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Detection Survey Protocol for *Dickeya chrysanthemi* (Burkholder et al., 1953) Samson et al. 2005 in Nepal

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Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
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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 *Dickeya chrysanthemi* 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 *Dickeya chrysanthemi* 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 *Dickeya chrysanthemi* (Burkholder et al. 1953) Samson et al. 2005 (formerly *Erwinia chrysanthemi*), a member of the soft rot enterobacteria group, is a known plant pathogen that can affect a variety of crops. While it is most commonly associated with plants like potatoes, ornamentals, and some vegetable crops, its occurrence in maize (*Zea mays*) is relatively rare. The pathogen is taken as non-quarantine for mainland in USA. The soft rot

Enterobacteriaceae (SRE) are recognized among the top 10 most important bacterial pathogens in agriculture limiting crop yield and quality (Mansfield et al., 2012). In potato, the virulent *Dickeya* strains reduced the yield of individual plants by up to 50% and caused rotting of the daughter tubers in the field and in storage (Laurila et al., 2010).

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

1.2.1 Identity

Preferred scientific name: *Dickeya chrysanthemi* (Burkholder et al. 1953) Samson et al. 2005

Preferred common name: Bacterial wilt of chrysanthemum

Other scientific names: *Dickeya chrysanthemi bv chrysanthemi*

Dickeya chrysanthemi pv chrysanthemi

Erwinia carotovora var chrysanthemi

Erwinia chrysanthemi

Pectobacterium chrysanthemi

EPPO code: DICKCC

1.2.2 Taxonomic tree of the pest

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Dickeya*

Species: *Dickeya chrysanthemi*

1.3 Host range

Banana (*Musa paradisiaca*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), sugarcane (*Saccharum officinarum*), onion (*Alium cepa*), garlic (*Allium satium*), potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), chrysanthemum (*Chrysanthemum* spp.), pink dianthus (*Dianthus* spp.), cole crop (*Brassica oleracea*), bell pepper (*Capsicum annuum*).

Corn stalk rot is a major disease of maize in tropical and subtropical countries. In temperate regions, corn stalk rot is often a problem with overhead irrigation. *Dickeya* spp. can affect a number of host species in different temperature zones (Toth et al., 2011). Over the last decade, most *Dickeya* spp. strains in Europe belong to biovars 1 and 7, and were classified as *D. dianthicola* (Janse &Ruissen, 1988). Subsequently, isolates belonging to the new genetic clade of biovar 3 have been found on potato in Europe (Sławiak et al., 2009). These strains constitute possibly a new *Dickeya* sp. named *D. solani*, which was found to be the prevalent *Dickeya* spp. on potato in Europe (Toth et al., 2011; van der Wolf et al., 2013).

1.4 Disease symptoms

D. chrysanthemi can generate a systemic rot that moves from leaves to heart (or vice versa), ignoring any stress factor displayed. *D. chrysanthemi* can affect any plant organ such as roots, stems, leaves and storage organs, depending on plant species and environmental conditions. The resulting symptoms vary from soft rot to wilting. It is particularly severe under conditions of high temperature and humidity. Even with high humidity, the disease failed to develop at 21°C; it was low at 24°C; infection was highest at 32°C. *D. chrysanthemi* can enter injured leaves, detasselling wounds, or through whorls sprayed under pressure. Infection is sometimes evident on the upper portion of the stem together with damage by *Chilo partellus*. However, when it does affect maize, the symptoms are often consistent with general *Dickeya* infections. These symptoms include:

Soft rot

- Water-soaked, soft, and mushy tissues, especially in stems or lower parts of the plant.
- A foul odor from decomposing tissues, indicative of bacterial activity.

Wilting

- Sudden wilting of leaves, even when soil moisture is sufficient.
- The plant may collapse entirely if the vascular system is severely affected.

Discoloration

- Browning or blackening of the vascular tissues.
- Yellowing of leaves as the infection progresses, particularly starting from the edges

Lesions

- Soft, sunken lesions on stems, leaves, or ears.

- These lesions can expand and lead to extensive tissue breakdown.

Stunting

- Infected plants may exhibit stunted growth due to compromised vascular function.

Secondary Infections

- The initial damage caused by *Dickeya chrysanthemi* often opens the door for secondary infections by fungi or other bacteria.

1.5 Epidemiology

- **High humidity and warm temperatures**: These conditions favor the growth and spread of *Dickeya chrysanthemi*.
- **Mechanical injuries or insect damage**: These create entry points for the pathogen.
- **Poorly drained soils**: Promote conditions for bacterial proliferation

Figure 1. Early stage of maize stalk rot – *Dickeya chrysanthemi*
(Source: Bull et al., 2010)

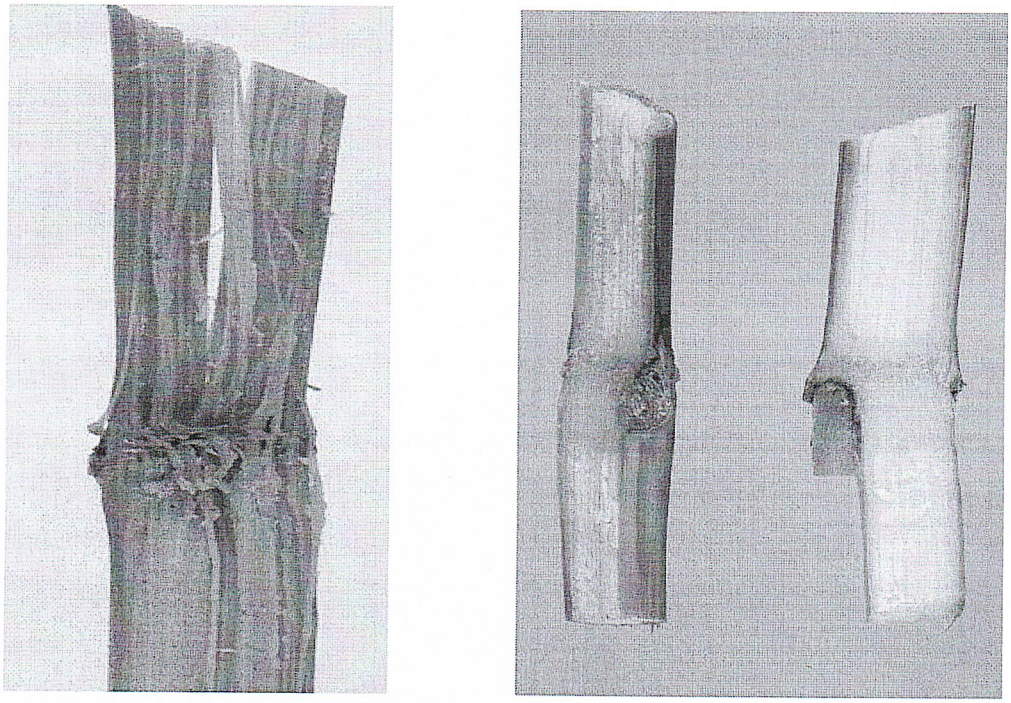

Figure 2. Stem section of maize stalk rot – *Dickeya chrysanthemi*
(Source: Bull et al., 2010)

1.6 Mode of dispersion / Pathway

The *Dickeya* are primarily spread through infected seed or by using contaminated equipment.

a) **Natural dispersal:** In the areas where *D. chrysanthemi* prevails, alternative hosts, such as weeds and volunteer host plant (tomato, potato, sugarcane, etc.), might act as an inoculum reservoir for the next cropping season.

b) **Accidental introduction:** The species is predominantly seedbornae and almost all new findings can be traced back to the movement of latently infected seed. The pathogen has also been found in irrigation water with infected crops grown in the vicinity. The machinery, transport/storage materials can serve as a source of infection if seed come into subsequent contact.

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 *D. chrysanthemi* 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. The priority will be given during the hot and humid months (May-July).

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 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. The decomposed tissue release foul odor indicating the presence of bacterial activity. On the basis of visual observation, collect infected sorghum or maize plants with typical soft, sunken lesions presence on stems or leaves and water-soaked, soft, and mushy tissues, especially in stems or lower parts of the plant. Also, collect ears if symptoms are present. 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.

2.8 Diagnostic laboratory

- National Plant Pathology Research Centre, 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 methods

Diagnostics can effectively be used to identify the presence of *Dickeya* spp., which help to avoid planting or exporting *Dickeya*-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 test and molecular approaches.

2.9.1 Isolation of the bacteria

- Collect maize stalk with water-soaked, softened, soft rot symptoms or a foul odour from different fields.
- Cut stem tissues from the margins between diseased and healthy areas with sterilized blades into 5-10 mm pieces.
- Surface sterilized the cut tissues using 70% ethanol for 30 sec and 2% NaOCl (sodium hypochlorite) for 1 min, followed by three rinses with sterile water.
- Crush the sterilized tissue pieces in sterile distilled water using a sterile mortar and pestle or by shaking in a small test tube to release bacteria for 30 minutes.
- Prepare serial dilutions of the homogenate (e.g., 1:10, 1:100, and 1:1000). Spread 100 µL of each dilution onto the surface of the recommended media (see section 2.9.2) and incubate for 24-48 h at 28-30°C.
- Look for single colony of bacteria and transferr to a new medium for purification.

Note: Crystal violet pectate (CVP) is selective medium for pectolytic bacteria like *Dickeya* spp.

2.9.2 Cultural identification

Nutrient media	**Colony characteristics**
NA medium	Smooth, opaque, cream-colored/ gray colonies with round convex raised centers with a diameter of 2 to 3 mm
King's B medium	White/ off-white, slimy and shiny colonies
Logan's medium	Red to pink colored colonies with deep red centre, colourless border
CVP medium	Characteristic pitting or clear zones due to pectolytic activity/ small, circular, concave, creamy white, or yellowish colonies with pitting

Nutrient glycerol manganese chloride medium (NGM)	Translucent, slightly convex colonies with regular edges producing blue pigment indigoidine. Blue pigment (indigoidine) used as chemotaxonomic trait for rapid identification of *Dickeya zeae.*

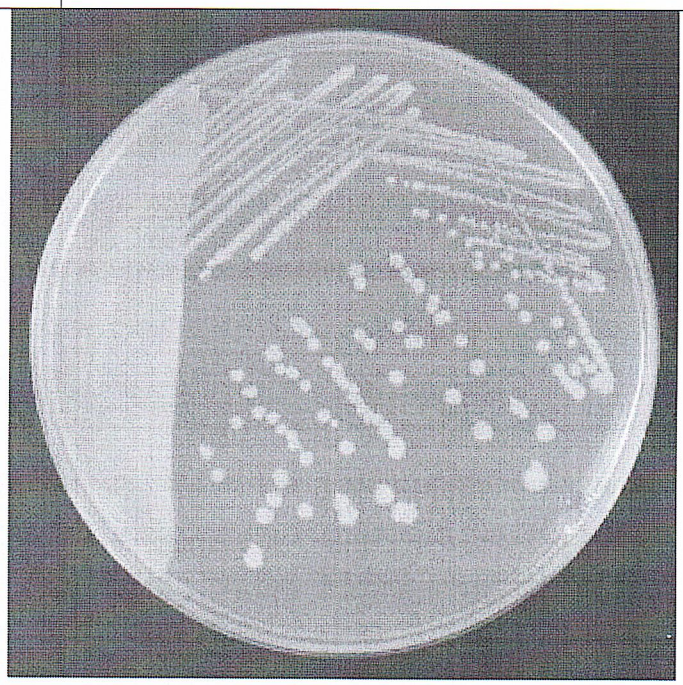

Figure 3. Colony morphology of *Dickeya chrysanthemi*
(Source: Bull et al., 2010)

2.9.3 Morphological identification

D. *chrysanthemi* is a gram-negative, facultative anaerobic, straight rod-shaped bacterium measuring 1.1-3.8 x 0.5-1 µm, usually single, motile with several peritrichous flagella, catalase positive and oxidase negative. *Dickeya* are the only plant pathogenic bacteria that are facultative anaerobes. The strains of *D. chrysanthemi* produce β-galactosidase, reduced nitrate, produce H_2S and produce acid from L-(+) –arabinose, D-(-)-ribose, L-(+)-rhamnose, D-(-)-fructose, D-(+)-galactose, D-(+)-mannose, D-(+)-cellobiose, glycerol, D-mannitol, D-sorbitol, esculin and salicin. Colony characteristics: round, white, convex, the opaque colony on YPA medium at 24 hours after inoculation. At 48 to 72 hours after inoculation, the colony shape turned to nearly round with irregular edges. Microscopic observations showed that the cells were straight rods with rounded ends, occurred singly or in pairs, and non-spore forming (Figure 3).

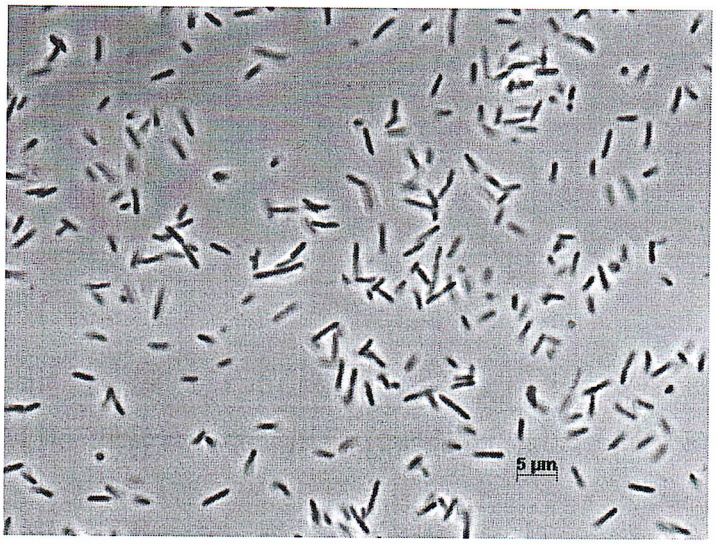

Figure 4. *Dickeya chrysanthemi* morphology
(Source: https://www.dsmz.de/collection/catalogue/details/culture/DSM-4610)

2.9.4 Biochemical identification

Test	Results
3% KOH test	A string of slime lifted with sterile loop, due to the liberation of cellular DNA, leading to viscous slime formation, confirming Gram negative nature
Oxidase test	Negative, No colour changes produced within 30 seconds of bacterium transferred aseptically on disk. Due to absence of cytochrome oxidase enzyme, *Dickeya zeae* failed to oxidizes the test reagent.
Catalase test	Positive
Gelatin liquefaction	Positive, bacterium liquefied gelatin and growth surrounded by a clear zone when agar surface flooded with 0.2% mercuric chloride solution in 20% HCl.

See Annex 3 for testing procedures.

2.9.5 Pathogenicity test

- Inoculate four-week-old corn plants (susceptible) with 100 µl of cell suspensions containing 10^8 cfu/ml.

- For inoculation, pinprick at the base of an internode of the stem and then incubate in a greenhouse at 28°C and at 80% relative humidity.
- Observe stem infection and leaf blight similar to symptoms observed in natural infections two weeks after inoculation.
- Maintain negative controls using distilled water.
- Re-isolate the bacteria from the inoculated plants showing typical symptoms and compare with original cultures *in vitro*.

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 Aeny et al. (2020) could be applied for molecular diagnostic of the *D. chrysanthemi,* 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

Inoculate bacteria into 5 mL yeast peptone (YP) medium (Suharjo et al., 2014). Incubate in a shaking incubator (185 rpm) at 27°C overnight. Harvest the bacterial cells by centrifugation (14,000 rpm for 10 min). Extract DNA from the bacterial cells using the cetyltrimethylammonium bromide method (Ausubel et al., 2003). For molecular analysis, the DNA can be used at the concentration of ~ 1 µg/µL.

Perform PCR using 25 µL total volume of the mixture 16SrDNA, *rec*A, and *dna*X using MyTaq™ Red Mix (Bioline, USA) according to the manufacturer's instruction. The PCR can be carried as follows: 1 cycle of an initial denaturation at 94°C for 5 min, 30 cycles consisted of denaturation at 94°C for 1 min, annealing at 58°C (16S rDNA), 57 °C (*dna*X) or 56°C (*rec*A) for 1 min, primer extension at 72°C for 1 min, and final extension at 72°C for 5 min. The primers could be used in this study are listed in Table 1.

2.9.6.2 DNA sequencing and analysis

The PCR products of 16S rDNA, *dna*X and *rec*A can be electrophoresed in 0.5% agarose gels containing ethidium bromide (10 mg ml^{-1}) with Tris-Boric Acid-EDTA (TBE) buffer (pH 8.0) at 50 Volt for 70 min. The result can be visualized under DigiDoc UV transilluminator

(UVP, USA). The PCR products of 16S rDNA, *rec*A, and *dna*X were sent to reliable DNA sequencing service provider laboratory for sequencing.

The sequencing results can then be analyzed using BioEdit for Windows program ver. 7.2.6 (Hall, 1999). The phylogenetic tree can be constructed based on sequences of 16S rDNA, *rec*A and *dna*X using the neighbor-joining method (Jukes and Cantor model) with MEGA7 for Windows (Kumar et al. 2016) or with different packages of R. Sequence data of *Dickeya* species reference strains can be obtained from NCBI GenBank (https: //www.ncbi.nlm.nih.gov/).

Table 1. Polymerase chain reaction (PCR) primer sequences that could be used in study

Locus	Primer	Sequence (5'-3')	Reference
16S rDNA	fD1	CCGAATTCGTCGACAACAGAGTTTGATCCTGGC TCAG	Weisburg et al., 1991
	rP2	CCCGGGATCCAAGCTTACGGCTACCTTGTTACG ACTT	
dnaX	dnaXf	TATCAGGTYCTTGCCCGTAAGTGG	Sławiak et al., 2009
	dnaXr	TATCAGGTYCTTGCCCGTAAGTGG	
recA	RS1	GGTAAAGGGTCTATCATGCG	Suharjo et al., 2014
	RS2	CCTTCACCATACATAATTTGGA	

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 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.