DETECTION AND IDENTIFICATION METHODS AND NEW TESTS AS DEVELOPED AND USED IN THE FRAMEWORK OF COST873 FOR BACTERIA PATHOGENIC TO STONE FRUITS AND NUTS

Xanthomonas arboricola pv. *corylina*

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SUMMARY

Timley and reliable detection of the hazelnut pathogen *Xanthomonas arboricola* pv. *corylina (Xac)* is essential for the production of good quality disease-free planting material. In order to improve knowledge on diagnostic tools for this quarantine bacterium, the effectiveness of laboratory methods recomended by EPPO and additional modified procedures developed within COST873 was compared. Methods included the classical, biochemical, serological, pathogenicity and molecular approach. To confirm the presence of the pathogen in plant tissue, a combination of different techniques is necessary. For successful isolation of *Xac* cells from different hazelnut organs, general nutrient agar or semi-selective glucose amended media are recommended. Traditional biochemical techniques are still necessary for the identification of isolates at the species level. ELISA and IF are useful as screening assays for the early and rapid pathogen detection. In addition, PCR-based methods proved to be useful for the rapid detection of *Xac* cells, although highly pathovar-specific genetic tool is not available. Identification at the pathovar level requires pathogenicity tests on hazelnut plants that enable confirmation of strains' pathogenic nature and reproduction of symptoms. However, a choice of various pathogenicity testing procedures should be made based on testing material available and type and dynamics of symptom development.

Key words: diagnostic procedures, isolation, culture media, biochemical techniques, immunofluorescence, ELISA, PCR, pathogenicity, *Xanthomonas arboricola* pv. *corylina*.

INTRODUCTION

Xanthomonas arboricola pv. *corylina (Xac)* (Miller *et al.*, 1949) Vauterin *et al.* 1995, (syn. *Xanthomonas campestris* pv. *corylina*) is a Gram-negative bacterium that causes bacterial blight of hazelnut. The disease was initially described and the pathogen isolated from *Corylus maxima* in Oregon (USA) in 1913 [Barss (1913), in EPPO Standards, PM 7/22]. *Xac* is now widely distributed and it causes a potentially serious disease, resulting in economic losses to hazelnut production worldwide (Anonymous, 1986). Representing a phytosanitary risk for the EPPO region, the pathogen is regulated as an EPPO A2 quarantine pest of potential economic importance (Anonymous, 2004). Yield reduction is caused by dieback of fruit-bearing twigs and branches and premature fruit drop, which affects the vitality and further development of the trees (Miller *et al.*, 1949; Obradovic *et al.*, 2008). In severe cases, infections can cause from 10 to 100% tree death, especially in young plantations and nurseries (Miller *et al.*, 1949; Moore, 2002; Pulawska *et al.*, 2010). In countries where the hazelnut industry is small and of limited importance, this pathogen has received limited attention or has remained undetected (Calic *et al*., 2010). Fast and effective strategies for preventing, detecting and diagnosing the pathogen are not available (Anonymous, 2004; Janse, 2010). The current diagnostic procedures consist mainly of methods developed for *Xanthomonas* spp. Diagnostic procedures available for *Xac* were reviewed and evaluated within the framework of COST873 in order to increase their effectiveness, as a prerequisite for an effective and timely protection strategy. These procedures include the classical, biochemical, serological and pathogenicity approach (still the main methodology for detection and identification of *Xac*) and molecular PCR-based methods.

HOST RANGE

Xac damages both cultivated and wild hazelnut (*Corylus* sp.) plants (Scortichini *et al.,* 2002). Bacterial blight is most significant on European hazelnut (*Corylus avellana*) which is commercially grown for nut production, but *C. pontica, C. maxima* and *C. colurna* are also susceptible as well (Anonymous, 1986).

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DETECTION AND IDENTIFICATION

Isolation. Surface-sterilize symptomatic plant material and blot dry. Excise small pieces of tissue at the border between apparently healthy and diseased tissue (water-soaked angular spots and lesions on leaves and bracts of flowers or fruits, necrotic buds, oily lesions or dark brown discoloration on woody tissue) using a sterile scalpel. Homogenize the sample in a few drops of sterile distilled water, using pestle and mortar or cut open with a sterile scalpel and leave for *ca*. 30 min in a tube with 2-5 ml sterile PBS 0.01 M, pH 7.2. Streak an aliquot (10-100 µl) of the resulting homogenate on culture media, preferably YPGA (yeast extract 5.0 g, bacteriological peptone 5.0 g, glucose 10 g, agar 20 g, distilled water to 1 liter, pH 6.5-7.0) or GYCA (yeast extract 5.0 g, D-glucose 10 g, $CaCO_3$ 30 g, agar 20 g, distilled water to 1 liter) and incubate 3-4 days at 25-28°C. Colonies of *Xac* on YPGA are round, shiny, domed, mucous and yellow in colour, reaching 2-3 mm in diameter in 3-4 days. Since culturing may fail, especially from material collected in advanced stages of infection due to the low number of bacterial cells or to competition with and overgrowth by saprophytic bacteria, sampling should be repeated from pooled fresh symptomatic samples.

Culture media. The most frequently used media for isolation are standard non selective (NA, King's B) and semi-selective media generally used for *Xanthomonas* spp. (GYCA, YDC, YNA) (Lelliott and Stead, 1987; Schaad *et al.,* 2001). Yeast-peptone-glucose agar (YPGA) is a rich medium used in most European laboratories for isolation of *Xanthomonas* spp. (J. Janse, personal communication). In order to avoid production of extracellular polysaccharides on nutrient rich media, use nutrient agar [NA (Difco, USA)] or otherwise add 23.3 g of nutrient broth (containing g l^{-1}, peptone 15 g, meat extract 3.0 g, NaCl 5.0 g, K_2HPO_4 0.3 g) and 18 g agar to 1000 ml distilled water, adjust pH to 7.0 and autoclave for 20 min at 121°C. A yeast extract nutrient agar (YNA) is recommended as general purpose medium for culturing and short-term maintenance of strains during research.

On NA medium *Xac* forms small (1-2 mm), pale-yellow, circular, convex and glistening colonies with regular edges (Fig. 1A). Glucose-containing media are recommended to facilitate the isolation as well as for presumptive diagnosis. A larger amount of mucoid polysaccharide (xanthan gum) produced on this media, differentiates *Xac* strains from other often commonly occurring saprophytes with dark-yellow colony pigment (Fig. 1B). Wilbrink´s medium (Sands *et al*., 1986) is also recommended for successful isolation and culturing of pure strains, as it counteracts acid formation in the medium, thus enabling fast growth and better bacterial survival (J. Janse, personal communication). Other (semi-selective) media such as brilliant cresyl-blue starch (BS), modified tween medium, or SX agar can also be employed for isolation and further identification (Mulrean and Schroth, 1981; Scortichini, 2002; Schaad *et al.*, 2001). These media are based on the ability of *Xac* to hydrolyze starch or tween and form typical translucent haloes in the medium surrounding the colony growth. In this way *Xac* colonies can be distinguished from yellow non-xanthomonads and other bacteria isolated from infected material.

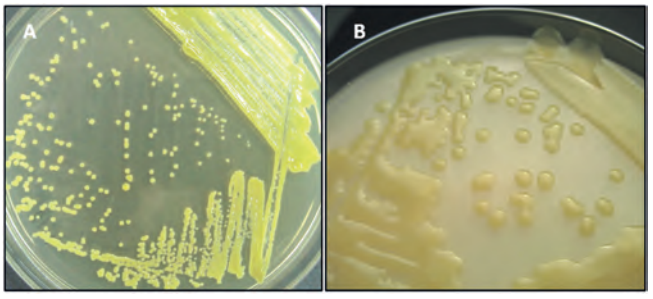

Fig. 1. Appearance of *Xanthomonas arboricola* pv *corylina* colonies on different nutrient media. A. Small, round, glistening colonies on NA medium. B. Mucous, domed growth on GYCA medium.

Storage of isolates/strains. Bacterial cultures can be maintained at room temperature in sterile tap water as a working culture for several months (personal observation), but this needs to be checked since quality of tap water can vary in different areas. For a long-term storage stock cultures can be preserved at -80°C in nutrient broth medium (NB) supplemented with 30% glycerol.

Reference strains. Pathovar reference strains of *Xac*

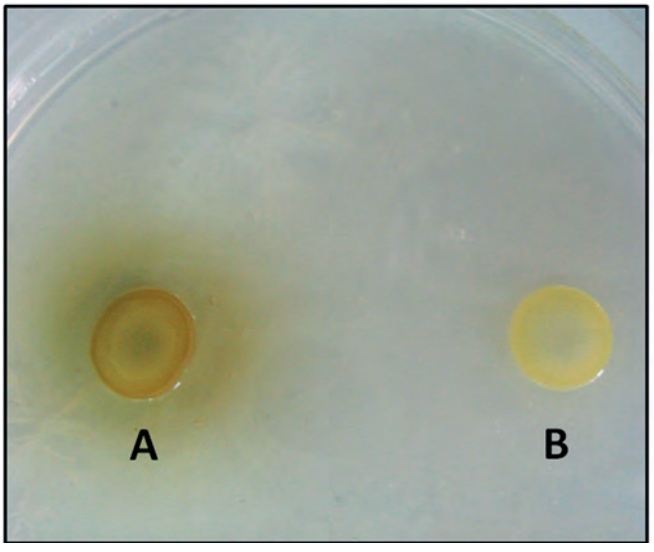

Fig. 2. Quinate metabolism test. A - positive reaction: production of a deep green discoloration around colony growth, B - negative reaction.

are: CFBP 1159; NCPPB 935; ATCC 19313; CNBP 1159; DSM 50854; DSM 60239; ICMP 5726; ICPB XC12; LMG 689; Miller 5262; NZRCC 10380; PDDCC 5726; Starr XC12; VdM 42; XC 12.

However, the type strain isolated from *Corylus maxima* in the USA was found to be distinct from *X. a.* pv. *corylina* strains isolated from *Corylus avellana* as far as phenotypic and genetic features are concerned (Pulawska *et al.*, 2010; Scortichini *et al.*, 2002). Therefore, other *Xac* strains can be used as a reference: NCPPB 2896 (*X. a.* pv. *corylina*, *Corylus avellana*-UK, 1976); NCPPB 2898; ICMP 7080; LMG 8659; Roberts A724a (*X. a.* pv. *corylina*, *Corylus avellana*-UK, 1976); NCPPB 3037; ICMP 7081; LMG 8660; Barnes 69-8 (*X. a.* pv. *corylina*, *Corylus avellana*-UK, 1978); NCPPB 3870 (*X. a.* pv. *corylina*, *Corylus avellana*, Italy, 1991); NCPPB 3339 (*X. a.* pv. *corylina, Corylus avellana*, France, 1984).

Biochemical tests. Traditional biochemical techniques can be used after isolation and pure culturing to support the identification of isolates at the genus or species level (Table 1). They are not discriminative enough to adequately identify *Xac* isolates, so further identification is necessary. *X. arboricola* species may be differentiated from the other *Xanthomonas* spp. by the ability to degrade quinate in succinate-quinate (SQ) medium (Lee *et al.*, 1992) where *X. arboricola* colonies produce a deep green colour, diffusing around the bacterial streak, which is recorded after 5-6 days of growth on the medium. Other *Xanthomonas* spp. will grow on this medium, but no colour change is produced (Fig. 2).

Serological techniques. *Xac* can also be identified by serological methods following the procedures described in EPPO protocols (Anonymous, 2009, 2010b) or a suitable procedure supplied with a commercial kit. Polyclonal antibodies for use in immunofluorescence (IF) and/or ELISA assays are available from Loewe Biochemica (Germany). These immunological tests are useful as screening for early and rapid pathogen detection,

Table 1. Bacteriological tests for the identification of *Xanthomonas arboricola* pv. *corylin*a.

Bacteriological techniques	Reaction	References
Isolation NA, YNA, KB, YDC, YPGA, GYCA		Schaad *et al.*, 2001; Lelliott and Stead, 1987.
Gram reaction	-	Schaad *et al.*, 2001.
Growth on GYCA medium	+	Anonymous, 2004.
Growth at 35°C	+	Schaad *et al.*, 2001.
Tolerance of NaCl, (2% and 5%)	+/-	Schaad *et al.*, 2001.
Oxidase reaction	-	Schaad *et al.*, 2001.
Starch hydrolysis	+	Lelliott and Stead, 1987.
Esculin hydrolysis	+	Lelliott and Stead, 1987.
Gelatin liquefaction	+	Lelliott and Stead, 1987.
Quinate metabolism	+	Lee *et al.*, 1992.
HR on tobacco/tomato[1]	+/+	Lelliott and Stead, 1987.
Oxidative/fermentative metabolism of glucose	+/-	Lelliot and Stead, 1987
Carbon source utilization		Anonymous, 2004; Pulawska *et al.*, 2010.
l-arabinose	+(-)	
d-arabinose	+	
glucose	+	
galactose	+	
mannose	+	
sucrose	+	
maltose	+ (-)	
trehalose	+	
cellobiose	+	
glycerol	+(-)	
l-xylose	-	
d-xylose	-(+)	
rhamnose	-	
lactose	-(+)	
raffinose	-(+)	
adonitol	-	
mannitol	-	
inuline	-	
sorbitol	-	
dulcitol	-	
erythritol	-	

+ positive reaction; - negative reaction; (-), (+), results recorded from Pulawska *et al.* (2010)

Table 2. Reaction mix based on a modified protocol for *X.a.* pv. *pruni* from Pagani *et al.* (2004).

PCR Mix	Final concentration	Volume (µl)
Sterile water (molecular grade)		9.96
TaqBuffer with KCl (10X) [100 mM Tris-HCl (pH 8.8 at 25°C pH 7.5), 500 mM KCl and 0.8% (v/v) Nonidet P40]	1X	2
$MgCl_2$ (25 mM)	2 mM	1.6
dNTP (10mM)	0.2 mM	0.4
DMSO	4%	0.8
Primer Y17CoF (10 µM)	0.5 µM	1
Primer Y17CoR (10 µM)	0.5 µM	1
Primer Xarb-F (10 µM)	0.5 µM	1
Primer Xarb-R (10 µM)	0.5 µM	1
Taq Polymerase (5 U/µl)	1.2 U	0.24
DNA template		1
Total		20 µl

but they lack sufficient specificity and/or sensitivity. Because of the risks of obtaining false positive and false negative recordings, positive samples should be subjected to further confirmatory tests, using the pure culture(s) obtained.

DNA-based techniques. DNA can be extracted from pure cultures using a slightly modified method from Scortichini *et al.* (2002), i.e. wash a small amount of a 24-48 h old bacterial culture grown on NA at 25-27°C with 0.85% NaCl in a 1.5 ml micro-centrifuge tubes. Centrifuge the suspension at 8,900 *g* for 2 min, discard supernatant and resuspend the pellet in 0.85% NaCl at an optical density corresponding to 10^8 CFU ml^{-1} (OD_{600} = 0.3). Place the tubes in boiling water for 10 min, than rapidly cool on ice for 20 min and store the extract at -20° C until use. DNA extraction from plant samples can be done according to the protocol by Llop *et al.* (1999).

PCR-based methods. Molecular tools that can be used for rapid diagnosis of *Xac* colonies include methods specific for *Xanthomonas* genus (Maes *et al.*, 1993) and *X. arboricola* species (Pothier *et al.*, 2011), while procedures for determination at pathovar level are based on rep-PCR or partial sequence alignments (see below). However, primers designed for *X. a.* pv. *pruni* (XapY17-F/ XapY17-R) based on a sequence encoding putative protein (*ftsX* gene), have proven useful for accurate identification of this pathogen, as they cross react with *corylina* strains (Pothier *et al.*, 2011). Moreover, *X. arboricola* specific primers (XarbQ-F/ XarbQ-R) corresponding to *qumA* gene sequence encoding for quinate

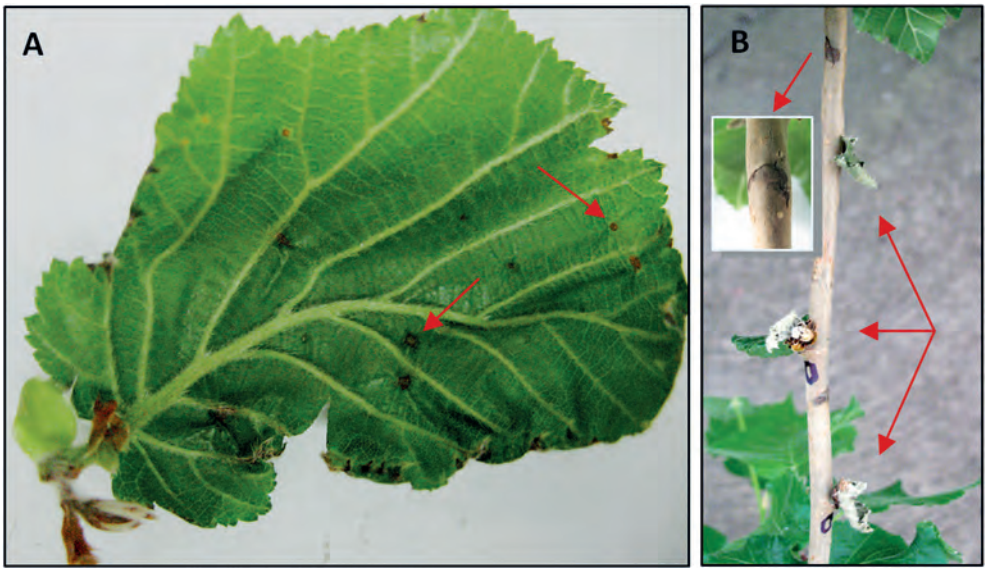

Fig. 3. Pathogenicity tests. A. water-soaked angular spots and lesions on leaves 30 days after inoculation. B. bud necrosis and dieback and necrotic lesions on the stem 6 months after inoculation.

metabolism and *Xap* specific primers used in a duplex PCR detect simultaneously both genes in *pruni* and *corylina* genotypes, enhancing diagnostic confidence (Pagani *et al*., 2004; Pothier *et al*., 2011; Pulawska *et al*., 2010). Names of the primers, oligonucleotide sequences and literature data are summarized in Table 2.

Duplex PCR (Pothier *et al*., 2011). PCR amplification can be carried out in a final volume of 20 µl using HotStarTaq Master Mix or multiplex PCR kits (e.g. Qiagen, Switzerland) and 0.2 µM (for specificity tests) or 0.5 µM (for sensitivity tests and *in planta* detection) of each primer. Amplification reaction can also be obtained using the following master mix reported in Table 3. The PCR reaction consists of an initial denaturation step for 15 min at 95°C, followed by 30 cycles (35 for DNA extracted from plant samples) of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and DNA extension at 72°C for 60 sec and final extension at 72°C for 7 min. PCR products (5 µl) are separated by agarose gel (1.5%) electrophoresis in Tris-acetate-EDTA (TAE) buffer, stained with ethidium bromide (1 µg ml^{-1}) and visualized under UV light.

Repetitive extragenic palindromic (Rep) PCR with BOX, REP and ERIC primers generates specific genomic fingerprints useful in identifying and differentiating *Xac* strains (Schaad *et al*., 2001; Anonymous, 2010a). ERIC and BOX fingerprints were found more discriminatory than REP for differentiation of *Xac* strains (Scortichini *et al*., 2002; Calic *et al*., 2009). Furthermore, partial sequence alignments of conserved *gyrB* gene proved to be reliable method for identification of *Xac* strains (Pulawska *et al*., 2010).

Pathogenicity tests. *Hypersensitive reaction (HR)***.** A rapid indicative test for pathogenicity is the HR assay on tobacco, tomato, sunflower, pepper or pelargonium leaves or on been pods. Introduce a bacterial suspension (10^6 CFU ml^{-1}) from a 24-48 h culture grown on NA at 26°C into the parenchyma between the upper and the lower epidermis of a fully expanded leaf of the test plant. Characteristic tissue collapse at the inoculation site can be observed within 24 h from inoculation, indicating the ability to induce a hypersensitivity reaction. HR on tobacco can be influenced by environmental factors (temperature, light, bacterial concentration), therefore tomato should be preferred for this test for *Xanthomonas* spp. (Lelliott and Stead, 1987).

Inoculation of the host plant (modifications of the pathogenicity test developed by Gardan and Devaux, 1987; for original pathogenicity testing protocol see Anonymous, 2004). In all tests, use a *Xac* reference

Table 3. PCR- based methods and primer sequences available for *Xac* identification.

PCR procedure	Primer pair sequence (5'-3')	Product size (bp)	References
Xanthomonas spp.	X1: AAGGATCGGGTATI'AAC X2: AGAGTTTGATCITGGCTCAG	480	Maes *et al*., 1993
X. arboricola	XarbQ-F: GCGAGATCAATGCGACCTCGTC XarbQ-R: GGTGACCACATCGAACCGCGCA	402	Pothier *et al*., 2011
X. a. pv. *pruni*	XapY17-F: GACGTGGTGATCAGCGAGTCATTC XapY17-R: GACGTGGTGATGATGATCTGC	943	Pothier *et al*., 2011
Rep PCR	Boxair: CTACGGCAAGGCGACGCTGACG Rep1R-1: III ICG ICG ICA TCI GGC Rep2-1: ICG ICTTATCIGGCCTAC Eric1R: ATGTAAGCTCCTGGGGATTCAC Eric2: AAGTAAGTGACTGGGGTGAGCG		Schaad *et al*., 2001
*gyr*B	XgyrPCR2F: AAGCAGGGCAAGAGCGAGCTGA Xgyrrsp1: CAAGGTGCTGAAGATCTGGTC		Parkinson *et al*., 2007

strain as a positive and sterile water as a negative control.

Inoculation of buds. Grow one-year-old susceptible hazelnut plants in a greenhouse in 15 cm pots containing soilless medium (Floragard, Floradur potting substrate). For testing, use plants entering the dormant phase. One day before inoculation, water the plants and cover with PVC bags to provide high humidity. Prepare the inoculum by suspending bacterial cells of a 48 h culture grown on NA agar at 26°C in sterile distilled water to an approximate concentration of 10^8 CFU ml^{-1} (OD_{600}= 0.3). Inoculate the buds of young lateral shoots by puncture-inoculation with a hypodermic syringe, applying about 0.5 ml of bacterial suspension until run off. Inoculate several buds with each strain. Cover the treated plants with the plastic bags for two days, then remove. Appearance of disease symptoms requires from 14 days to one month (Fig. 3A).

Inoculation of buds and stem. Prepare bacterial inoculum and plant material as described above. Cut back the plants entering the dormant phase after leaf fall to 80-100 cm of height before inoculation. Using a sterile sharp scalpel, make two cuts 0.5 mm deep in the parenchyma tissue of the upper part of the stem, at about 45° angle with the stem axis, and longitudinal cuts on three randomly selected buds in the same part of the stem. Plant tissue along the stem and buds are wounded for giving the pathogen the chance of penetrating not only throughout natural openings. For favouring penetration, add a surfactant [e.g. Silwet 77 (500 µl l^{-1})] in the bacterial suspension just prior to inoculation and mix cautiously (since the surfactant tends to produce foam if agitated). Perform the inoculation by immersing the upper part of the plant into the inoculum in a 0.5 liter graduated cylinder for 10 sec. After inoculation seal the wounds on the stem with parafilm for protection and prevention from fast dessication. Cover the plants with plastic bags for two days and maintain in the greenhouse until the next spring. Observe for appearance of bud and stem necrosis and bud dieback (Fig. 3B) after the incubation period. Drawbacks related to this method are the long duration of the whole process, the development of symptoms restricted to the site of inoculation and the possibilty of inoculation failure.

Inoculation of leaves (Pulawska *et al*., 2010). Infiltrate young leaves of one-year-old hazel shoots (e.g. cv. Webb's Prize Cob) under greenhouse conditions, with a 10^7 CFU ml^{-1} suspension of a 48 h culture grown on NA. Cover the shoots with plastic bags for 48 h. Observe the appearance of brown, necrotic spots at the place of infiltration.

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