4.

Detection survey protocol for *Xanthomonas albilineans* (Ashby, 1929) Downson (1943) in Nepal

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NPPO-Nepal, 2025

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1. Background information

With entry into the WTO, Nepal has the opportunity to export its produce to the international markets. However, the exports from Nepal have not escalated to the same proportion as trade between developed nations. Developed countries have increased exports by using the rules of the SPS Agreement. At the moment, the Government of Nepal is obliged to use the SPS rules to exclude commodities that are posing a threat to the related industries within the country. Nepal should provide an adequate description of the health status of plant-based industries, while negotiating access to foreign trade. Prospective importers of Nepalese agriculture-related commodities assess the risk of introducing new pests based on the authentic pest information provided. Prospective importers also assess the phytosanitary measures being practiced in Nepal to reduce risk to an acceptable level. Extensive specimen-based records are the key for Nepal to negotiating with importing countries on a fair trading system. This document gives detailed guidelines for detection surveys of the pathogen *Xanthomonas albilineans* in the field of agriculture. Besides, it will be applicable for monitoring, surveillance, import inspection and export certification and is the basis for specimen-based records to be developed by the NPPO-Nepal.

Under the Plant Quarantine and Protection Act, 2064, article 6(2), survey and surveillance functions and responsibilities are designated to NPPO-Nepal as per the sub-clause (i) "To perform such other functions as prescribed". This technical guideline for undertaking a pest detection survey of *X. albilineans* has been prepared with a view to guiding the survey activity. This protocol is prepared for researchers, plant protectionists, teachers, and other concerned professionals. This document will be a guide to submitting specimens to a laboratory for diagnosis and preservation.

1.1. About the target pest (pathogen)

The bacterium *Xanthomonas albilineans* (Ashby, 1929) Downson (1943), is a known plant pathogen that can affect a variety of crops. It causes leaf scald disease in sugarcane crops around the world. It is considered of global interest due to its destructive capacity and ability to cause severe economic losses (Rott et al., 1995; Rott and Davis, 2000). Since the first report of leaf scald in 1911 in Australia (Martin and Robinson, 1961), the presence of the disease has been confirmed in at least 65 countries worldwide (CABI, 2020). It has a major negative

economic effect on the global sugarcane sector. Leaf scale reduces juice quality, especially in the ratoon crop, leading to high cane losses measured in tons per hectare (Gutierrez et al., 2018).

Note: The pest is not reported in Nepal.

1.2 Identity and taxonomy of the target pest (CABI, 2021)

1.2.1 Identity

Preferred scientific name: *Xanthomonas albilineans* (Ashby 1929) Downson (1943)

Preferred common name: Leaf scald of sugarcane

Other scientific names: *Agrobacterium albilineans* (Ashby) Savulescu 1947

Bacterium albilineans Ashby 1929

Phytomonas Martin & Robinson

Phytomonas albilineans (Ashby) Magrou 1937

Pseudomonas albilineans (Ashby) Krasil'nikov 1949

Xanthomonas albilineans var. *paspali* Orian 1962

EPPO code: XANTAB

1.2.2 Taxonomic tree of the pest

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Lysobacterales

Family: Lysobacteraceae

Genus: *Xanthomonas*

Species: *Xanthomonas albilineans*

1.3 Host range

Sorghum (*Sorghum bicolor*), maize (*Zea mays*), sugarcane (*Saccharum officinarum*), Bamboo (*Bambusa* spp.).

1.4 Disease symptoms

Foliar infection induced by airborne inoculum of *X. albilineans* is characterized by cream to yellow stripes starting at the tip or occasionally the margin of the leaf (Fig. 1). Three phases are associated with the symptomatology of the disease: **latent, chronic and acute phases**.

- **Latent infection** or the absence of symptoms is a characteristic feature of the disease, which occurs in tolerant varieties and under favourable conditions for plant growth.
- **In the chronic phase**, a typical white pencil-line stripe (1-2 mm wide) runs from several centimetres to almost the entire length of the leaf (Fig. 2). At a later stage (Fig. 3), the sharp margins of the stripe may become diffuse, and a red pencil line may be formed in the middle of the stripe. Partial or total chlorosis of leaves occurs and is accompanied by an inward curling of the leaves (scalding). Affected stalks may be stunted with the development of axillary buds (side shoots) bearing symptoms of the chronic phase. A longitudinal section of the stalk shows a reddish discoloration of the vascular bundles, particularly at the nodes. As the disease progresses, lysogenous cavities may be formed and the stalk dies.
- **In the acute phase** of the disease, sudden plant death occurs with few or no symptoms.

Foliar symptoms of the disease in maize are shown in figure 4.

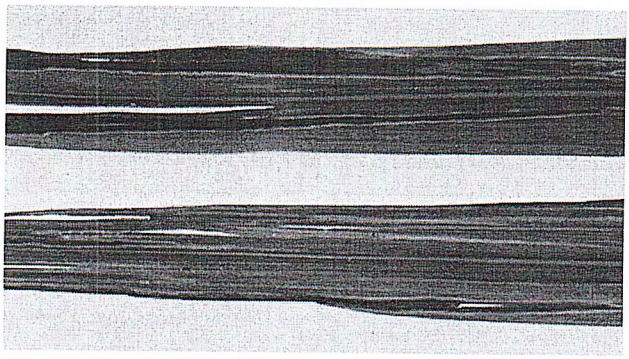

Figure 1. Foliar infection induced by airborne inoculum of *X. albilineans* is characterized by cream/yellow stripes starting at the tip, or occasionally at the margin of the leaf (Source: CABI, 2021)

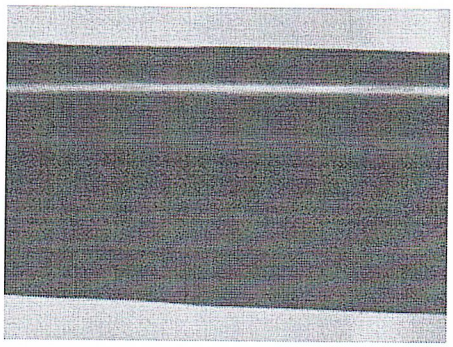

Figure 2. In the chronic phase of infection, a typical white pencil-line stripe (1-2 mm wide) runs from several centimetres to almost the entire length of the leaf (Source: CABI, 2021)

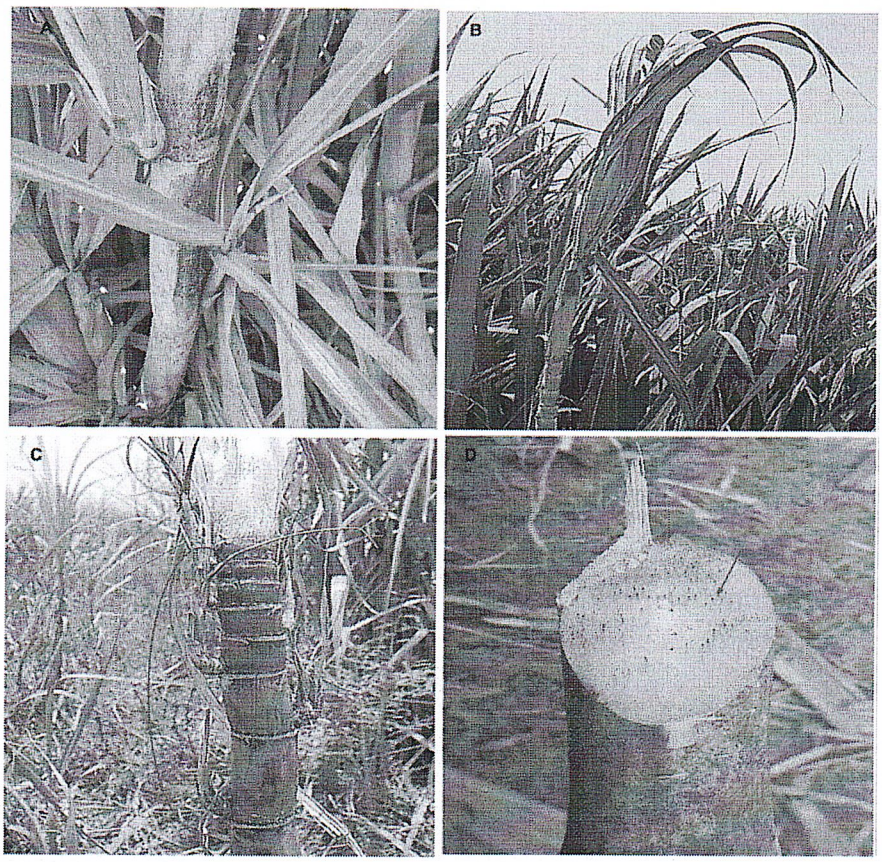

Figure 3. Sugarcane plants with leaf scald symptoms caused by *Xanthomonas albilineans*. A) Stalk exhibiting side shoots with white pencil lines (red arrow); B) Plant with bleached leaves and partial defoliation (red arrow); C) Stalk with shortened internodes (red arrow); D) Transversal section of a diseased stalk showing red-brownish vascular bundles (red arrow).

(Source: Cervantes-Romero et al., 2021)

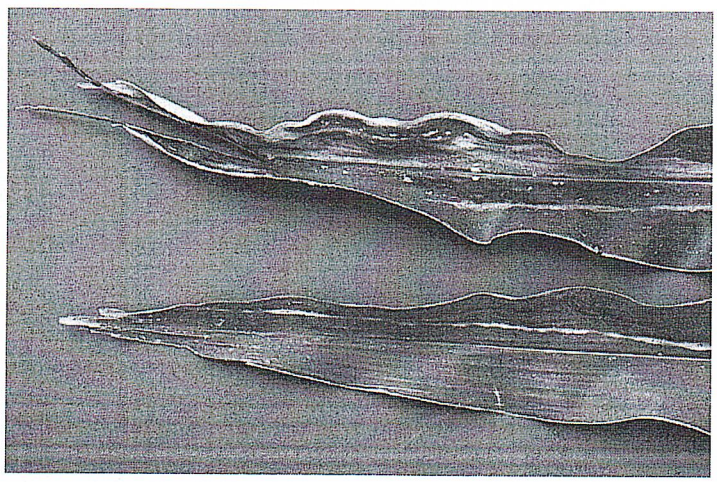

Figure 4. Foliar stripes (aerial infection) on maize. At later stages of infection, partial or total chlorosis of leaves occurs and is accompanied by an inward curling of the leaves (scalding). (Source: CABI, 2021)

1.5 Epidemiology

The disease emergence favored by water-stressed plants. High humidity (80%) and warm temperatures (25-35°C) favors the pathogen's spread. *X. albilineans* produces albicidin, a specific toxin that is responsible for the development of foliar symptoms of leaf scald by blocking chloroplast differentiation (Birch, 2001; Pieretti et al., 2015). This blockage results in the characteristic white foliar stripe symptoms, although a toxin-deficient mutant of the pathogen can still produce disease symptoms (Rott et al., 2011). *X. albilineans* multiplies in the xylem and colonizes the entire plant, including leaves, roots and stalks (Birch, 2001; Klett and Rott, 1994).

1.6 Mode of dispersion /Pathway

The pathogen primarily spreads through infected cutting or by using contaminated equipment.

a) **Primary transmission:** Transmission occurs primarily through the use of infected seed pieces and contaminated tools during field work or at harvest.

b) **Aerial transmission:** The transmission and spread may also occur by the combination of strong wind and strong rain, which can break the infected tissue of stalks, allowing exposure of the bacterium, which is dragged by water and wind. This type of transmission has also been linked to infections that occur through the hydathodes in the guttation process.

2. Detection survey

A detection survey is conducted in an area to determine if pests are present (FAO, 1990; revised FAO, 1995). These surveys are more frequently carried out to determine pest status in an area, and they follow a definite survey plan, which is approved by NPPO-Nepal. These surveys are carried out either seasonally or annually and/ or following the eradication measures applied to a pest in a given area or production sites. These surveys are organized following a definite survey methodology based on statistical sampling, which is determined after taking into account the biology of the pest and employing appropriate detection techniques such as field diagnostic kits, traps etc. The results of the survey are documented and communicated (PPD/NPPO-Nepal, 2071 BS).

2.1 Purpose and scope of detection survey

The purpose of the detection survey is to determine the presence or absence of *Xanthomonas albilineans* in a given area or production sites. The scope will be limited to maize

and other defined crops to be grown for haylage/silage production for export to China and other concerned countries.

2.2 Timing of survey

The survey can be carried out round the year. Priority will be given during the hot and humid months (June-September) during monsoon or early post-monsoon period (October).

2.3 Selection of survey area

As per the requirements of NPPO Nepal (to begin with maize and sorghum growing districts).

2.4 Materials required for survey

Paper bags (envelopes), scissors, hand lens, test tubes with a holder (ooze test in the case of systemic infection), gloves, face mask, forceps, tags, permanent markers, GPS, camera, and data sheets.

2.5 Number of plants sampled for identification

All the specific host fields should be monitored. For initial disease detection, the minimum sample size should be based on the area covered by maize. Normally, 10 plants for 100 m^2 should be inspected for pathogen surveillance. Three or more plants can be chosen randomly from every row to be inspected (FAO, 2023).

2.6 Plants parts to be observed

- Leaves
- Stems
- Whole plants

2.7 Sample collection and preparation from the disease-suspected host plant

For the proper collection and preparation of samples, identify plants showing typical symptoms of the disease. Collect samples from various parts of the field, especially from high-risk zones like field edges, low-lying areas, and regions near water sources. On the basis of visual observation, collect infected sorghum or maize plants with typical white leaf stripes with necrotic zones at leaf margins, extensive chlorosis of emerging leaves, vascular reddening and cavity formation in invaded stems, rapid and wilting and death plant. Place each sample in a separate plastic bag or paper envelope to avoid cross-contamination. Clearly label each sample with information related to field location, date of collection, crop variety, symptoms observed,

and collector's name. Avoid plastic bags as this causes the samples to sweat and promotes secondary infection. Transport samples to the diagnostic lab as soon as possible.

2.8 Diagnostic laboratory

- National Plant Pathology Research Center, Nepal Agricultural Research Council, Khumaltar, Lalitpur
- Central Agricultural Laboratory, Department of Agriculture, Hariharbhawan, Lalitpur
- National Herbarium and Plant Laboratories, Department of Plant Resources, Godawari, Lalitpur
- Natural History Museum, Swayambhu, Kathmandu
- Department of Plant Pathology, Agriculture and Forestry University, Rampur, Chitwan
- Department of Plant Pathology, TU/IAAS, Kathmandu
- Central Department of Botany, Tribhuvan University, Kathmandu
- Private laboratories – Center for Molecular Dynamics Nepal (CMDN), Thapathali, Kathmandu Nepal Plant Disease and Agro Associates (NPDA), Balaju, Kathmandu, and others, identified if any.

** Biosecurity protocol to handle the quarantine sample should be followed in each laboratory.*

2.9 Identification method

Diagnostics can effectively be used to identify the presence of *Xanthomonas* spp., which helps to avoid planting or exporting *Xanthomonas*-infected stocks. Isolation of suspected bacteria will be done for confirmation and the pathogen will be identified by uisng a set of morphological, biochemical, physiological tests and molecular approaches.

2.9.1 Isolation of bacteria

- Cut stalks exhibiting side shoots (in sugarcane) into pieces.
- Wash with tap water, disinfect with a 1.5% sodium hypochlorite solution (w/v) for 1 minute, and rinse them three times with sterile distilled water.
- Dry with absorbent paper in a laminar flow chamber to remove excess moisture.
- Flame briefly the stalk pieces with 99.9% ethanol.
- Take out 0.5-cm pieces of internal tissue with a sterile scalpel, place in a sterile Petri dish and squeeze the tissue to release the cane juice.

- Streak the juice using a bacteriological loop onto the Petri dish containing modified Wilbrink's agar (MW) medium.
- Incubate the Petri dish at 28°C for 5 days.
- Look for single bacterial colonies and transfer them to fresh medium to obtain pure cultures for further analysis.

Note: Bacterial cells can also be observed under electron microscopy.

2.9.2 Cultural identification

Nutrient media	Colony characteristics
Modified Wilbrink's agar (MW)	Small bacterial colonies start to be visible on MW agar medium after 3 days of growth. At 7 days, these colonies become pale yellow in colour, convex, often with smooth margins, and non-mucoid.
XAS Agar medium (Selective medium)	A single circular, convex, smooth, shiny, translucent, and yellow-pigmented bacterial colony.

Note:

- MW agar medium containing sucrose (10 g), peptone (5 g), K_2HPO_4-$3H_2O$ (0.50 g), $MgSO_4$-$7H_2O$ (0.25 g), Na_2SO_3 (0.05 g), agar (15 g), and distilled water (1 liter), pH 6.8 to 7.0.
- XAS (*Xanthomonas albilineans* selective) medium consists of a modification of Wilbrink's medium that is supplemented with 5g KBr, 100 mg of cycloheximide, 2 mg of benomyl, 25 mg of cephalexin, 30 mg of novobiocin, and 50 mg of kasugamycin per liter.

2.9.3 Morphological Identification

- Transfer a single colony into a tube containing 1 ml of XAS liquid medium.
- Incubate the bacterial suspension at 28 °C for 12–24 h with continuous shaking at 200 rpm.
- Centrifuge the bacterial growth in the tube at 3000×g for 2 min to obtain bacterial pellets.
- Wash the bacterial pellet two times with 1 ml sterile H_2O and suspend in 200 µL sterile water.

- Place a loop of the suspension on a copper mesh and top of a filter paper for 1 min to absorb the excess liquid.
- Stain the bacterial cells with a solution of 2% phosphotungstic acid (PTA) (pH 6.7) for 1 min, and absorb the excess liquid using blotting paper.
- Observe morphological characteristics of single cells of *X. albilineans* under transmission electron microscope, and capture images.

Method	**Results**
Gram staining	Gram -ve bacteria show pink to reddish-colored cells under compound microscope
Shape	Rod-shaped cells with a single polar flagellum under a compound microscope or an electron microscope

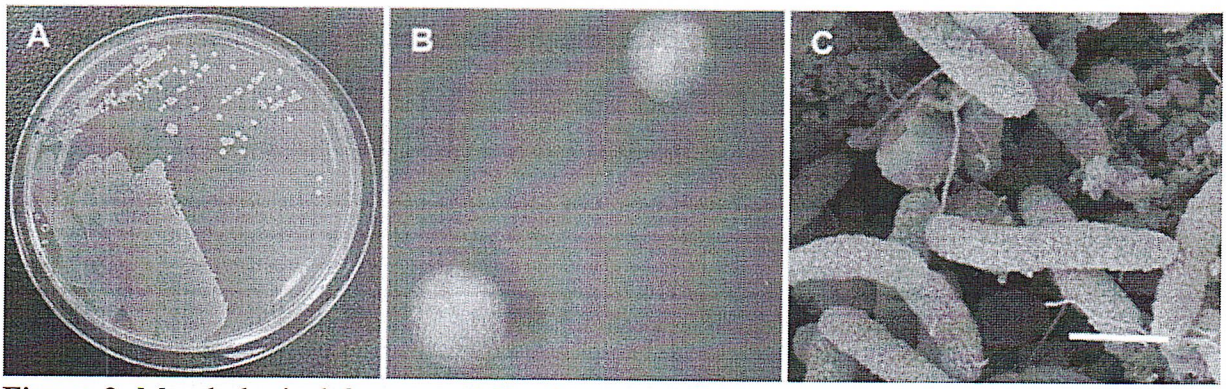

Figure 3. Morphological features of *Xanthomonas albilineans* grown for 7 days on modified Wilbrink's agar medium. A) Pale yellow colonies; B) Convex colonies with smooth margins C) single polar flagellum observed by scanning electron microscopy. (Source: Cervantes-Romero et al., 2021)

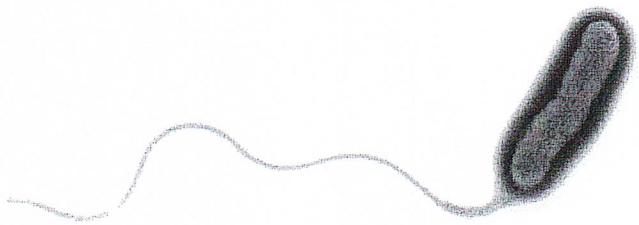

Figure 4. Transmission electron micrograph of a negatively stained single cell (Source: García-Ochoa et al., 2000)

2.9.4 Biochemical Identification

Test	Results
3% KOH test	String of slime lifted with a sterile loop, due to the liberation of cellular DNA, leading to viscous slime formation, confirming Gram-negative nature
Oxidase test	Negative
Catalase test	Positive
Gelatin Liquefaction	Positive

See Annex 3 for testing procedures.

2.9.5 Pathogenicity test

- Grow healthy maize plants up to 3–5 leaves and approximately 15–20 cm in pots in a growth chamber at 28°C and 60% humidity with 16 h light and 8 h dark per day. Inoculate the plant by cutting leaf blades at mid-length with scissors previously dipped in a bacterial suspension of 10^8CFU/ml.
- Keep untreated control plant inoculated with sterile water or the medium used for bacterial growth.
- Examine leaves 7 days post-inoculation for leaf scald symptoms and reisolate the bacteria for confirmation.

2.9.6 Molecular identification

A number of methods are available for molecular diagnosis of plant bacteria. They involve different steps, starting from genomic DNA extraction to their sequencing. The procedure applied by Ntambo et al (2019) could be applied for molecular diagnostic of the *X. albilineans*, which is described below. However, the method is not necessarily mandatory to follow. Any other established/adopted methods may be used alternatively.

2.9.6.1 DNA extraction and PCR amplification

Collect maize/sorghum leaf samples exhibiting leaf scald symptoms from commercial maize/sorghum-growing areas and store the collected leaf tissues at -80°C until DNA extraction and further processing. Extract total genomic DNA from maize/sorghum leaf samples using the standard CTAB protocol (Doyle & Doyle, 1987). Culture bacteria in XAS liquid medium. Extract bacterial genomic DNA from the culture of *X. albilineans* strain Xa-FJ1 (Lin et al., 2018)

with the TIAMamp Bacterial Genomic DNA extraction kit. (Tiangen Biotech Co., Ltd) following the manufacturer's instructions and elute in 50 µL sterile water. ACC 10416 and DSM 3586 strains can be purchased from the Agricultural Culture Collection of China (ACCC). Determine DNA quality and DNA quantity by gel electrophoresis (1% agarose gel) and a UV absorbance assay using a Synergy H1 Multi-Mode Reader (BioTek), respectively. Adjust the final concentration of all DNA samples for PCR amplification to 100 ng μL^{-1} with sterile water.

Obtain five genes namely *abc* (encoding an ABC transporter), *rpoB* (encoding the b subunit of the bacterial RNA polymerase), *glnA* (encoding a citrate synthase), *gyrB* (encoding the b subunit of the DNA gyrase) and *atpD* (encoding the b subunit of ATP synthase) for multi-locus sequence analysis. The primers use to amplify these housekeeping genes and the PCR conditions are given in Table 2.

Table 2. Polymerase chain reaction (PCR) primer sequences that could be used for amplification of five housekeeping genes and the intergenic transcribed spacer (ITS) region of the 16S-23S rRNA genes in *X. albilineans*

Gene	Primer	Sequence (5'–3')[a]	Annealing temperature (°C)	Fragment size (bp)	PCR programme	Reference
abc	XAF1	CCTGGTGATGACGCTGGGTT	56	608	94 °C for 1 min; 35 cycles at 94 °C for 30 s, 56 °C for 1 min, 72 °C for 30 s; 72 °C for 5 min	Wang *et al.* (1999)
	XAR1	CGATCAGCGATGCACGCAGT				
atpD	atpD-F	GGGCAAGATCGTTCAGAT	50	868	94 °C for 2 min; 30 cycles at 94 °C for 1 min, 50 °C for 90 s, 72 °C for 90 s; 72 °C for 10 min	Fischer-Le Saux *et al.* (2015)
	atpD-R2	GTTCTTGGTGGAGGTGAT				
glnA	glnA-F2	GGTTAAGGACAACAAGATCG	52	1095	94 °C for 2 min; 30 cycles at 94 °C for 1 min, 52 °C for 90 s, 72 °C for 90 s; 72 °C for 10 min	Ntambo et al., 2019
	glnA-R2	GCGGCRAAGGTCAGGTAG				Ntambo et al., 2019
gyrB	XgyrB1F	ACGAGTACAACCCGGACAA	52	904	94 °C for 2 min; 30 cycles at 94 °C for 1 min, 52 °C for 90 s, 72 °C for 90 s; 72 °C for 10 min	Fischer-Le Saux *et al.* (2015)
	XgyrB1R2	CCCATCAAGGTGCTGAAAAT				Ntambo et al., 2019
rpoD	rpoD-F1	ATGGCCAACGAACGTCCTGC	56	1298	94 °C for 2 min; 30 cycles at 94 °C for 1 min, 56 °C for 90 s, 72 °C for 90 s; 72 °C for 10 min	Fischer-Le Saux *et al.* (2015)
	rpoDR3	AACTTGTAGCCGCGACGGTATTC				Ntambo et al., 2019
ITS	16Suni1330	GTTCCCGGGCCTTGTACACAC	55	1112	94 °C for 1 min; 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; 72 °C for 7 min	Honeycutt *et al.* (1995)
	23Suni322antI	GGTTCTTTTCACCTTTCCCTC				
	16Suni1330	GTTCCCGGGCCTTGTACACAC	55	1112	94 °C for 1 min; 35 cycles at 94 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min; 72 °C for 7 min	Honeycutt *et al.* (1995)
	Xa23S-R	GGTTCTTTTCGCCTTTCCCTC				Ntambo et al., 2019
	PGBL1	CTTTGGGTCTGTAGCTCAGG	52	288	94 °C for 2 min; 30 cycles at 94 °C for 1 min, 52 °C for 90 s, 72 °C for 90 s; 72 °C for 10 min	Pan *et al.* (1997)
	PGBL2	GCCTCAAGGTCATATTCAGC				

[a]The bold and underlined nucleotides were revised according to the published sequences of 13 *Xanthomonas albilineans* strains from the GenBank database.

2.9.6.2 DNA sequencing and analysis

Gel-elute the PCR amplicons using the E.Z.N.A kit (Omega Bio-Tek) following the manufacturer's instructions. Clone the purified DNA into plasmid vector pMD19-T (Takara Biotech) and subsequently transform into chemically competent cells of *Escherichia coli* DH5α (Tiangen Biotechnology Co., Ltd), following the manufacturer's recommended procedure. Send three PCR positive clones per amplicon to a molecular lab (eg. Sangon Biotech Co., Ltd.) for bidirectional sequencing using universal primers M13F and M13R. Assemble and analyse sequences using DNAMAN v.8.0 (Lynnon Biosoft). Compare each consensus sequence to the NCBI database (https://www.ncbi.nlm.nih.gov) for sequence identity. Align the sequences using the CLUSTALW algorithm implemented in MEGA v. 6.0 (Tamura et al., 2013). Construct MLSA-based phylogenetic tree using the neighbour-joining algorithm of Kimura's 2-parameter model (Kimura, 1980) and the bootstrapping algorithms contained in MEGA v. 6.0 (Kumar et al., 2001).

2.7 Bacterial culture preservation

Storing bacterial cultures for long periods requires preserving their viability while minimizing genetic or physiological changes. Common methods include:

(a) Refrigeration (Short-term storage, weeks to months)

- 4°C in a refrigerator.
- Use agar slants or plates sealed with parafilm or stored in airtight containers to prevent desiccation.
- Periodically subculture to fresh media to maintain viability.

(b) Freezing (Medium- to long-term storage, months to years)

- −20°C to −80°C in a freezer.
- Mix bacterial cells with a cryoprotectant like glycerol (15–20%) or Dimethyl sulfoxide DMSO (5–10%) to prevent ice crystal damage.
- Aliquot the mixture into sterile cryovials and freeze quickly to preserve cell integrity.
- Ideal for maintaining pure cultures for long periods.

2.8 Sample analysis and reporting

Concerned laboratories, or an independent surveyor who analyse and identify the bacteria, should submit the report to the NPPO-Nepal for the reporting/declaration of bacteria. The reports should also include infestation maps, photographs and field observations.

2.9 Record keeping

NPPO, in collaboration with responsible laboratories, should preserve the disease specimen and keep all the record safely. The documentation system should be well maintained by the NPPO and member institutions should have easy access to it.

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ANNEXES

Annex- 1: Field datasheet

1. Name of field/Site visited:
2. Date/Time of visit:
3. GPS reference point Latitude:
   Longitude: Altitude:
4. Province: District:
   Municipality: Ward no./Place:
5. Climate data of locality: Average min. temp (in °C):
Average max. temp (in °C): Rainfall (in mm)
6. Survey/Field plot no.
7. Host plant species inspected: Variety:
8. Phenological stage of the plant:
7.1 Description of habitat (such as aspect, slope, vegetation type, soil type)

7.2 Alternate host plant species found infected, if any:
9. Sampling method:
10 Contact details of the local informant involved in the survey:
11. Details of pest recorded

SN	Scientific name	Common name	Plant parts affected	Symptom & Sign	Disease incidence	Severity % / Score

10. Any additional information (including collection of specimens for investigation):

11. Name/Signature of surveyor with date:

Annex 2: Format for forwarding specimens

1. Collection number: 2. Date of Collection:
3. Submitting organization:
4. Name/Address/Contact no. of the sender:
5. Locality of collection (Province / District / Municipality / Ward No. / Place):

6. Reasons for identification:
7. Name of the host plant species (Scientific name / Common name / Variety:

8. Origin of host/commodity (Source of seed/planting materials, if applicable):

9. Plant parts affected:	[ ] roots; [ ] stems; [ ] leaves; [ ] inflorescence; [ ] fruits; [ ] seeds/nuts [ ] others (_____)
10. Category of pest specimen/organism submitted:	[ ] insects; [ ] mites; [ ] nematodes; [ ] fungi; [ ] bacteria; [ ] virus; [ ] others (_____)
11. Life stage of the pest (Applicable to insects):	[ ] egg; [ ] larvae; [ ] pupae; [ ] adult; [ ] nymphs; [ ] juveniles; [ ] anamorphic [ ]; cysts; [ ]others (_____)
12. Type of pest specimen/organism submitted:	[ ] preserved specimen; [ ] pinned/card board mounted specimen; [ ] dry specimen with host; [ ] culture; [ ] disease specimen (fresh); [ ] disease specimen (partially dry); [ ] slide mount; [ ] others (_____)

14. Number of specimens submitted per each collection:
15. Signature/stamp/office seal of the sender with date:

_____

For identifier use

16. Name &address of Diagnostic/Referral Laboratory:
17. Remarks of identifier (condition of receipt of specimens):
18. Pest identification (Common/Scientific name/Taxon):
19. Description notes, if any:

Place: _____

Date: _____

_____

(Signature/Name/Designation of Identifier)

Note: This form should be prepared in duplicate by the sender and forwarded to the identifier/referral laboratory along with each collection of specimens. The identifier should return the original copy after

entering the particulars of the pest identified along with description notes and remarks if the identifier will retain any to the sender of the specimen and duplicate the copy.

Annex 3. Protocols for some common biochemical and bioassay tests

Protocol of Oxidase test (Test Tube Method)

- Grow a fresh culture (18 to 24 hours) of bacteria in 4.5 ml of nutrient broth (or standard media that does not contain a high concentration of sugar.
- Add 0.2 ml of 1% α-naphthol, then add 0.3 ml of 1% paminodimethylaniline oxalate (Gaby and Hadley reagents).
- Observe for color changes.
- Microorganisms are oxidase positive when the color changes to blue within 15 to 30 seconds.
- Microorganisms are delayed oxidase positive when the color changes to purple within 2 to 3 minutes.
- Microorganisms are oxidase negative if the color does not change.

Protocol of catalase test (Tube method)

- Add 4 to 5 drops of 3% H2O2 to a 12 x 75-mm test tube.
- Using a wooden applicator stick, collect a small amount of organism from a well isolated 18- to 24-hour colony and place into the test tube. Be careful not to pick up any agar.
- Place the tube against a dark background and observe for immediate bubble formation (O2 + water = bubbles) at the end of the wooden applicator stick.
- Positive reactions are evident by immediate effervescence (bubble formation).
- Use a magnifying glass or microscope to observe weak positive reactions.
- No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

Protocol of Gelatin Liquefaction

- Gelatin hydrolysis is the nutrient gelatin plate method. In this method, a heavy inoculum of an 18- to 24-hour-old test bacteria is stab-inoculated onto culture plates prefilled with nutrient gelatin (23 g/liter nutrient agar, 8 g/liter gelatin). Inoculated nutrient gelatin plates are incubated at 35°C for 24 hours. Gelatin hydrolysis is indicated by clear zones around gelatinase-positive colonies

Protocol of indole production test

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole. Indole test is also important in subspecies identification, as *P. stewartii* subsp. *indolegenes* distinguished and identified from *P. stewartii* subsp. *stewartii* through the positive result of indole test with Kovac's reagent.

- Inoculate the tube of tryptone broth with a small amount of a pure culture.
- Incubate at 35°C (+/- 2°C) for 24 to 48 hours.
- To test for indole production, add 5 drops of Kovács reagent directly to the tube.
- A positive indole test is indicated by the formation of a pink to red color ("cherry-red ring") in the reagent layer on top of the medium within seconds of adding the reagent.
- If a culture is indole negative, the reagent layer will remain yellow or be slightly cloudy.

2.9.5 Hypersensitivity reaction test

- Grow *Pantoea stewartii* isolate on a suitable medium at 28–30°C for 24–48 hours.
- Scrape bacterial growth from the culture plate and suspend it in sterile distilled water.
- Adjust the bacterial suspension to an optical density of approximately OD600 = 0.2–0.3 (equivalent to ~10^8 CFU/mL).
- Select a non-host plant such as tobacco (*Nicotiana tabacum*).
- Detach a healthy leaf, rinse with sterile distilled water, and disinfect the surface with 70% ethanol.
- Using a sterile syringe, inject the bacterial suspension into the intercellular spaces (underside of the leaf).
- As a control, inject sterile water into another area of the same leaf.
- Incubate the inoculated leaf in a moist chamber at room temperature.
- Check for the development of a hypersensitive reaction within 24–48 hours.

  Positive HR: The leaf tissue around the injection site shows necrosis or browning, indicating the bacterial strain is pathogenic and eliciting an HR.

  Negative HR: No visible reaction indicates the bacterium may not be pathogenic or lacks the ability to trigger an HR.
- Inoculate maize seedlings with the bacterial suspension using a sterile needle or syringe.
- Observe for wilting symptoms and necrotic lesions over 5–7 days to confirm virulence.