NPPO-Nepal, 2025

3.

Detection Survey Protocol for *Xanthomonas axonopodis* pv. *vasculorum* (Cobb 1894) Vauterin et al. 1995 in Nepal

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Ministry of Agriculture and Livestock Development
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March, 2025

Approved
March 12, 2025

NPPO-Nepal, 2025

Endorsed by NPPO-Nepal on March 12, 2025

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 axonopodis* pv. *vasculorum* 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 *Xanthomonas axonopodis* pv. *vasculorum* 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 axonopodis* pv. *vasculorum* (Cobb 1894) Vauterin et al. 1995, is a known plant pathogen that can affect a variety of crops. In the past, gumming was a major disease of sugarcane in Australia, Mauritius and Reunion. Severe epidemics occurred in

Australia where reductions of 30-40% in cane tonnage and 9-17% in sugar content were reported between 1893 and 1899 (North, 1935). In the 1890s, heavy losses were also observed in Mauritius and susceptible cultivars were abandoned. Besides the losses in the field, gumming causes problems in the milling process, resulting in a lower sugar recovery.

Note: The pest is not reported in Nepal.

1.2 Identity and taxonomy of the target pest (CABI, 2022)

1.2.1 Identity

Preferred scientific name: *Xanthomonas axonopodis* pv. *vasculorum* (Cobb 1894) Vauterin et al. 1995

Preferred common names: Sugarcane gumming disease, bacterial leaf streak of corn

Other scientific names: *Bacillus vasculorum*

Phytomonas vasculorum

Xanthomonas campestris pv. *vasculorum*

Nepali name: व्याक्टेरियल पाते धर्से रोग

EPPO code: XANTVA

1.2.2 Taxonomic tree of the pest

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: *Xanthomonas*

Species: *Xanthomonas axonopodis* pv. *vasculorum*

1.3 Host range

Sugarcane (*Saccharum* spp.), maize (*Zea mays*), Guatemala grass (*Tripsacum laxum*), broom bamboo (*Thysanolaena latifolia*), hurricane palm (*Dictyosperma album*), royal palm (*Roystonea regia*), nut palm (*Areca catechu*), sorghum (*Sorghum bicolor*), Johnson grass (*Sorghum halepense*), guinea grass (*Panicum maximum*), elephant grass (*Pennisetum purpureum*)

Sugarcane appears to be the most susceptible host, infection of other host plants being induced under conditions of high inoculum potential.

1.4 Disease symptoms

There are two distinct phases: the foliar stage and the systemic stage.

- On leaves, 3-6 mm wide streaks arise from the leaf margin and develop along the vascular bundles towards the base.
- Streaks are yellow to orange with red flecks, becoming necrotic and grey with age.
- The necrosis may involve large areas of the leaf.
- A few short streaks are observed in resistant cultivars or when environmental conditions are not favourable for the disease.
- On highly susceptible cultivars and when conditions are favourable, the bacterium progresses down the lamina and the sheath, and infects the stalk.
- The systemic stage of the disease is characterized by a reddish discoloration of the vascular bundles at the nodes, and by a bacterial slime.
- Gum pockets are formed and the slime exudes from the cut surface of the stalks. Stalk deformation and knife-cut lesions due to transverse splits in young elongating tissue can also be observed.
- When conditions are not favourable for cane growth, the formation of the gum pockets near the apex and in the leaf spindle results in the death of the growing point.

- Another characteristic of the systemic stage of the disease is the partial or total chlorosis of new leaves in mature canes. Chlorosis can also occur in young ratoons as a result of transmission by contaminated leaves.

Figure 1. Foliar symptoms of gumming disease in sugarcane (Source: CABI, 2022)

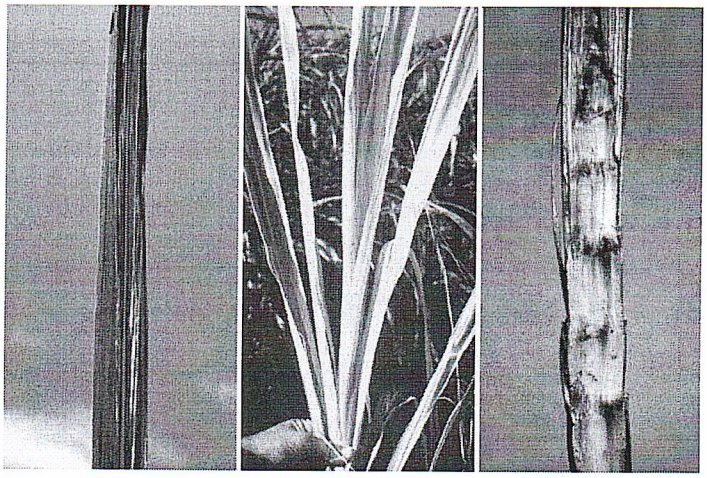

Figure 2. Yellow stripes on sugarcane leaf (left), chlorosis of sugarcane leaves (middle) & necrosis of nodes and growing point of sugarcane stalk (right) (Source: CABI, 2022)

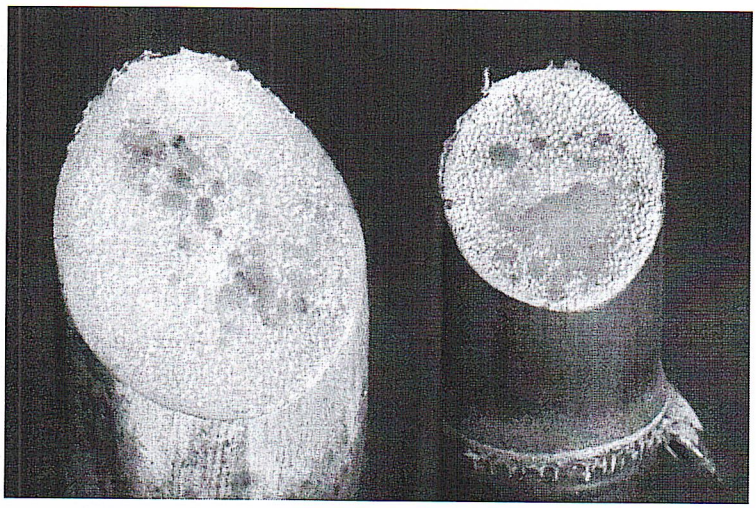

Figure 3. Yellow bacterial slime exuding from sugarcane stalks infected with *X. axonopodis* pv. *vasculorum* (Source: CABI, 2022)

Note: As the disease is not reported in Nepal and no symptoms described in maize are available in literature, the symptoms described/reported in sugarcane (main host) are given.

1.5 Epidemiology

The violent wind and rain during cyclones are ideal conditions for infection. High temperatures during the growing season favours the disease. Epidemics are particularly severe when late cyclones immediately precede the early start of a dry and cool maturing season (Ricaud and Autrey, 1989). These latter conditions appear very favourable to systemic infection as they lower plant resistance.

1.6 Mode of dispersion / Pathway

The *Xanthomonas axonopodis* pv. *vasculorum* is primarily spread through infected cutting or by using contaminated equipment.

a) **Primary transmission:** Transmission occurs primarily through the use of infected seed pieces and contaminated tools (cane knives) during field work or at harvest.
b) **Aerial transmission:** The transmission and spread from plant to plant may occur by wind-driven rain. High levels of humidity and warm temperatures are needed for the exudation of inoculum from infected leaves as well as for the entry of the pathogen into new leaves wounded by the shearing action of their saw-tooth edges and the wind.

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 axonopodis* pv. *vasculorum* 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 the 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 $100m^2$ should be inspected for pathogen surveillance. Three or more plants can be chosen randomly from every row to be inspected (FAO, 2023).

2.6 Plant 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 3-6 mm wide streaks arising from the leaf margin and develop along the vascular bundles towards the base. The leaves may show yellow to orange with red fleck streaks that become necrotic and grey with age. Place each sample in a separate 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.

Microscopic examination of a leaf portion mounted in water will reveal a profuse concentric exudation of bacteria and slime from the cut end of vascular bundles. The systemic stage can be easily recognized by the presence of gum pockets and the exudation of slime from the cut surface of infected stalks.

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
- 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.

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

2.9 Identification methods

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 using a set of morphological, biochemical, physiological tests and molecular approaches.

2.9.1 Isolation of bacteria

X. axonopodis pv. *vasculorum* may be isolated from leaf blade portions showing the streak symptoms.

Sample Preparation

- **Surface Sterilization:** Dip samples in 70% ethanol for 30 seconds or 1% sodium hypochlorite for 60 sec, followed by rinsing with sterile distilled water.
- **Homogenization:** Crush 1-2 cm^2 of symptomatic tissue in sterile distilled water or phosphate-buffered saline (PBS).
- **Filtration:** Filter the extract through sterile gauze or centrifuge at low speed (5,000 rpm for 5 minutes) to remove debris.

Bacterial Isolation

- **Growth Medium:** Streak the supernatant onto a suitable medium like nutrient agar (NA), yeast extract peptone glucose agar (YPGA), or specialized agar media for *Xanthomonas* spp.
- **Incubation:** Incubate plates at 28°C for 48-72 hours.

2.9.2 Cultural identification

Nutrient media	Colony characteristics
Nutrient Agar (NA)	Yellow, smooth, and shiny colonies, moderately slimy appearance

Yeast extract peptone glucose agar (YPGA)	Development of pale yellow mucoid colonies within 3-4 days

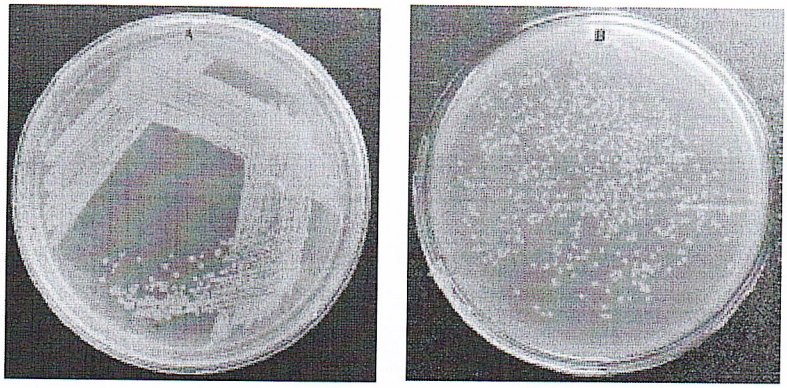

Figure 4. Colony forming by *X. axonopodis* on LB Medium
(Source: Sultana et al., 2018)

2.9.3 Morphological identification

Generally, *Xanthomonas* cells are straight rods, 0.4–1.0 by 1.2–3 micrometers, and are motile by means of a polar flagellum.

Method	**Results**
Gram staining	Gram –ve bacteria, binocular compound microscope performing gram staining showed pink to reddish-colored
Shape	Rod-shaped cells via compound microscope
Flgella	Single polar flagellum

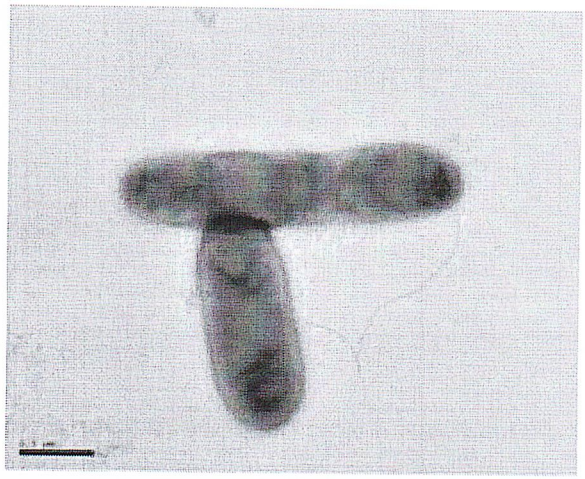

Figure 5. Transmission electron micrograph of *X. axonopodis*
(Source: Meena et al., 2017)

2.9.4 Biochemical identification

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

See Annex 3 for testing procedures.

2.9.5 Pathogenicity test

Grow 16 maize plants in the greenhouse at 28-30 °C.

At V4-V5 stage, inoculate 8 plants with sterile water (16 control plants) for each trial. Inoculate plant with 200 µl of bacterial suspension adjusted to 10^7 CFU/ml, using sterile 1 ml

syringes and hypodermic needles. Inoculate remaining plants with sterile water. Record the appearance of symptoms and take photographs of any suspected visible symptoms weekly.

For the maize pathogenicity trial, the symptom severity scores are as follows: 0 - no visible symptoms, 1 - water-soaked-like streaks, 2 - yellow or brown or white streaks, 3 - brown lesions, and 4 - deformation of plant or stunted growth. After completion of the experiment, autoclave the experimental specimen before disposal.

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 Sultan et al. (2018) & Ntambo et al. (2019) could be applied for molecular diagnostics 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 Bacteria culture and DNA extraction

Collect maize/sorghum leaf samples exhibiting bacterial streak symptoms from commercial maize/sorghum-growing areas and surface sterilize them with 75% ethanol for 1-2 minutes. Wash the leaves with distilled water as described by Zam et al. (2016). Place the sterile leaf sample on a mortar pestle and homogenize it with distilled water. Prepare a ten-fold-dilution series from leaves to extract and spread the liquid culture on a LB agar plate. Incubate cultured samples overnight at 28°C.

Bacterial genomic DNA can be extracted from cultures of *X. axonopodis* pv. *vasculorum* samples using the CTAB (Cetyl-trimethyl-ammonium-bromide) method (Gnat et al., 2017). Briefly resuspend the bacterial cells in a warm extraction buffer (20 mM EDTA, pH 8.0, 1.4 M NaCl, 10 mM Tris–HCl, pH 8.0, 3% CTAB and 0.3% mercaptoethanol) and incubate at 65°C for 30 min. Extract the suspension with chloroform/isoamyl alcohol (24:1). Add 0.6 volumes of ice-cold isopropanol to precipate the DNA. Then wash the pellet with 70% ethanol, dry and resuspend in sterile water and finally quantify using a spectrophotometer (Zamani et al., 2011).

Alternatively, DNA can be efficiently isolated using commercial extraction kits following the manufacturer's protocols.

2.9.6.2 PCR amplification and electrophoresis

The primer sets XCV1-FP and XCV1-RP are specific to *Xanthomonas axonopodis* pv. *vasculorum* and can be utilized for its detection (Table 2). This primer set amplifies 208 nucleotides-long fragments from the *PelL* gene from this bacterium with no off-target amplification (Marabella et al., 2024). For this diagnostic PCR, prepare the reaction tubes consisting of 10 µl of Master Mix (2X), 7 µl of nucleus-free water, 1 µl of each forward and reverse primer, and 1 µl of DNA template with a total volume of 20 µl.

Run the PCR with the initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 3 minutes (Marabella e al., 2024). Conduct the electrophoresis on 1.5% agarose gel in a Tris–borate-EDTA (TBE) buffer for 40 min at 100 V. Stain the gel with 0.5µl ethidium bromide/ml and visualize the DNA band/s. Production of a 208 bp long fragment on the gel confirms the sample to be positive for *Xanthomonas axonopodis* pv. *vasculorum*.

Table 2. Polymerase chain reaction (PCR) primer sequences suggested for the diagnostic test

Primer	Sequence (5'-3')	Reference
XCV1-FP	CTTGCACACGAAACGCTTCC	(Marabella et al., 2024)
XCV1-RP	ACGACATCACCAAGGACGG	

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 cryoprotectants 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.
- This method is ideal for maintaining pure cultures for long periods.

2.8 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-Nepal, in collaboration with responsible laboratories, will preserve the diseased specimens and the cultures and keep all the records safe. The documentation system will be well maintained by the NPPO-Nepal and the collaborating institutions will have easy access to it.

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Sultana, R., Islam, Md. S., Islam, Md.A., & Sikdar, B. (2018). Identification of pathogen causing common bacterial blight (CBB) of bean through the biochemical and molecular pathway and their management system. *Journal of Entomology and Zoology Studies*, 6(3), 752-757.

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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~~

Annex 2: Format for forwarding specimen

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.