et al.* (2011), described under "Identification

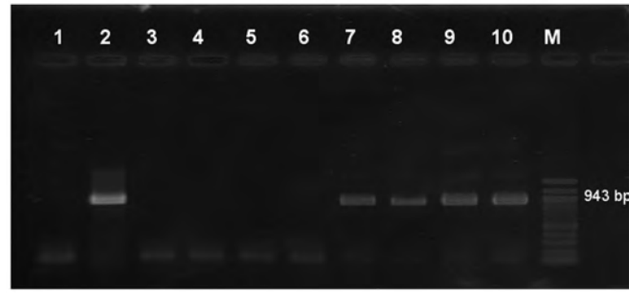

Fig. 1. Detection of *Xap* on symptomatic almond leaves naturally infected by conventional PCR (on DNA extracts) using *Xap*Y17F/*Xap*Y17R primers (Pagani, 2004). Lane 1, negative control (master-mix-only); lane 2, positive control (*Xap* strain ISPaVe B4 heat-treated suspension); lanes 3 to 6, healthy almond leaves; lanes 7 to 10, symptomatic almond leaves; M, molecular marker (100 bp DNA ladder, BioLabs).

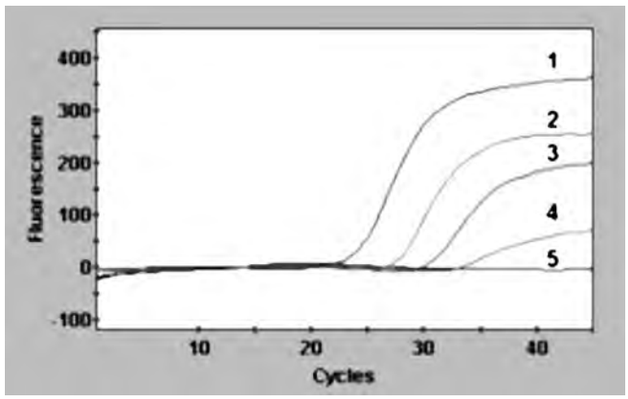

Fig. 2. Detection of *Xap* on symptomatic almond leaves naturally infected by quantitative real-time PCR (Palacio-Bielsa *et al.*, 2011) (on washed tissues). 1, positive control (*Xap* strain ISPaVe B4 heat-treated suspension); 2, undiluted leaves washing; 3, leaves washing dilution (10^{-1}); 4, leaves washing dilution (10^{-2}); 5, negative control (healthy leaves washing).

by molecular techniques", enables specific and sensitive detection of *Xap* from symptomatic or symptomless *Prunus* spp., at a level of sensitivity of 10^2 CFU ml^{-1} (Fig. 2). In symptomless leaf and bud samples, this protocol is more efficient after a DNA extraction with isopropanol procedure (Llop *et al.*, 1999). Although in comparative studies, CTAB and isopropanol methods showed similar sensitivity and efficiency, the latter is advised to avoid the use of toxic compounds such as phenol or chloroform (Palacio-Bielsa *et al.*, 2011). The DNeasy Plant minikit (Qiagen, USA) did not provide as satisfactory results as the other DNA extraction methods from stone fruit trees and almond leaves (Palacio-Bielsa *et al.*, 2011). Plum and cherry tissues can be an especially problematic material for DNA-based methods, probably due to the presence of high concentrations of PCR inhibitors even at the leaf surfaces (Pich and Schubert, 1993; De Boer *et al.*, 1995; López *et al.*, 2003, 2006, 2009; Palacio-Bielsa *et al.*, 2011).

STORAGE OF ISOLATES/STRAINS AND CONTROL STRAINS

Storage. After purification on a general medium (YPGA, YDC, Wilbrink) isolates should be stored as soon as possible (-80°C in 30% v/v glycerol) since they usually survive on these media only for a few days. Check viability by thawing and plate some suspension after one week storage, and thereafter periodically.

Reference strains suggested for use as positive controls in diagnostic/identification tests. Xap pathotype type strain CFBP 2535^T (*Prunus salicina*; Auckland, New Zealand, 1953) is advised by the EPPO standard protocol (Anonymous, 2006a). Other collection accession numbers assigned to this strain are: ATCC 19316; CFBP 3894; ICMP 51; ICPB XT 103; LMG 852; NCPPB 416. As the complete genome of the *Xap* strain CFBP 5530 (*Prunus persica*; Vento Villa Franca, Italy, 1989); fFALP Group A, according to Boudon *et al.* (2005) and Zaccardelli *et al.* (1999) has been sequenced (Pothier *et al.*, 2011c), this strain is also advised as a control.

IDENTIFICATION BY CONVENTIONAL BIOCHEMICAL TESTS

Biochemical characterization should be performed on pure cultures. According to Fahy and Persley (1983), Schaad and Stall (1988), Schaad *et al.* (2001) and COST 873 results, the following tests are considered universal for *Xap*: Gram negative; oxidase negative; catalase positive; oxidative metabolism of glucose (Hugh-Leifson); negative for nitrate reduction, arginine dihydrolase (Thornley medium), urease, indole, growth in 5% NaCl and Nutrient broth at 37°C; and positive for casein digestion, hydrolysis of gelatine, Tween 80 and esculin, Simmons' citrate, growth in 2% NaCl and Nutrient broth at 35ºC. With API 20 NE miniaturized strips (bioMérieux, France) used according to the manufacturer's instructions (except a change in incubation temperature and time, namely 25ºC for 48 h), the following positive reactions are observed: esculin and gelatine hydrolysis, β-galactosidase activity and oxidative metabolism of glucose, mannose, N-acetylglucosamine, malate and citrate (Roselló, 2007). With API 50 CH miniaturized strips (bioMérieux, France) performed using Dye's C medium (Dye, 1968) with 0.08 ‰ bromothymol blue, and after incubation at 25ºC for 72 h, esculin hydrolisis and N-acetylglucosamine are positive in 100% of the tested strains, and variable reactions (more than 85% of *Xap* strains positive) are obtained for acid production from galactose, D-glucose, D-fructose, D-mannose, cellobiose, sucrose, trehalose, D-fucose and L-fucose, as well for alkalinization of 2-ketogluconate (Roselló, 2007).

IDENTIFICATION BY SEROLOGICAL TECHNIQUES

Although IF and ELISA commercial kits for *Xap* identification are currently available (Neogen Europe, UK; Sediag, France; Agdia, USA), none of these polyclonal antisera is specific for *Xap* since, in the authors experience, reactions are also obtained with other *Xanthomonas* species such as *X. campestris* pv. *campestris*, *X. hortorum* pv. *pelargonii*, *X. axonopodis* pv. *vitians*, *X. vesicatoria* and *X. cynarae*, and probably also with other *Xanthomonas* species. Consequently, they could be used for a rapid screening of the samples, or for detecting the presence of *Xanthomonas*-like bacteria, but not for the accurate identification of *Xap* (López *et al.*, 2010).

IDENTIFICATION BY FAME, BIOLOG AND MALDI-TOF MASS SPECTROMETRY ANALYSES

Fatty acids methyl ester profile (FAME) analysis. After growth for 48 h at 28°C on trypticase soy agar, putative colonies of *Xap* can be analyzed with an appropriate FAME procedure (Sasser, 1990). A positive FAME test is achieved when the profile of the presumptive culture compared to the library database has a Similarity Index of 0.500 or higher, with a separation of 0.100 between the first and the second choice (MIDI-Inc., USA; online: http://midi-inc.com/pdf/MIS_Similarity_Index.pdf) to that of the positive control. Scortichini *et al.* (1996) reported that the more abundant fatty acids are the branched ones 15:0 ISO and 15:0 ANTEISO, and the unsaturated fatty acid 16:1 w7c, after comparing the profiles of different *Xap* isolates. Although when comparing results by the MIS identification database TSBA.40, according to Stead *et al.* (1992) misidentification could occur since this database uses data of profiles obtained from *Xap* isolates cultured for 24 h, in our experience the tested strains were correctly identified using this database.

BIOLOG system. The Biolog GN microplate and the current OmniLog systems permit to differentiate the *Xanthomonas arboricola* genomic group from the other genomic groups belonging to the *Xanthomonas* genus, on the basis of several tests of metabolic activity on different carbon substrates (Vauterin *et al*., 1995).

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). In the framework of COST 873, whole-cell MALDI-TOF mass spectrometry has been applied to *Xap* and related pathovars or species in order to derive a comprehensive spectral reference database (MABRITEC AG, Switzerland) (Pothier *et al*., 2011b). Based on cross-referencing fingerprints with discriminatory peptide masses, rapid and reliable identification at the species, but also at the pathovar level is possible (Pothier *et al*., 2011b). This technique was successfully applied to putative *Xap* colonies grown for 48 h on peptone-yeast extract-glycerol agar (NYGA) (Turner *et al.*, 1984).

IDENTIFICATION BY MOLECULAR TECHNIQUES

Heat treatment can be used to prepare DNA from *Xap* from pure cultures for PCR amplification. Suspend a loop of bacteria (from a 72 h culture on YPGA or another medium) in 1 ml of sterile distilled water in a 1.5 ml Eppendorf vial (approximately 10^8 CFU ml^{-1}). Vortex and get a homogeneous suspension, treat it for 10-15 min at 95°C, cool on ice and use for amplification techniques. In some special cases (when the exopolysaccharide content is very high or unexpected negative amplification) it is convenient to perform DNA extraction (around 10^7 CFU ml^{-1} bacterial suspension) using the simple isopropanol procedure described above (Llop *et al.*, 1999).

Conventional PCR protocols. Several PCR protocols developed for *Xap* specific identification are detailed. Amplicons obtained by these conventional PCR protocols are visualized under UV light after electrophorosis on 1.5% agarose gels after staining with ethidium bromide solution (0.5 µg ml^{-1}) for 15-20 min.

PCR protocol from Pothier *et al.* (2011a). This protocol uses primers developed by Pagani (2004) for detection of a DNA fragment of a gene sequence encoding a putative protein related to an ABC transporter ATP-binding system in *Xap*. It has been evaluated in the frame of COST 873. No cross-reactions were observed with 68 saprophytic and epiphytic assayed isolates, associated with *Prunus* plants. However, cross-reactions were obtained with *Xanthomonas arboricola* pv. *corylina*, a quarantine pathogen of hazelnut; *X. arboricola* pv. *celebensis*, a banana pathogen; *X. arboricola* pv. *poinsettiicola* type C strains, a poinsettia pathogen; as well as with *X. arboricola* pv. *juglandis*, a walnut pathogen (Pulawska *et al.*, 2010; Ballard *et al.*, 2011; Pothier *et al.*, 2011a), none of which have been reported from *Prunus* species. It should be considered that the pathovars *corylina*, *juglandis*, *pruni*, and type C strains of pv. *poinsettiicola*, reclassified as *X. arboricola*, form a highly related group (Vauterin *et al.*, 1995). Moreover, it has been recently reported that these primers may fail to identify some non-European *Xap* strains (Ballard *et al*., 2011).

Primer sequences are: *Xap*Y17-F (forward) 5´-GAC GTG GTG ATC AGC GAG TCA TTC-3´, *Xap*Y17-R (reverse) 5´-GAC GTG GTG ATG ATG ATC TGC-3´. DNA is amplified in a final reaction volume of 20 µl using HotStarTaq Master Mix, or multiplex PCR kits (Qiagen, USA); 0.2 µM of each primer and 1 µl of sample. Similar results are obtained using other commercial *Taq* DNA polymerase (i.e., Promega, France; Quanta Biosciences, USA). PCR conditions: an initial activation step of 15 min at 95°C; 30 cycles (35 cycles for DNA extracted from plant samples), each consisting of 30 sec at 95°C, 30 sec at 55°C, and 60 sec at 72°C, and a final extension of 7 min at 72°C. The expected product is a 943 bp fragment.

Duplex-PCR protocol (Pothier *et al*., 2011a). A duplex-PCR assay was developed by combining the above mentioned pathovar-level primers (Pagani, 2004) with species-level primers based on species-specific regions of the quinate metabolic gene *qumA* (*Xarb*Q) (Pothier *et al*., 2011a). In this duplex assay, cross-reactions are again observed with *X. arboricola* pv. *celebensis*, *X. arboricola* pv. *poinsettiicola* and *X. arboricola* pv. *corylina*.

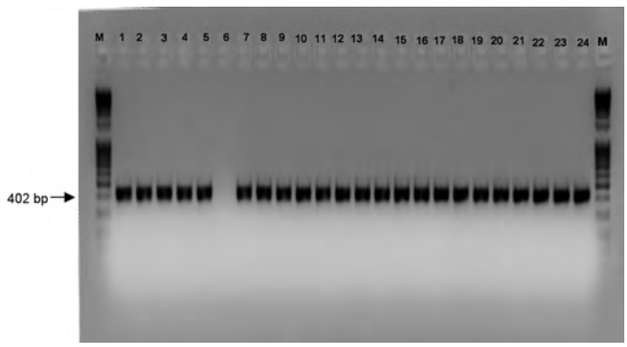

Fig. 3. Species-level simplex-PCR analyses (*Xarb*Q-F/*Xarb*Q-R primers) (Pothier *et al.,* 2011a) performed on *X. arboricola* pathovars (heat-treated pure cultures) Lane 1, *X. arboricola* pv. *celebensis*; lane 2, *X. arboricola* pv. *corylina*, lane 3, *X. arboricola* pv. *fragariae*; lane 4, *X. arboricola* pv. *juglandis*; lane 5, *X. arboricola* pv. *pruni*; lane 6, negative control (master-mix-only); lanes 7 to 24, *Xap* strains; M, molecular marker (100 DNA bp ladder extended, Roth).

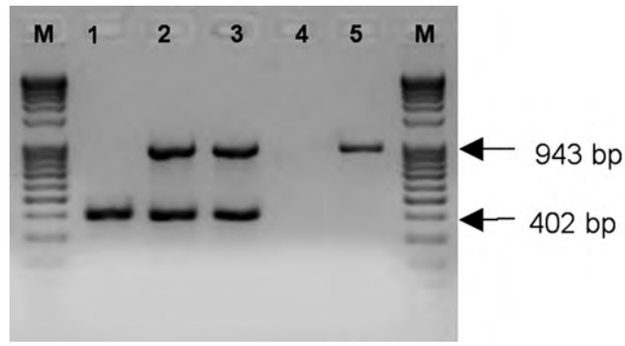

Fig. 4. *Xap* strain analysis. Lane 1, species-level simplex-PCR (*Xarb*Q-F/*Xarb*Q-R primers); lanes 2 and 3, duplex-PCR (*Xap*Y17-F/*Xap*Y17-R and *Xarb*Q-F/*Xarb*Q-R primers); lane 4, negative control (master-mix-only); lane 5, pathovar-level simplex-PCR (*Xap*Y17-F/*Xap*Y17-R primers); M, molecular marker (100 bp DNA ladder extended, Roth).

However, such cross-reactivity will probably not interfere with accurate diagnosis of stone fruits bacterial spot, since these related pathovars have been not reported from *Prunus* and the assay offers, in this way, a molecular test for several *X. arboricola* pathovars.

Primer sequences are: *Xap*Y17-F (indicated above), *Xap*Y17-R (indicated above), *Xarb*Q-F (forward) 5´-GCG CGA GAT CAA TGC GAC CTC GTC-3´, *Xarb*Q-R (reverse) 5´-GGT GAC CAC ATC GAA CCG CGC A-3´. PCR reaction mix and cycling conditions are the same as those of Pothier *et al.* (2011a) described above. Expected products are a 943 bp and a 402 bp fragment for *Xap*Y17 and *Xarb*Q primers, respectively (Fig. 3 and 4).

Multiplex-PCR protocol (Pothier *et al.,* 2011d). Based on the DNA sequence of the ubiquitous plasmid pXap41, whose presence was confirmed by plasmid profiles for eight representative *Xap* strains with a broad geographical origin, years and hosts, a multiplex-PCR assay was established by designing primers targeting genes spread over the plasmid and involved in its replication and mobilization (*repA1*, *repA2* and *mobC*). The assay confirmed the presence of pXap41 also on 35 additional *Xap* strains, thus covering the full range of genotypes described by Boudon *et al.* (2005) and Zaccardelli *et al.* (1999) (Pothier *et al*., 2011d). Pathovar-level discriminatory power of this PCR method was shown by the amplification of a geographically and genetically representative collection of *Xap* isolates, whereas no amplification was obtained with six other *X. arboricola* pathovars assayed, i.e. pvs *juglandis*, *corylina*, *fragariae*, *celebensis*, *populi* and *poinsettiicola* (type C strains). There is no available information regarding sensitivity level of this protocol.

Primers sequences are: pXap41repA1-F (forward) 5' GCG AGG ACA TGG CTT TCA C 3'; pXap41repA1-R (reverse) 5' GCG GCC AAG GCG TGC ATC TGC 3'; Xap41repA2-F (forward) 5' TAC CAA GAG CGG CAA CAT CTG C 3'; Xap41repA2-R (reverse) 5' TTT GGC CTT GCT GTA GAG CGT 3'; Xap41mob-F (forward) 5' GCC TAT CTG GCG AAG GTC GAG 3'; Xap41mob-R (reverse) 5' GCT TGT AGC TCG GCC AGG ATG 3'. Amplifications are carried out in a final volume of 20 µl using AccuStart PCR Supemix (Quanta Biosciences, USA) and 0.2 µM of each primer. PCR parameters are: an initial activation step of 5 min at 95°C; 30 cycles, each consiting of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, and a final extension of 7 min at 72°C. Expected products are DNA fragments of 343 bp (repA1 primers), 451 bp (repA2 primers), and 245 bp (mob primers).

PCR and Bio-PCR (Park *et al.*, 2010). This protocol has not been evaluated in the frame of the COST 873. It uses *Xap* specific primers targeting the *hrp* gene cluster region and the size of the expected product is 243 bp.

Real-time for Xanthomonas arboricola species (adapted from Weller *et al*., 2007). A modification of the protocol from Weller *et al.* (2007) was used during the COST 873 Short term Training Mission-Plant Bacteriology 2008 (Central Science Laboratory, York, UK, http://www.cost873.ch/_uploads/_files/CLS_COST873_TrainingManual.pdf). Primers and probe (*Xaf pep*) were designed within regions of the prolyl endopeptinase housekeeping gene (*pep*) by sequencing copies of this gene from both *X. fragariae* and *X. arboricola* pv. *fragariae*. Homologues of the *pep* gene have been found in a large number of bacteria and sequence divergence

between the *X. arboricola* and other *Xanthomonas* allowed the design of an assay specific for the *X. arboricola* species. The real-time PCR with *Xaf pep* primers and probe also detects strains of *X. arboricola* pathovars *pruni*, *corylina* and *fragariae*.

Primers and probe sequences are: *Xaf-pep*-F (forward) 5' GCG TGC CGC AGC CGC 3'; *Xaf-pep*-R (reverse) 5' CCG GTG GGC TTG GCG CCG 3'; *Xaf-pep*-P (probe) 5' FAM CCG GAA ACC GGC AAG AAG GCA TAM RA 3'. The standard 25 µl volume reaction mixture consists of 4 µl cell DNA extract; 1 x PCR buffer; 3.5 mM $MgCl_2$; 200 µM dNTPs; 300 µM primers; 100 nM probe; 0.63 U *Taq* DNA polymerase. PCR parameters are: an initial step of 10 min at 95°C; 40 cycles, each consiting of 15 sec at 95°C, 60 sec at 60°C (annealing time can be reduced to 30 sec if using the SmartCycler system). This programme was optimised for use with the ABI 7700 and 7900 sequence detector TaqMan systems and the Cepheid SmartCycler system. The expected amplicon is 64 bp.

Quantitative real-time (Palacio-Bielsa *et al.,* 2011), Primers and probe utilised in this protocol were based on primers from Pagani (2004). As for some of the conventional PCR protocols, positive signals were also obtained for *X. arboricola* pv. *corylina* and *Xanthomonas citri* subsp. *citri*, a citrus pathogen never reported on *Prunus* species.

Primers and probe sequences are: Xap-2F (forward) 5' TGG CTT CCT GAC TGT TTG CA 3'; Xap-2R (reverse) 5' TCG TGG GTT CGC TTG ATG A 3'; Xap-2P (probe) (5'-6-carboxyfluorescein [FAM]-TCA ATA TCT GTG CGT TGC TGT TCT CAC GA 6-carboxytetramethylrhodamine [TAMRA]-3'). A 25 µl volume reaction mixture contains: 2.5 µl sample, 0.4 µM each primer, 12.5 µl master mix (QuantiMix Easy Probes kit, Biotools) and 150 nM TaqMan probe. PCR parameters are: an initial denaturation step at 95ºC for 5 min, followed by 45 cycles, each one consisting of 1 min at 95°C and 1 min at 59ºC. The expected product is a DNA fragment of 72 bp. This programme was optimised for use with a Smartcycler (Cepheid Inc., USA), but similar results are also obtained with other real-time thermal cyclers such as LightCycler 480 (Roche, USA) (see López *et al.*, this issue).

Real-time SYBR Green I assay and Bio-PCR (Ballard *et al.*, 2011). This protocol has not been evaluated in the framework of COST 873. The authors used suppression substractive hybridization to generate a *Xap* substracted DNA library, using *X. arboricola* pv. *corylina* as the driver strain. The primer pair 29F/R designed from the cloned sequence amplifies a 344 bp amplicon apparently capable of differentiating *Xap* from all other *X. arboricola* pathovars.

Fingerprinting PCR protocol (REP-PCR). This rapid and inexpensive fingerprinting method utilises primers against repetitive elements in the bacterial genome such as the repetitive extragenomic palindromic elements (REP elements) (Stern *et al.*, 1984; Louws *et al.*, 1994). Pure cultures of *Xap* can be identified by REP-PCR (Anonymous, 2006a; Dreo *et al.*, this issue).

IDENTIFICATION BY PATHOGENICITY TESTS

Detached leaf assay (Randhawa and Civerolo, 1985*).* This test is advised for a rapid screening of putative *Xap* isolates and is recommended in the EPPO protocol (Anonymous, 2006a). Use young fully expanded leaves (3rd-6th leaf from the top) detached from glasshouse-grown peach or plum seedlings or rootstocks susceptible to *Xap,* such as peach cvs Sunhigh, Barrier, Catherine, Parade, Royal Glory or Reach Lady, or plum cvs 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 hosts and cultivars (Du Plessis, 1988; Scortichini *et al.*, 1996). Wash the leaves with sterile water and disinfect for 40-60 sec with 70% ethanol. Rinse repeatedly in sterile water and use immediately for inoculation. Bacterial suspensions from 48-72 h cultures at a concentration of about 10^7 CFU ml^{-1} in sterile distilled water or PBS can be utilised. Leaves, or parts of them, abaxial side upward, are placed on several layers of sterile blotting paper. Distal leaf portions are preferred for inoculation because of the ease of infiltration. Inoculum is infiltrated using a syringe without needle and applying a constant pressure against the leaf until an area of mesophyll tissue 2 to 4 mm in diameter is water-soaked. Inoculate 8-10 sites on each leaf approximately 1 cm apart. The leaves can be lightly blotted to remove any excess of inoculum. Place all inoculated leaves in Petri dishes containing 0.5% water agar and incubate for two weeks at 25°C under fluorescent lights (60-75 µE S^{-1} m^{-2}), with a 16:8 h photoperiod. The test is positive if after 6-9 days all inoculated sites exhibit confluent water soaking, becoming dark brown and brittle necrotic spots often surrounded by a margin.

Plant inoculation assay. Following Randhawa and Civerolo (1985), infiltrate a bacterial suspension of about 10^7 CFU ml^{-1} in sterile distilled water or PBS from a 48 h culture in young leaves on young shoots using a syringe without needle, as indicated for detached leaves. Following Du Plessis (1988), plants should be maintained at 25-27°C and 95-100% relative humidity (RH) for 8 h before being sprayed with a bacterial suspension on the young but fully expanded leaves from the tip of the shoots on the abaxial side with a spray gun connected to an air compressor. Plants should be main-

tained under glasshouse conditions at about 25°C under plastic bags for 48 h, then at high humidity. Lesions can be recorded 1-4 weeks after inoculation. In all assays, the negative control is treated with sterile distilled water or PBS and the positive control with a suspension of about 10^7 CFU ml^{-1} of a known *Xap* strain. Re-isolations must be performed from lesions to confirm the presence of *Xap* colonies and identify them using the methods mentioned above to fulfill Koch's postulates.

Hypersensitive reaction (HR) assay. Perform HR reaction on tobacco (e.g., cvs Samsun or Xanthi) or tomato (e.g., cvs Moneymaker or Roma) leaves with a bacterial suspension from a 24-48 h culture at a concentration of about 10^9 CFU ml^{-1} in sterile distilled water or PBS (Klement *et al*., 1964). Typical HR in tomato leaves is observed after 24-48 h, but in tobacco usually only after 3 days. In some cases, an atypical HR in tobacco can be observed as a loss of turgidity in the infiltrated area after 24 h, which becomes chlorotic after 48 to 72 h. Five days after inoculation, infiltrated sites show a collapse of the tissue surrounded by a chlorotic area. An HR in tobacco is sometimes obtained at 48 h using bacterial cultures grown on King´s B medium (King *et al.*, 1954) for 24-48 h at 25ºC (Roselló, 2007).

CONCLUDING REMARKS

There are several methods currently available for *Xap* detection and identification. As to isolation procedures, there is a lack of selective media with good recovery of the target from different types of samples, and of comparative assays for evaluating them. According to our experience, specific antibodies are not commercially available. Regarding the diverse PCR protocols, further assays using symptomatic and symptomless material from different hosts are still necessary to comparatively evaluate their sensitivity, specificity and accuracy. All PCR protocols based on the gene sequence expressing a putative protein related to an ABC transporter ATP-binding system in *Xap* (Pagani, 2004) show non-desired reactions with other *Xanthomonas* species. Nevertheless, they can still be used for *Xap* identification since no other xanthomonads have been reported from *Prunus* species up to now. There are many different techniques available for *Xap* identification, and a combination of biochemical, PCR and pathogenicity tests usually provides a good accuracy. Rapid techniques, as fatty acids profile or Biolog and OmniLog systems, or the recently developed MALDI-TOF MS, can also afford specific identification.

The availability of the genome sequence of the representative European *Xap* strain CFBP 5530, the first of this species, will be of great value for improving detection and identification methodologies for this quarantine pathogen. For example, the multiplex PCR developed by Pothier *et al.* (2011d) based on the complete sequence of the ubiquitous plasmid pXap41 could be a new potential tool for specific *Xap* detection.

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