Diagnostics
Diagnostic

Xanthomonas oryzae

Specific scope

This standard describes a diagnostic protocol for *Xanthomonas oryzae* pathovars *oryzae* and *oryzicola*.

Specific approval and amendment

Approved in 2007-09.

Introduction

The species *Xanthomonas oryzae* includes two pathovars, namely, *oryzae* and *oryzicola* (Swings *et al*., 1990). These bacterial pathogens are closely related organisms and were earlier named as pathovars of *Xanthomonas campestris*. Rice is the main host for both pathogens, which are seed-borne and seed-transmitted.

Bacterial leaf blight of rice (BLB) was first reported in Fukuoka Prefecture, Japan, during 1884 in rice affected by *X. oryzae* pv. *oryzae*. This disease is considered one of the most serious rice diseases worldwide although it has declined in incidence in Japan since the mid 1970s. Nevertheless it is still prevalent worldwide. The disease was reported in South East Asia in the early 1960s, where it is currently widespread, and it still affects the rice crop in its severe form (Goto, 1992). It has also been reported in several African countries, in Australia, North America (Lousiana and Texas, US), Central and South America (OEPP/EPPO, 2006a) but it is only of economic importance in Asia. Other reported hosts include wild or minor cultivated Poaceae: *Brachiaria mutica, Cenchrus ciliaris, Cyperus difformis, C. rotundus, Cynodon dactylon, Echinochloa crus-galli, Leersia* spp. (*Leersia hexandra, L. oryzoides), Leptochloa chinensis, Oryza* spp., *Panicum maximum, Paspalum scrobiculatum, Zizania aquatica, Z. latifolia, Z. palustris,* and *Zoysia japonica* (Li *et al*., 1985; Bradbury, 1986; Valluvaparidasan & Mariappan, 1989; EPPO/CABI, 1997; Saddler, 2002a). Cyperaceae (sedges) that are naturally infected include *Cyperus difformis* and *C. rotundus*. Variation in virulence has been observed between isolates, and many races have been described; however, the different races have not been clearly defined with specific reactions being assigned to each variety (Mew, 1987). The diversity of the pathogen has been analysed based on virulence and PCR-based DNA fingerprinting (George *et al*., 1994). Low virulence strains have been reported in the United States and India (Jones *et al*., 1989; Gnanamanickam *et al*., 1993).

Bacterial leaf streak of rice (BLS) is caused by *X. oryzae* pv. *oryzicola*. The disease is present in Tropical Asia, West Africa and Australia (OEPP/EPPO, 2006b). *X. oryzae* pv. *oryzicola* has reached epidemic proportions in recent years in China. Other hosts affected by the pathogen are: *Oryza* spp., *Leersia* spp., *Leptochloa filiformis*, *Paspalum orbiculare*, *Zizania aquatica, Z. palustris* and *Zoysia japonica*. No races have been recorded, however, differences in virulence of strains have been observed in many countries (Vera Cruz *et al*., 1984; Adhikari & Mew, 1985; Saddler, 2002b).

Further information on the biology and ecology of the species can be found in the EPPO data sheet on *X. oryzae* (OEPP/EPPO, 1997)

Identity

Taxonomic position: Kingdom: Bacteria; Phylum: Proteobacteria; Class: Gammaproteobacteria; Order: Xanthomonodales; Family: Xanthomonodaceae; Genus: Xanthomonas (Dowson, 1939)
Name: *Xanthomonas oryzae* pv. *oryzae* (Ishiyama 1922) Swings *et al*. (1990)
Synonyms: *Xanthomonas campestris* pv. *oryzae* (Ishiyama 1922) Dye 1978; *Xanthomonas oryzae* (Ishiyama, 1921) Dowson 1943; Other synonyms are *Xanthomonas itoana, Xanthomonas kresek*, *Xanthomonas translucens* f. sp. *oryzae*
EPPO computer code: XANTOR
Phytosanitary categorization: EPPO A1 list no. 2, EU annex II/A1 as *Xanthomonas campestris* pv. *oryzae*.

Xanthomonas oryzae pv. *oryzicola*

Name: *Xanthomonas oryzae* pv. *oryzicola* (Fang *et al*., 1956) Swings *et al*. (1990)

Fig. 1 Symptoms of *X. oryzae* pv. *oryzae* (bacterial leaf blight). (a) Rice field plants with *X. oryzae* pv. *oryzae* symptoms; (b) *X. oryzae* pv. *oryzae* symptoms in rice nursery beds; (c) Rice seedlings showing *X. oryzae* pv. *oryzae* symptoms; (d) Wilting of transplanted seedlings; (e) Kresek symptoms in field plants; (f) Progressive development of bacterial leaf blight in rice plants grown from infected seed.

Synonyms: *Xanthomonas campestris* pv. *oryzicola* (Fang *et al.,* 1956) Dye 1978; *Xanthomonas oryzicola* (Fang *et al*., 1956) Dowson 1943; *Xanthomonas translucens* f. sp. *oryzicola* (Fang *et al*., 1956) Bradbury 1971
EPPO computer code: XANTTO
Phytosanitary categorization: EPPO A1 list no. 3, EU annex II/A1 as *Xanthomonas campestris* pv. *oryzicola*.

Detection

Disease symptoms

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* can be clearly distinguished by symptoms, which reflect the differences in their modes of infection.

Bacterial leaf blight of rice (BLB)
X. oryzae pv. *oryzae* enters either through wounds or hydathodes, multiplies in the epitheme and moves to the xylem vessels where active multiplication results in blight on the leaves. The symptoms of the disease include leaf blight, wilting (kresek), (Fig. 1) and pale yellow leaves. Leaf blight is characterized by wavy elongated lesions, which develop along the leaf margins. They start as small water-soaked stripes from the tips where water pores are found and rapidly enlarge in length and width, forming a yellow lesion with a wavy margin along the lead edges. Later on, diseased areas turn white to grey. These lesions can develop on one or both sides of the leaf and occasionally along the midribs, and leaf blight symptoms generally occur from maximum tillering stage and onwards. In young lesions, drops of bacterial ooze can be observed early in the morning. On panicles the disease causes grey to light brown lesions on glumes that result in infertility and low quality of the grains. Kresek is the result of systemic infection that is common in the tropics in young plants and during the tillering stage of susceptible cultivars. Leaves of infected plants wilt, roll up, turn grey-green and whither, and entire plants finally die. Surviving plants look stunted and yellowish. Yellow or pale yellow leaves are due to systemic infections that appear at tillering stage; the youngest leaves become uniformly pale yellow or show a broad yellow stripe, and bacteria are found in the internodes and crowns of affected stems, but not in the leaf itself (Ou, 1985; Goto, 1992).

Bacterial leaf streak of rice (BLS)
X. oryzae pv. *oryzicola* is a foliar disease that appears at any growth stage of the host. Cells of *X. oryzae* pv. *oryzicola* enter

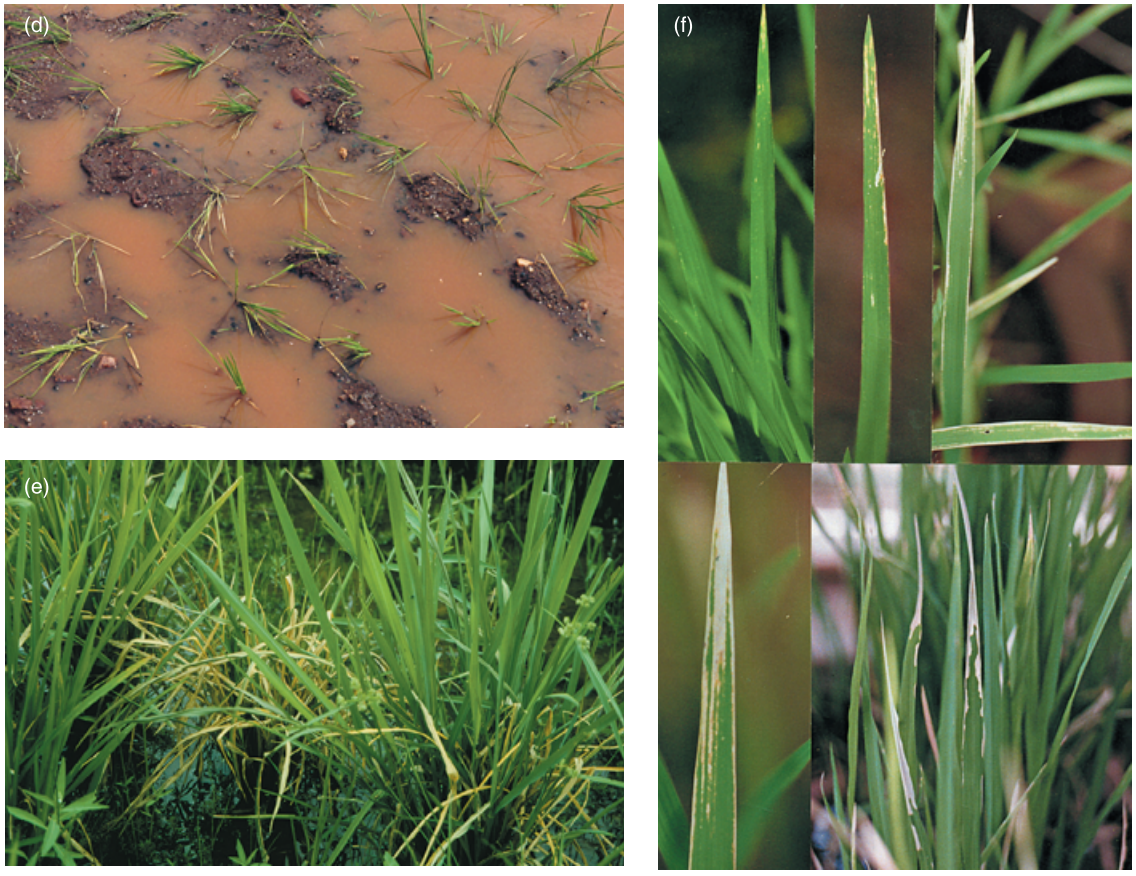

Fig. 1 *Continued*

Fig. 2 Symptoms of bacterial leaf streak in rice plants.

through the stomata and multiply in the parenchyma tissues of the leaves. *X. oryzae* pv. *oryzicola* infects mainly the parenchyma of the cells of the leaves, but is not systemic. Initial symptoms are small water-soaked, transparent interveinal streaks (Fig. 2), which may elongate and darken. The transparent streaks differentiate leaf streak lesions from those of *X. oryzae* pv. *oryzae* that are opaque against the light. Bacterial exudates can be observed as tiny yellow beads. The narrow, long, translucent lesions may coalesce, forming large patches, and severely affected fields appear burnt. It is at this stage that leaves wither, turn brown and eventually die, and the disease can be difficult to distinguish from bacterial leaf blight (Ou, 1985).

Isolation

Symptomatic samples are processed individually or in small groups. Precautions are made to avoid cross contaminations when collecting the samples and during the extraction process. The samples should be processed as soon as possible after collection and conserved at 4–8°C until use. Freshly prepared sample extracts are necessary for a successful isolation. Several procedures have been used for the isolation of *X. oryzae* pv. *oryzae* from symptomatic and asymptomatic plant parts, including seeds. Most of the procedures described for the isolation of *X. oryzae* pv. *oryzae* from rice plants, can also be applied for the isolation of *X. oryzae* pv. *oryzicola*. However, it is known that both bacteria grow slowly on the isolating media and can be overgrown by fast growing contaminants. Often these contaminants are of yellow colour (e.g. *Pantoea agglomerans* and *Xanthomonas*-like saprophytic bacteria) thus making difficult the observations of colonies of the target organisms and

are often visually undistinguishable in colony morphology, growth, and colour from strains of both pathogens. Culturing may fail from advanced stages of infection due to competition or being overgrown by saprophytic organisms and also from seeds with low levels of inoculum or under bad conditions of storage. If disease symptoms are typical but isolation is negative, the isolation step should be repeated.

Direct plating from symptomatic and asymptomatic leaves collected from the field

Bacterial leaf blight in temperate regions can usually be observed during the latter part of the seed bed stage (Ou, 1985). The bacteria can be detected from the upper part of infected leaves before symptoms appear (Goto, 1965; Misukami & Wakimoto, 1969; Sakthivel *et al*., 2001). Tabei (1967) reported that symptomless rice seedlings may carry the pest.

The isolation of *Xanthomonas* from symptomatic material is preferable and can be performed using Peptone sucrose agar (PSA), Nutrient Broth Yeast Extract agar medium (NBY), Growth Factor (GF) agar (Agarwal *et al*., 1989; Sakthivel *et al*., 2001). (Appendix 1). The bacteria can also be isolated on nutrient agar (NA) but with a very slow growth; semi-selective agar substrates like modified XOS agar medium (mXOS) (Di *et al*., 1991; Gnanamanickam *et al*., 1994). *X. oryzae* pv. *oryzicola* colonies are also isolated on modified Wakimoto's agar (Mew & Mistra, 1994) developed for the detection of *X. oryzae* pv. *oryzae,* but with the omission of ferrous sulfate.

Surface disinfection with 70% alcohol for 15 s followed by two to three times rinsing with sterile water is used for isolation from field plants. Plant parts showing fresh symptoms and with exudates are selected whenever possible. Sections (2 mm × 7 mm) from infected tissue and preferably from the advancing portion of lesions are selected. The exudates can be processed separately in 1 mL of sterile water. Narrow sections of infected tissue are cut with a sterilized razor blade or scalpels, placed in a drop of sterile water and covered with a cover slip before the observation under the compound microscope for bacterial streaming, an indication of bacterial presence. Drops of leaf extract are streaked onto the selected media plates and incubated at 27 ± 2°C (Agarwal *et al*., 1989; Mew & Mistra, 1994). Characteristics of the colonies are described in the section: general characteristics (Fig. 3). These selected colonies are transferred to NBY, PSA or Nutrient Agar (NA) plates for 1–2 days for purification and further identification. Isolates are maintained for longer periods at −80°C, e.g. in bacterial preservers (i.e. Protect, Technical Service Consultants Ltd., GB).

Direct isolation from seeds

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* are found in the glumes and occasionally within the endosperm of seed collected from heavily infected fields (Fang *et al*., 1956; Srivastava & Rao, 1964).

Samples of 400 seeds are crushed to coarse flour that is suspended in 200 mL of sterile sodium chloride (0.85%) (sterile saline solution) (Agarwal *et al*., 1989; Mortensen *et al*., 1994). Similar recovery values of *X. oryzae* pv. *oryzae* have been obtained from extractions conducted with samples of 400 or 10 000 seeds (Dikin, 1992). The suspension is incubated for 2 h at room temperature. Samples are stirred every 30 min in order to favour aeration; a sample of 1 mL is taken from the seed extract to prepare three (1:10) serial dilutions in Nutrient broth (NB) or sterile saline solution. Aliquots of 50 µL or 100 µL of the undiluted and diluted suspensions are streaked onto the selected agar plates (GF or mXOS) by duplicate. Plates are incubated at 27 ± 2°C for 3–5 days. Colonies on GF are very small, yellow and shiny and, on mXOS colonies, have a characteristic rose-pink colour, glistening and mucous (Di *et al*., 1991) (Fig. 3).

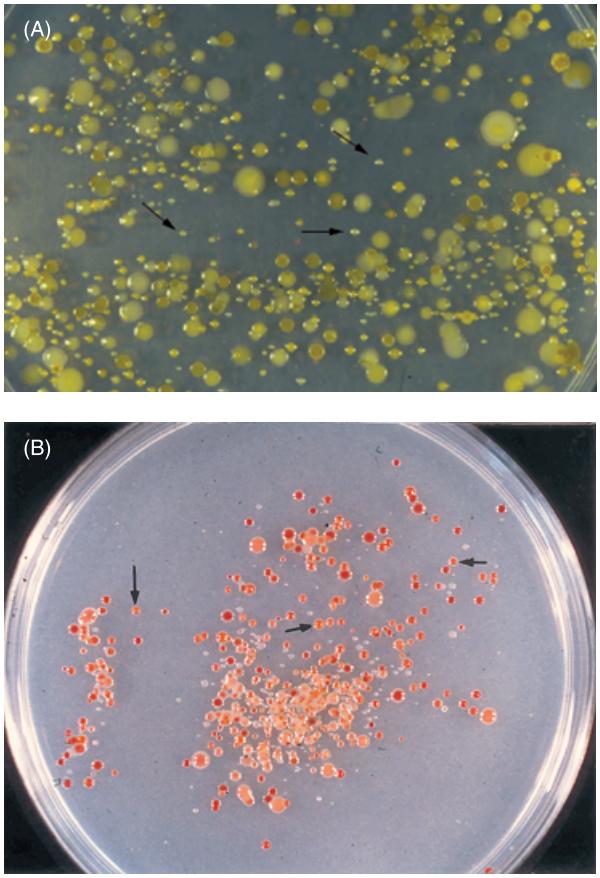

Fig. 3 A: Small, shiny, raised colonies of *Xanthomonas oryzae* pv. *oryzae* on GF medium; B: colonies of the bacterial leaf blight organism on mXOS agar medium.

Rapid screening tests (Sakthivel *et al*., 2001)

Detection from asymptomatic leaves by PCR

Samples of one gram from asymptomatic leaves or seedlings are taken from individual plants for the detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Genomic DNA from plants is extracted and used for the PCR assay using primers

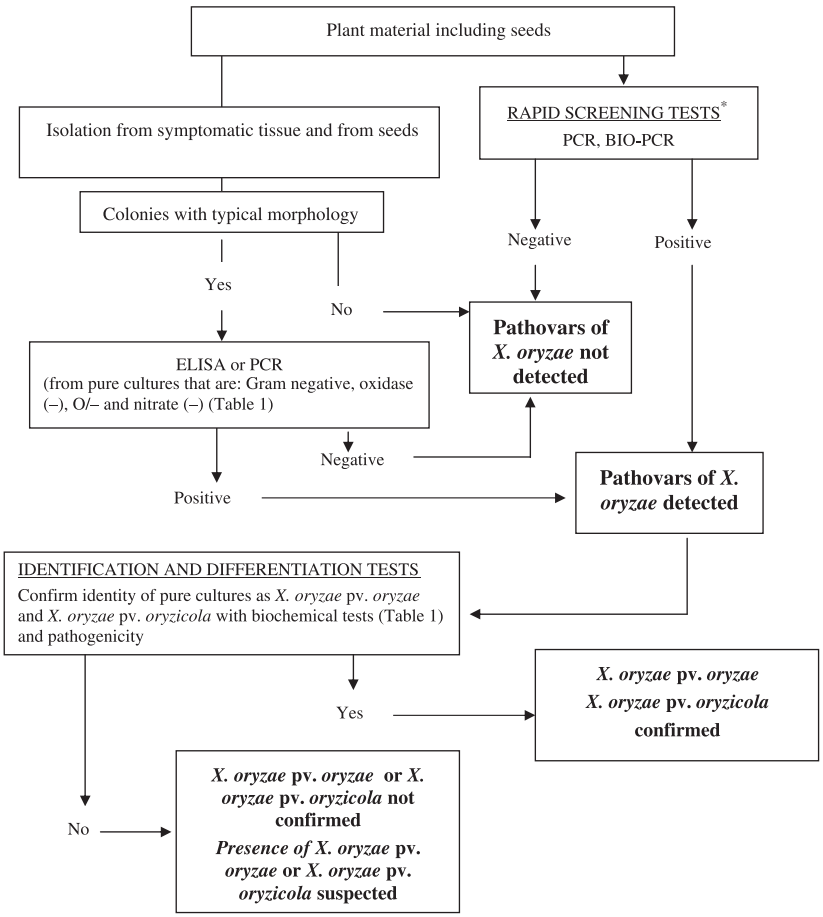

Fig. 4 Flow-diagram for diagnosis of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from leaves and seed of host plants. *Rapid screening tests are recommended when no differentiation of the pathovars of *X. oryzae* is needed, but not in the case of new findings.

TXT and TXT4R (Sakthivel *et al*., 2001) described in Appendix 2. These primers amplify a 964-bp fragment of an insertion squence (IS1113). Suspensions of 10^8 CFU mL^{-1} (0.1 OD_{600}) of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* should be used for positive controls, and a healthy plant extract and a sample of the ultra pure water (PCR reagent) for negative controls.

Direct detection from seeds by BIO-PCR

The main difference between this method and the method of direct isolation from seeds is that the incubation time is shorter and no morphological identification is required. In addition, a larger number of seeds can be tested.

BIO-PCR is an enrichment in solid medium combined with PCR and is recommended with primers TXT and TXT4R (Appendix 2). 500 g of seeds are soaked overnight at 4°C in 750 mL of 0.01% Tween 20 in sterile water. Sub-samples of 100 µL of seed extract are plated onto PSA in duplicates and incubated at 27 ± 2°C for 2 days or until the appearance of pin-point colonies. Plates are then washed three times each with 1 mL of sterile distilled water and 35 µL of the washings are used for the PCR assays (see Appendix 2). Suspensions of 10^7 CFU/mL of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* should be used as positive controls and a sample of the ultra pure water (PCR reagent) as negative control.

Identification

The procedure for identification of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* is summarized in the flow diagram in Fig. 4.

The two pathovars of *X. oryzae* differ in the symptoms induced (Ou, 1985), phenotypic characters (Vera Cruz *et al*., 1984; Vauterin *et al*., 1995), polyacrylamide gel electrophoresis protein fingerprints (Mew & Vera Cruz, 1979; Kersters *et al*., 1989), serological behaviour (Benedict *et al*., 1989) and phage typing (Swings & Civerolo, 1993).

Diagnosis can be made from pure cultures of putative yellow colonies of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* isolated from leaf, stems or seeds or directly from the seed or plant extracts. Note that isolates of the pathogen(s) maintained in artificial substrates may lose virulence over a period of time by successive sub-culturing (personal observations by the author). Identification of pure cultures of presumptive *X. oryzae*

Table 1 Discriminating bacteriological tests for preliminary identification and differentiation of pathovars of *Xanthomonas oryzae*

Tests[b]	*X. o.* pv. *oryzae*	*X. o.* pv. *oryzicola*
Gram staining	–	–
Oxidase test	–[a]	–[a]
2-ketogluconate production	–	–
Fluorescence on King's B medium	–	–
Nitrate reduction	–	–
Oxidation-fermentation of glucose	O/–	O/–
Gelatin hydrolysis[c]	–/v	+/v
Starch hydrolysis[c]	–	+
Sensitivity to 0.001% cupric nitrate	+	–
Growth on L-alanine as carbon source	–	+/v
Acetoin production[d]	–	+
Growth on 0.2% vitamin-free casamino acids[d]	–	+

+ = positive; – = negative; O = oxidative; v = variable.

[a]Weak positive reactions can be observed.

[b]Mew & Misra (1994); [c]Bradbury (1986); [d]Vera Cruz *et al*. (1984).

isolates is conducted using at least two different tests of the pathogens (nutritional profile, serological or molecular). An appropriate host test is used as final confirmation of pathogenicity. If this test is negative, the presence of *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola* is suspected but cannot be confirmed. Reference strains and negative test controls are used where appropriate for performing the tests.

For confirmation of diagnosis of isolated bacteria (see Fig. 4), follow the tests recommended in the identification section and conduct a pathogenicity test in rice plants. The biochemical tests results and pathogenicity tests observations are used in the differentiation of pathovars of *Xanthomonas oryzae*. Rapid screening tests can be recommended when no differentiation of the pathovars of *X. oryzae* is needed. Additional information on these tests can be found in the literature (Vera Cruz *et al*., 1984; Lelliott & Stead, 1987; Mew & Mistra, 1994; Schaad *et al*., 2001).

General characteristics

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* are Gram negative, rods ($0.4–0.6 \times 1.1–2.0$ µm), frequently capsulated, occurring singly, rarely in pairs, but not in chains, motile with a single polar flagellum. Colony morphology is examined on the third day or when growth has appeared on the selected agar medium. Colonies of *X. oryzae* pv. *oryzae* grow more slowly on NA than those of *X. oryzae* pv. *oryzicola*. After 3–4 days colonies of *X. oryzae* pv. *oryzae* on NA are circular, entire, smooth, convex, opaque, and pale yellow at first, straw yellow colour later. Colonies reach 1–2 mm after 5–7 days for *X. oryzae* pv. *oryzae*, and their survival on solid media is short. Colony formation from a single cell is poor and frequently fails to grow in many media, but can be improved by the addition of beef extract, methionine, or glutamic acid. On potato, sucrose agar growth is faster, reaching 2 mm in 3–4 days, honey yellow, and longer-lived. *X. oryzae* pv. *oryzicola* grows faster than *X. oryzae* pv. *oryzae* on NA, producing smooth, opaque, glistening, circular, convex and entire colonies; whitish at first, becoming straw to pale yellow later; about 1 mm in diameter in three days. Colonies of both pathogens on NBY are pale yellow, circular, raised and mucoid; on PSA, colonies are pale yellow, mucoid and shiny; on GF, colonies are very small, yellow and shiny. On mXOS, colonies have a characteristic rose pink colour, mucoid, raised and glistening after 3–5 days (Fig. 3).

Optimum temperature for growth is 25–30°C for *X. oryzae* pv. *oryzae* and the growth range is 5–40°C while for *X. oryzae* pv. *oryzicola* is 25–28°C and with a growth range from 8–38°C. For further details of the strains, see Vera Cruz *et al*. (1984) and IMI Descriptions of Fungi and Bacteria No. 1457 and 1458 (Saddler, 2002a). Diagnosis is confirmed with pathogenicity tests on 4–6 weeks-old rice plants. For preliminary identification of isolates follow the tests recommended in Table 1.

Fatty acid profiles

Fatty acid profiles allow identification at genus level only (Swings *et al*., 1990) so it is not recommended as a diagnostic method. This is given by the presence of 11:0 iso3OH, 13:0 iso3OH, 12:0 iso3OH. The fatty acid profiles of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* are very similar, but with differences in the relative amount of 12:0 iso3OH, 12:0 3OH, and 15:0 anteiso fatty acids.

PCR

Presumptive *X. oryzae* colonies from agar platings are used to prepare bacterial suspensions of approximately 10^8 CFU mL^{-1} in molecular grade sterile water. The PCR test allows identification at species level. Appropriate PCR procedures should be applied without DNA extraction. (see Appendix 2). Bacterial suspensions of 25 µL are used in the PCR tests. Pathovar-specific primers have been described in the literature but they have not been tested in Europe. They are presented for reference in Appendix 2.

ELISA

Presumptive *X. oryzae* colonies (yellow, raised, nitrate, oxidase, gelatine and starch negative), are tested by an indirect-ELISA procedure. A commercial Indirect ELISA kit based on *X. oryzae* monoclonal antibodies (Agdia Inc.) is available and can be used in the confirmatory identification of the isolates. For conducting the tests follow guidelines and procedures described by the supplier of the kit.

Pathogenicity tests

Isolates are tested on susceptible rice cultivars. For *X. oryzae* pv. *oryzae* use 30–45 days old IR24 or IR8 (International Rice

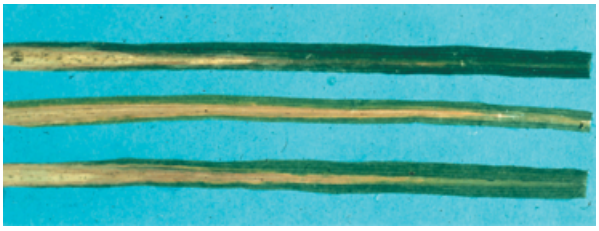

Fig. 5 Rice leaves showing symptoms after inoculation with *Xanthomonas oryzae* pv. *oryzae* by the lead 'clipping' method.

Research Institute, Manila, Philippines) or TN1 (Taichung native 1) rice plants; for *X. oryzae* pv. *oryzicola* IR24 or IR50 are recommended. Local popular rice varieties from the European region with known susceptibility to the disease are recommended to be included in the tests (Mew & Mistra, 1994). Pathogenicity grouping of isolates is based in several countries on the reaction of specific differential cultivars. For additional information consult Ou (1985). The leaf clipping method is widely used for the inoculation tests with *X. oryzae* pv. *oryzae* strains and spray inoculations for *X. oryzae* pv. *oryzicola*. For all inoculations grow the plants in potting mixture with a weekly fertilization of 1–2 g urea L^{-1}. Keep inoculated plants under high moisture conditions with 12 h light/dark cycle at an optimum temperature of 28–32°C/22°C. Include a negative control (e.g. plants inoculated with sterile saline solution alone) and a positive control to monitor false negative reactions caused (e.g. by technical failure).

The clip-method. The test developed by Kauffman *et al.* (1973) for *X. oryzae* pv. *oryzae* with slight modifications is conducted: Cutting 2–3 cm of the tips of 30–40 leaves of rice plants with a pair of scissors while still immersed in the bacterial suspension (10^8 CFU mL^{-1}) prepared in sterile saline solution. Alternatively spray the clipped leaves with the bacterial suspension (particularly useful for *X. oryzae* pv. *oryzicola*). The inoculated plants are covered for 24 h with a polythene bag, and incubated at 30°C with 12 h light cycle, with care taken that the plants are not in direct contact with the bag. Plants are observed for symptoms after 48–72 h up to 14 days. Plants are checked for water-soaked areas in the inoculated leaves, usually beginning from the inoculated ends as water-soaked stripes, an indication of *X. oryzae* pv. *oryzae* symptoms. Lesions enlarge and may turn yellow within a few days (Fig. 5). Milky drops of exudates can be observed. Make sure that the symptoms are not localized, but extend downwards. Appearance of reddish stripes from the point of inoculation of clipped leaves may be an indication of *X. oryzae* pv. *oryzicola* (Dr. M. Machmud, Bogor Research Institute, Indonesia, personal communication). These symptoms usually appear after ten days. Lesion margins caused by *X. oryzae* pv. *oryzicola* pathogen remain linear rather than wavy as in the case of *X. oryzae* pv. *oryzae*. Some non-pathogenic yellow bacteria may induce formation of localized yellow lesions at the inoculation point (1 cm), but do not spread along the lamina with time.

Spray inoculation. Rice plants can be inoculated by spray inoculation with putative *X. oryzae* pv. *oryzicola* bacteria (Cottyn *et al.*, 1994). A bacterial suspension is prepared from pure cultures of the suspected bacterial colonies on modified Wakimoto's medium after 72 h; other substrates of general composition can be used with good results. The concentration of the suspension is adjusted to 10^8–10^9 CFU mL^{-1}. A drop of Tween 20 is added and the suspension is atomized evenly onto IR24 or IR50 plants (35–40 d-old), but taking care that the suspension does not run off. The plants are labelled and maintained at 28–30°C with 12 h daylight cycle under moist conditions in a growth room or greenhouse and examined for bacterial streak lesions 10 days after inoculation. The transparent streaks differentiate leaf streak lesions from those of the bacterial blight, which are opaque against the light at earlier stages of infection.

Interpretation of pathogenicity test results. Bacterial cultures that do not induce characteristic symptoms may result from a loss of pathogenicity in the laboratory or may be considered as saprophytes. Production of bacterial leaf blight or bacterial leaf streak lesions confirm the pathogenicity of the bacterial colonies isolated from infected seeds or plants. Bacteria should be re-isolated from the lesions and their identity confirmed by appropriate tests (i.e., ELISA or PCR).

Reference material

X. oryzae pv. *oryzae* NCPPB 3002. ATCC 35933; CFBP 2532; ICMP 3125; LMG 5047 has been indicated as the pathovar reference strain (Saddler, 2002a).

X. oryzae pv. *oryzicola* NCPPB 1585; ATCC 49072, CFBP 2286, ICMP 5743, LMG. (Saddler, 2002b).

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

Further information

Further information on this organism can be obtained from:

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Acknowledgements

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It was further revised by Elena G. Biosca, Dpt. Microbiología y Ecología, Universidad de Valencia, 46100 Burjassot, Valencia (ES).

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Appendix 1

Growing media and buffers

Agar media

The following media are used for the isolation and purification of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from seeds and leaves. GF, PSA, and XOS agar media are useful for the preliminary isolation from seeds or leaves. NBY, PSA, Wakimoto's agar and NA are useful in the general isolation from leaves and for purification of strains for identification. YDC is often used in the determination of pigment production of the strains. However, some strains of the bacterial blight organism may not grow well on this media, which has often been used for the short-term storage of the bacterial cultures.

Growth Factor (GF) Agar: KH_2PO_4 0.4 g; $MgSO_4$. $7H_2O$ 0.05 g; NaCl 0.1 g; $NH_4H_2PO_4$ 0.5 g; $FeCl_3$ 0.01 g; yeast extract 0.01 g; agar 18.0 g; distilled water 1000.0 mL. Dissolve by heating and autoclave for 15 min at 121°C.

Nutrient Broth (NB): Peptone 5.0 g; beef extract 3.0 g; distilled water 1000 mL. Dissolve, distribute into test tubes and autoclave for 15 min at 121°C. Can be purchased in dehydrated form from Difco.

Nutrient Broth Yeast Extract Agar Medium (NBY): *Sol. 1*. nutrient broth 8.0 g; yeast extract 2.0 g; K_2HPO_4 2.0 g; KH_2PO_4 0.5 g; agar 18.0 g; distilled water 950.0 mL. *Sol. 2*. glucose 5.0 g; distilled water 50.0 mL. *Sol. 3*. $MgSO_4$. $7H_2O$ 1.0 mL of 1 M solution* (*Make 1 M sol. by dissolving 2.46 g in 10 mL distilled water). Dissolve and autoclave (121°C for 15 min) the three solutions separately and mix well when the temperature has reached 45–50°C four pouring plates.

Peptone Sucrose Agar (PSA): sucrose 10.0 g; peptone 10.0 g; Na-glutamate 1.0 g; agar 17.0 g; distilled water 1000.0 mL. pH is adjusted 6.8–7.0, mix, heat to dissolve and autoclave at 121°C for 15 min.

Yeast Dextrose Chalk Agar (YDC): *Sol. 1*. yeast extract 10.0 g; precipitated chalk ($CaCO_3$), light powder 20.0 g; agar 18.0 g; distilled water 950.0 mL. *Sol. 2*. dextrosa (L-glucose) 20.0 g; distilled water 50.0 mL. Mix, dissolve and autoclave the two solutions separately for 15 min at 121°C; mix well when the temperature of the medium is about 40–50°C for pouring plates.

Modified XOS agar medium (mXOS): sucrose 20.0 g; peptone 2.0 g; monosodium glutamate 5.0 g; Ca $(NO_3)_2$ 0.2 g; K_2HPO_4 2.0 g; Fe (EDTA) 1.0 mg; agar 18.0 g; distilled water 1000.0 mL. Mix and dissolve, adjust pH of the medium to 6.8–7.0 and add, after autoclaving (15 min at 121°C), the following filter sterilised solutions: tetrazolium chloride (tetrazolium red) 10 mg (1 mL of a 1% stock solution); cyloheximide (Actidione) 100 mg (1 mL of a 100 mg mL^{-1} solution dissolved in 75% ethanol); cephalexin (20 mg) 20 mg (2 mL of a 10 mg mL^{-1} aqueous solution); kasugamycin 20 mg (2 mL of a 10 mg mL^{-1} aqueous solution); methyl violet 2B 0.3 mg (3 mL of a 0.01 g/100 mL aqueous solution).

Modified Wakimoto's medium: ferrous sulphate 0.05 g; calcium nitrate 0.5 g; sodium phosphate 0.82 g; bacto peptone 5.0 g; agar 17 g; sucrose 20 g; distilled water 1000 mL. Dissolve by heating and autoclave for 15 min at 121°C.

Potato sucrose agar: Cook 200 g of diced potatoes in 0.5 L of water for 10 min.

Filter through cheesecloth and add water to filtrate to 1.0 L. Add Agar (20.0 g) Sucrose (20.0 g). Autoclave at 121°C for 15 min.

DNA Extraction reagents

TE-buffer: Made from stock solutions of 1 M Tris-HCl, pH 8.0 and 0.5 M EDTA

10 mL 1 M Tris HCl, 2 mL 0.5 M EDTA. Bring up to 1000 mL with H_2O.

10% SDS: (Lauryl Sulfate / Sodium dodecyl sulfate): 10 g in 100 mL H_2O.

CTAB: (Hexadecyltrimethylammonium Bromide): 10 g in 100 mL 0.7 M NaCl. (0.7 M NaCl: 4.1 g NaCl in 100 mL H_2O).

5M NaCl: 29.2 g NaCl in 100 mL H_2O.

Chloroform:Isoamylalcohol 24:1: 24 mL Chloroform, 1 mL Isoamylalcohol. Mix well and store in poison cabin.

Protease: 10 mg mL^{-1}.

Buffer for electrophoresis

5X TBE buffer: 54 g Tris base, 27.5 g Boric Acid, 20 mL of 0.5M EDTA, pH 8.0, 2.5 mg EtBr = 2.5 mL of 10 mg mL^{-1} stock (optional), and adjust volume to 1 L distilled water and store in glass. Discard any buffers that develop a precipitate. Use 0.5X for agarose gels.

Loading buffer: 0.025 bromothymol blue, 3 g glycol, 10 mL distilled water.

*Buffer formulations for indirect ELISA (*Agdia Inc.*)*

PBS (PBS – phosphate buffered saline). Dissolve the following ingredients in 930 mL distilled water in the order listed. Add sodium phosphate slowly. 1.15 g Sodium phosphate, dibasic (anhydrous), 0.2 g potassium chloride, 0.2 g potassium phosphate, monobasic (anhydrous), 8.0 g sodium chloride, 0.2 g sodium azide; adjust pH to 7.4. Adjust final volume to 1000 mL with distilled water.

Coating buffer. Dissolve in distilled water to 1000 mL: 1.59 g sodium carbonate (anhydrous), 2.93 g sodium bicarbonate, 0.2 g sodium azide. Adjust pH to 9.6. Store at 4°C.

PBSTween (wash buffer). Dissolve in distilled water to 1000 mL: 8.0 g sodium chloride, 1.15 g sodium phosphate, dibasic (anhydrous), 0.2 g Potassium phosphate, monobasic (anhydrous), 0.2 g potassium chloride, 0.5 g Tween-20. Adjust pH to 7.4.

PNP buffer. Dissolve in 800 mL distilled water: 0.1 g magnesium chloride hexahydrate, 0.2 g sodium azide, 97 mL Diethanolamine. Adjust pH to 9.8 with hydrochloric acid. Adjust final volume to 1000 mL with distilled water. Store at 4°C.

Appendix 2

PCR tests

DNA extraction:

DNA extraction reagent composition are presented in Appendix 1.

Table 2 PCR master mix (15 µL)

Reagent	Initial concentration	Volume	Final concentration (in 50 µL)
Water (PCR grade)		5.9 µL	
DNA buffer (Promega)	10×	5 µL	1×
$MgCl_2$	25 mM	3 µL	1.5 mM
dNTPs	25 mM each	0.4 µL	200 µM
Primer TXT	100 pmol µL^{-1}	0.25 µL	0.5 pm µL^{-1}
Primer TXT4R	100 pmol µL^{-1}	0.25 µL	0.5 pm µL^{-1}
Taq polymerase [Promega][a]	5 units	0.2 µL	1 U

[a]A regular *Taq* polymerase.

Tissue samples from leaves are homogenized into emulsion in a small sterile mortar (normal glass or porcelain mortar with pestle, top diameter 5 cm) with 500 µL extraction buffer and transferred into a 1.5 mL centrifuge tube. The remaining steps are the same as that for normal scale DNA extraction.

From pure cultures of bacteria isolated from leaves or seeds, the strains are grown in nutrient broth (NB) overnight with shaking at 30°C and 1.5 mL is dispensed into an Eppendorf tube.

Suspensions from tissue macerate or bacterial suspension from pure cultures in NB are centrifuged at 12 000 rpm in a microcentrifuge for 10 min at room-temperature. The supernatant is discarded and the pellet is re-suspended in 500 µL TE-buffer and 75 µL of 10% SDS and 10 µL of 10 mg mL^{-1} protease is added. The suspension is gently shaken at 37°C for 1 h.

A volume of 120 µL 5M NaCl is added and the suspension is thoroughly mixed by inverting the tubes several times; 100 µL CTAB (10% C-TAB in 0.7 M NaCl) are added, mixed thoroughly and incubated at 65°C for 20 min.

An equal volume (approx. 700 µL) of chloroform: isoamylalcohol (24:1) is added and the sample is mixed and vigorously shaken for 30 min. Tubes are centriguged for 30 min at 15 000 rpm at room temperature and the aqueous phase is transferred to a clean tube.

An equal volume of isopropanol is added (centrifugation is omitted) and the precipitate is transferred to 750 µL of 70% ethanol. (Consider leaving in freezer overnight before washing in ethanol.)

The sample is centrifuged for 8 min at 14 000 rpm in a microcentrifuge, the supernatant is discarded and the precipitate is allowed to air dry. The pellet is dissolved in 50 µL TE and the resulting DNA is placed at 70°C to hasten the dissolution. The extracted DNA is stored at 4°C for the PCR assay. Samples of 200 ng are used for the PCR tests.

Alternatively several commercially available DNA extraction kit can be used (e.g. Easy-DNA isolation kit from Invitrogen or DNAeasy Plant kit from Qiagen).

Primers

TXT (5-GTCAAGCCAACTGTGTA-3) and TXT4R (5-CGTTCGGCACAGTTG-3)

PCR master mix (15 µL) (Table 2):

Sample volume for BIO-PCR: add 35 µL sample to 15 µL master mix

Sample volume for pure culture bacterial DNA: 4 µL of 50 ng µL^{-1} (total 200 ng/reaction) to 15 µL of mastermix; add 31 µL water.

PCR reaction conditions

The reaction conditions are: initial denaturation step of 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min and final extension of 10 min at 72°C. The amplification conditions are optimized for the Perkin Elmer 2400 themocycler, but reproducible results are obtained with other machines such as the Eppendorf mastercycler gradient, MJ thermocycler and Hybaid PCR express thermocycler (Cycling conditions should be adapted using Peltier-type thermocyclers.) *Taq* polymerase enzymes supplied by other manufacturers (Promega, Finnzyme, Bioline USA and Bangalore Geni) also yield similar PCR results.

After PCR, analyse samples of 10 µL on a 1% agarose gel using standard procedures (Sambrook *et al*., 1989).

Interpretation of the PCR results

A PCR product of 964 bp indicates the presence of *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola* in the sample. The PCR is negative if the *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* specific amplicon of expected size is not detected in the sample in question but detected for all positive controls. If one of the negative controls shows a band of the expected size, the PCR test is repeated with a new mix and several negative controls are included to monitor the contaminations. Inhibition of the PCR may be suspected if the expected amplicon is obtained from the positive controls of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, but negative from positive controls with suspensions of *X. oryzae* pv. *oryzae* or *X. oryzae* pv. *oryzicola* prepared in healthy plant extracts.

For the differentiation of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* strains Kim & Song (1996) used PCR primers R16-1 (CTTGTACACACCGCCGTCA) and R23-2R(TCCG-GGTACTTAGATGTTTC) targeted towards the ribosomal intergenic spacer region (16S and 23S). All Korean strains of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* tested showed a species-specific fragment of 880 bp. Both pathovars of *X. oryzae* were differentiated from each other based on

differences in a secondary fragment of 430 bp or 460 bp for *X. oryzae* pv. *oryzae* strains and of 440 and 370 bp. Other non-pathogenic *Xanthomonas-like* strains had a primary fragment of 860 bp and a secondary fragment of 460 bp. Adachi & Oku (2000) designed a set of primers from the spacer region between the 16S and 23S rDNA for the identification of pure cultures of *X. oryzae* pv. *oryzae*. The specific primers, XOR-F (5′-GCATGACGTCATCGTCCTGT-3′) and XOR-R2 (5′-CTCGGAGCTATATGCCGTGC-3′) amplified a 470 bp fragment from *X. oryzae* pv. *oryzae* strains isolated from Japan.

The PCR assays developed by Kim & Song (1996) appear to be useful in the differentiation of the strains, but are still to be evaluated with more strains of different geographical origin. Similarly the PCR studies for the specific identification and detection of *X. oryzae* pv. *oryzae* (Adachi & Oku, 2000) were based on Japanese strains alone. The PCR assays described in this protocol (Sakthivel *et al.*, 2001) do not differentiate between the pathovars of *Xanthomonas oryzae*, however, they can be applied in conjunction with a selected set of tests (PCR, serological test, biochemical characters) and host plant inoculation tests.