1.

Detection Survey Protocol for *Pantoea stewartii* (Smith 1898) Mergaert et al. 1993 in Nepal

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Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
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March 12, 2025

Detection Survey Protocol for *Pantoea stewartii* (Smith 1898) Mergaert et al. 1993

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 *Pantoea stewartii* 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 *Pantoea stewartii* 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 *Pantoea stewartii* (Smith 1898) Mergaert et al. 1993 (formerly *Erwinia stewartii*), cause Stewart's bacterial wilt (also called Stewart's disease, or bacterial wilt), which was first reported in the United States on Long Island, New York, in 1897. The nomenclature of the genus *Erwinia* was modified on the basis of chemotaxonomic and molecular approaches and the new genus *Pantoea* was proposed. Shortly thereafter, *E. stewartii* was assigned

to *Pantoea stewartii.* It is most commonly associated with plants like maize (*Zea mays*). It has been reported from many countries and probably exists throughout the world. In the United States the importance of the disease has declined with the availability of resistant corn hybrids, but the disease causes significant losses in developing countries. In sweetcorn, economic losses can be significant when susceptible or moderately susceptible hybrids are grown in an area where flea beetles occur. Losses do not occur or are minimal in resistant and moderately resistant hybrids; however, losses frequently range from 40 to 100% when susceptible sweetcorn hybrids grown under epidemic conditions are infected before the 5-leaf stage (Pataky and Eastburn, 1993).

1.2 Identity and taxonomy of the target pest (CABI, 2020)

1.2.1 Identity

Preferred scientific name: *Pantoea stewartii* (Smith 1898) Mergaert et al. 1993

Preferred common name: Bacterial wilt of maize

Other scientific names: *Aplanobacter stewartii* (Smith) McCulloch 1918

Bacillus stewartii (Smith) Holland 1920
Bacterium stewartii (Smith) Smith 1905
Erwinia stewartii (Smith 1898) Dye 1963
Pantoea stewartii subsp. *indologenes Mergaert* et al. (1993)
Pantoea stewartii subsp. *stewartii* (Smith 1898) Mergaert et al. 1993
Phytomonas stewartii (Smith) Bergey et al. 1923
Pseudobacterium stewartii (Smith) Krasil'nikov 1949
Pseudomonas stewartii Smith 1898
Xanthomonas stewartii (Smith) Dowson 1939

EPPO code: ERWIST

1.2.2 Taxonomic tree of the pest

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: *Pantoea*

Species: *Pantoea stewartii*

1.3 Host range

Maize (*Zea mays*), teosinte (*Zea mays* subsp. *mexicana*), Sudan grass (*Sorghum sudanense*), crabgrass (*Digitaria* spp.), rice (*Oryza sativa*), smooth meadow-grass (*Poa pratensis*), and Dracaena (*Dracaena sanderiana*).

1.4 Disease symptoms

There are two major phases of Stewart's wilt disease: (i) wilt and (ii) leaf blight. The wilt phase occurs when plants infected during early vegetative growth stages become systemically infected.

- Usually these plants rapidly wilt and may closely resemble plants with symptoms of drought, cold weather chilling injury, nutritional deficiency, or insect injury.
- Leaves have linear, pale green to yellow streaks with irregular wavy margins that follow veins and may extend the length of the leaf. Lesions rapidly desiccate and become necrotic. Plants that are not killed may produce bleached, dead tassels.
- Cavities may form near the soil line in the stalk pith of severely infected plants.
- Bacteria colonize the vascular system and may spread throughout the plant. Field or dent corn is generally not as susceptible as sweet corn to this phase of the disease, although some field corn inbred lines and hybrids are very susceptible and some sweet corn hybrids are quite resistant.
- Stalk rot might be appeared as secondary infection.

The second and more common phase of this disease occurs on the leaves at any growth stage and thus is called the leaf blight phase.

- It is typically associated with the local infection of leaf tissue due to vector transmission.
- Symptoms usually are most apparent after tasseling. Lesions on leaves are gray-green to yellow-green and develop as streaks with wavy margins along the veins.
- The streaked areas, which usually originate from feeding scars of the corn flea beetle, die and become straw colored.
- If the disease is severe, entire leaves may die and dry up. When leaves die prematurely, plants are predisposed to fungal stalk rots.

- However, the leaf blight phase is not as damaging as the seedling wilt phase, which can kill plants.

Important points to be taken during visual observation

A simple method to make a presumptive diagnosis of Stewart's wilt based on symptomatic leaf tissue is to observe, with the aid of a microscope, bacterial ooze streaming from vascular bundles of cut sections of symptomatic leaf tissue placed in a drop of water. Another bacterial disease, Goss's wilt, caused by *Clavibacter michiganensis* subsp. *nebraskensis*, might be confused with Stewart's wilt by simply checking for bacterial streaming. The leaf symptoms are slightly different in that Goss's wilt produces dark green to black spots, or freckles, in the leaf lesions. Look for the corn flea beetle which is the vector of Stewart's wilt. Adult is 1.3-3.5 mm long, shiny and black, oval, faint green or blue-bronze shine.

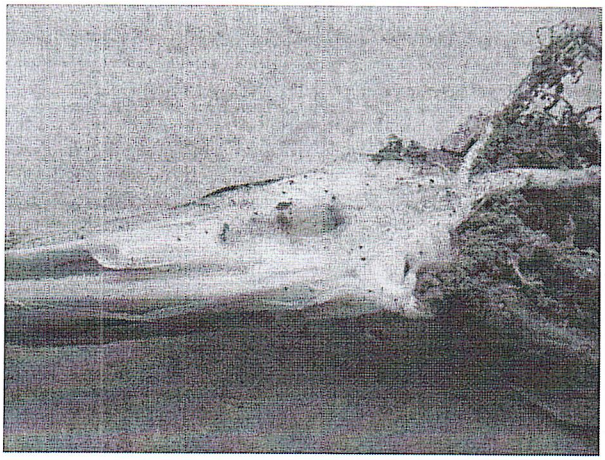

Figure 1. Internal cavity resulting from systemic infection by *Pantoea stewartii*.

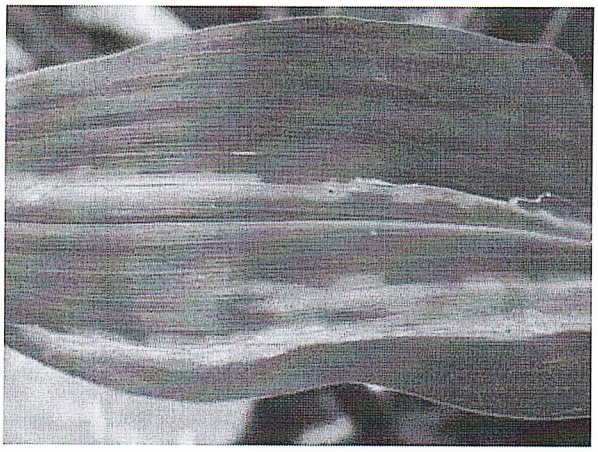

Figure 2. Early symptoms of Stewart's bacterial wilt, leaf blight phase

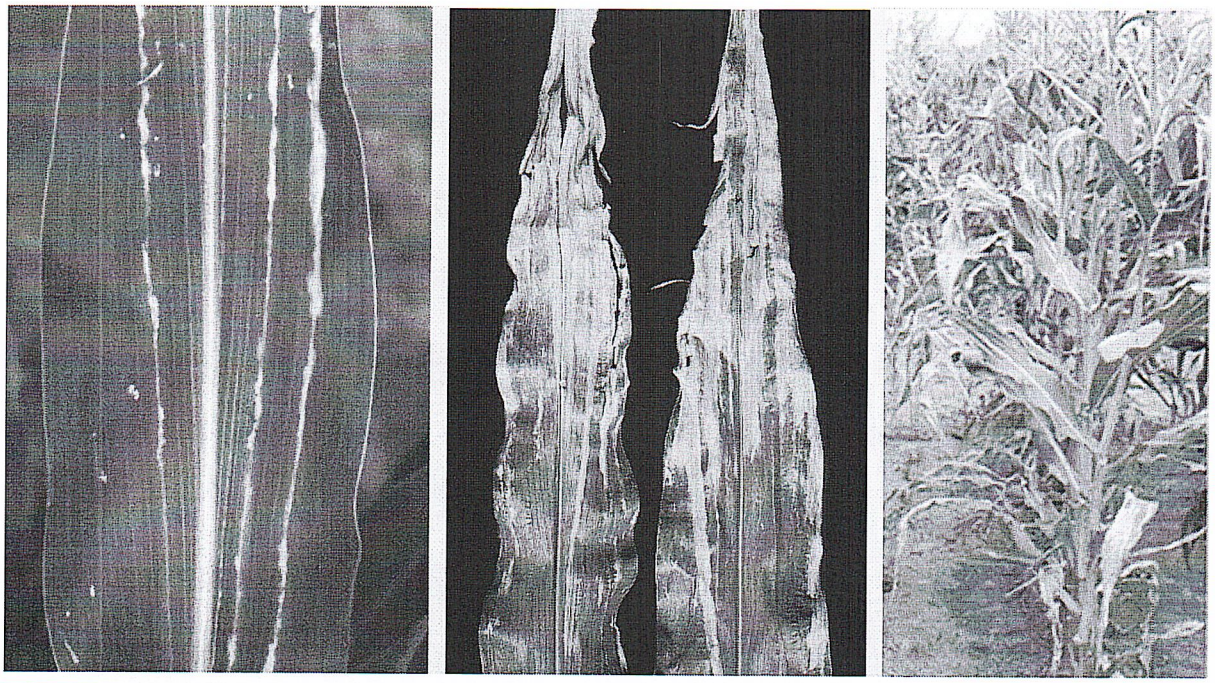

Figure 3. Typical leaf symptoms, close-up of infected leaves showing stripes of dead tissue and Leaf blight phase of disease (from left to right) (Source: Caroline Roper, 2011; CABI, 2020)

1.5 Epidemiology

Plant-to-plant spread does not occur without the presence of the corn flea beetle and disease incidence is correlated directly with the numbers of corn flea beetles present in corn fields (Claflin, 2000; Menclas *et al.*, 2006). There are two major cycles of Stewart's wilt infection throughout the growing season. The first cycle occurs when the emerged overwintering adults transmit the bacteria to young corn seedlings. This first cycle of Stewart's wilt is the most damaging, because corn is infected at the seedling stage, the most vulnerable developmental stage of the plant. The second cycle of infection occurs when the bacteria are transmitted by the first summer generation of the insect that have acquired the bacterium from the infected plants. The timing of the emergence of this population of flea beetles coincides with a decrease in the susceptibility of the corn plant that occurs after tasselling. The optimum temperature for growth of *P. stewartii* subsp. *stewartii* ranges from 27 to 30°C; the maximum temperature for growth varies between 32 and 40°C (Mergaert et al., 1993).

1.6 Mode of dispersion / Pathway

This pathogen can be seed borne but seed-transmission is very low. It is vectored by the corn flea beetle (*Chaetocnema pulicaria*). See Appendix 3.

Note: The beetle is not reported in Nepal.

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 *Pantoea stewartii* 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 warm temperature and humid months (June-September) for summer maize and March-April for spring maize. Monitor the field for corn flea beetles, as they play a critical role in the transmission of *Pantoea stewartii*. High beetle population indicate a greater risk of disease.

2.3 Selection of survey area

As per the requirement 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 field 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 where more corn flea beetle colonize. Collect tissue from infected maize showing typical symptoms (yellow streaks, wilting, and vascular discoloration). Cut small pieces (~1 cm) from the infected margins (transition zones between healthy and diseased tissue). 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. Transport the samples to the lab in sterile bags or containers.

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 *Pantoea* spp., which help to avoid planting or exporting *Pantoea*-infected stocks. Isolation of suspected bacterium will be done for confirmation and the pathogen will be identify by suing a set of morphological, biochemical, physiological test and molecular approaches.

2.9.1 Isolation of bacteria

Pantoea stewartii may be isolated from the plants showing the disease symptoms.

Surface Sterilization

- Surface-sterilize the sample by dipping in 70% ethanol for 30 seconds, followed by rinsing in sterile distilled water.

Tissue Homogenization

- Using a sterile scalpel, cut small sections from the vascular tissue of the infected maize.
- Crush the tissue in a small volume (1-2 ml) of sterile distilled water to extract the bacteria. Vortex or shake for 10–15 minutes to release bacterial cells.

Streaking on Agar

- Streak the extract onto nutrient agar or King's B medium.
- Incubate at 30°C for 24–48 hours.

2.9.2 Cultural identification

Nutrient media	Colony characteristics

NA media	Small, round, slow-growing and yellow. Nutrient agar streaks vary from thin, yellow, moist and fluid, to thin, dry, orange-yellow and not fluid. Broth culture shows feeble growth, a whitish ring and yellow precipitate.
King's B media	White or yellow pigment in culture produced around the colonies on King's B agar medium.
Yeast extract-dextrose-calcium carbonate agar	Colonies are yellow and convex.
Nutrient-glucose agar	Colonies on are cream-yellow to orange-yellow.

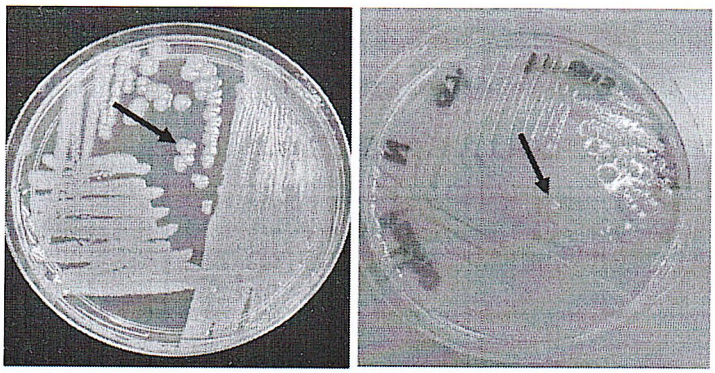

Figure 4. Colony morphology of *Pantoea stewartii* on a King's B agar medium (Source: Abidin et al., 2020)

Some strains grow at 4°C, some at 37°C, and no strain grows at 41°C. Colonies on yeast extract-dextrose-calcium carbonate agar are yellow and convex. Colonies on nutrient-glucose agar are cream-yellow to orange-yellow. *P. stewartii* produces extracellular polysaccharides that are associated with pathogenicity, i.e., virulence (Bradbury, 1967; Pepper, 1967). *P. stewartii* subsp. *stewartii* strains do not produce indole and do not hydrolyse esculin. No growth occurs on citrate, mucate, cis-aconitate and DL-4-aminobutyrate. No acid is produced from L-rhamnose, cellobiose, maltose, lactose, gentiobiose, a-methyl-D-glucoside, arbutin, salicin, amygdalin, glycerol, D-arabitol, dulcitol, *meso*-inositol, D-gluconate and 2-keto-D-gluconate. The main cellular fatty acids are octadecenoic acids ($C_{18:1}$), hexadecanoic acids ($C_{16:0}$) and *cis*-9-hexadecenoic acids ($C_{16:1}$).

2.9.3 Morphological identification

The bacterium is a facultative anaerobic, Gram-negative, yellow pigmented, mucoid, nonflagellate, nonspore-forming, rod measuring approximately 0.4-0.8 x 0.9-2.2 µm.

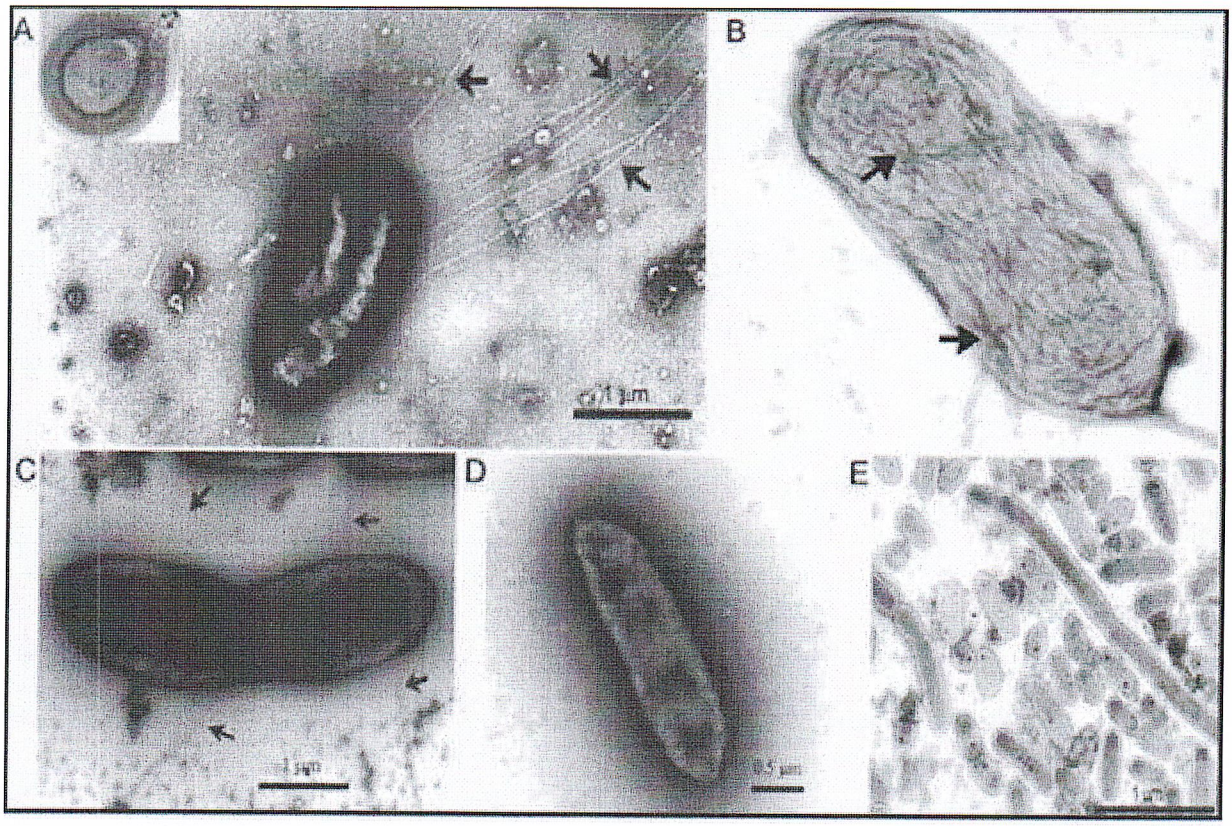

Figure 5. *Pantoea stewartii* morphology
(Herrera et al., 2008)

11.5 Motility test

Motility test is crucial because most bacteria under *Pantoea* genus are motile due to the presence of peritrichous flagella except for *P. stewartii* subsp. *stewartii* (Roper, 2011; Goszczynska et al., 1999 and Gutierrez, 2008). Indole test is also important in subspecies identification (Gehring et al., 2014; Nechwatal et al., 2018), as *P. stewartii* subsp. *indolegenes* distinguished and identified from *P. stewartii* subsp. *stewartii* through the positive result of indole test with Kovac's reagent. The outcome of Abidin et al. (2020) indole and motility test results from the 28 strains has also shown strong positivity towards the identification of *P. stewartii* subsp. *stewartii*.

2.9.4 Biochemical identification

Test	Results

3% KOH test	Positive, string of slime lifted with sterile loop, due to liberation of cellular DNA leading to viscous slime formation, confirming gram negative nature
Oxidase test	Negative, No colour changes produce 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 reaction	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.
Indole production	Negative

See Annex 4 for testing procedures.

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 Tambong et al (2008) could be applied for molecular diagnostic of the *P. stewartii.* 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

Culture strains of *P. stewartii* in Luria–Bertani (LB: 10g l^{-1} tryptone, 5g l^{-1} yeast extract, 10g l^{-1} NaCl) or nutrient broth. Harvest bacterial cells in late log phase, extract genomic DNA and purify DNA by the wizard SV Genomic DNA purification system (Promega, Madison, WI, USA). Determine the concentration of DNA using the Nano Drop ND-1000 Spectrophotometer (NanoDrop Technologist, Wilmington, DE, USA). Store purified DNA at -20°C. Use specific primers (CPSL1 and CPSR2c, Coplin et al. 2002) for the *cpsD* gene to amplify a 1.1-kb fragment using modified PCR conditions with 10ng of template DNA. Perform PCR amplifications with Titanium *Taq* DNA polymerase (Clontech, Inc., Palo Alto, CA, USA) in a thermal cycler (Biome tra GmbH i.L., Gottingen, Germany) with an initial denaturation at 95°C for 3 min,

followed by 40 cycles of 95°C for 45s, 60°C for 45s and 72°C for 90s and a final extension at 72°C for 8min. The primers could be used in this study are listed in Table 2.

Table 2. Polymerase chain reaction (PCR) primer sequences that could be used in study

Locus	Primer	Sequence (5'-3')	Reference
cpsD	CPSL1	CCTGTCAGTCTCGAACC	Coplin et al. 2002
	CPSR2c	ATCTCGAACCGGTAACC	
16S	Uni-BacF	GATCCTGGCTCAGGATGAAC	
rRNA	Uni-BacR	GGACTACCAGGGTATCTAATC	

2.9.6.2 DNA sequencing and analysis

Sequence the *cpsDE* fragment of isolated strains of *P. stewartii* as described by Tambong et al. (2006) by using ABI BigDye Terminator chemistry v3.0 (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3100 Avant automated sequencer (Applied Biosystems/Hitachi). Analyze the sequencing results using BioEdit for Windows program ver. 7.2.6 (Hall, 1999). Construct phylogenetic tree based on sequences using the neighbor-joining method (Jukes and Cantor model) with MEGA7 for Windows (Kumar et al. 2016) or with different packages of R. Obtain sequence data of *Pantoea* species reference strains from NCBI GenBank (https: //www.ncbi.nlm.nih.gov/).

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-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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The Plant Disease Diagnostic Clinic. Stewart's Wilt: *Pantoea (Erwinia) stewartii.* Cornell University.

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. Vector transmission, *Chaetocnema pulicaria* (Melsheimer)

The *Pantoea stewartii* is transmitted to plants *via* corn flea beetle (*Chaetocnema pulicaria*), where it first colonizes the apoplast causing water-soaked lesions and then migrates to the xylem and forms a biofilm that locks water transport. So, the proved corn flea beetle as a vector of *P. stewartii* should be taken into account in the detection survey along with the Stewart's wilt or bacterial wilt disease in maize.

Corn flea beetles are small, shiny, black beetles, approximately 1.3-2.5mm long, with enlarged hind legs. It feeds on corn leaves by stripping away the top layer of plant tissue. Feeding damage appears as gray to brown lines or tracks etched on the leaf surface. Heavily infested plants may appear gray as their leaves shrivel and dies (Cook and Weinzierl, 2004).

Life cycle of *Chaetocnema pulicaria* (Melsheimer) (Coleoptera: Chrysomelidae)

The corn flea beetle passes the winter as an adult, in the soil and debris in fencerows, roadsides, or the edges of woodlands. It becomes active early in the spring when temperatures warm to 18-21°C, although they may be seen feeding on grasses on warm days during the winter. Corn is the primary host of the corn flea beetle, but adults and larvae also feed on a number of secondary hosts such as orchard grass, Kentucky bluegrass, yellow foxtail, giant foxtail, fall panicum, and several other grasses. Wheat, barley, oats, and Timothy have also been identified as food plants for the flea beetle. In the spring, adults feed on corn and other hosts, mate and lay eggs on plant leaves, in the ground, or at the base of plants near underground stems and roots. The generation time of the corn flea beetle is approximately one month. Larvae complete their development, pupate, and adults emerge in June. The adults continue to feed on available host plants and lay eggs for another generation. Second-generation adults appear in early August and feed until late in the fall before overwintering.

Adults: Adults are easily disturbed and are known for their ability to jump long distances. They are small, oval shaped, shiny, black beetles, approximately 1.3-2.5mm long, with enlarged hind legs.

Egg: Eggs are white and pointed at one end. Female lay their eggs on leaves of weeds, corn, other cultivated crops, or in the ground, on or near underground stems and roots of the host plant. The eggs are typically scattered in the soil on the base of the host plant.

Larvae: The larvae are small, white, slender grubs and not very active. Full-grown larvae are 3.2-8.5mm long and most body segments are non-pigmented. Only the prothorax and the last abdominal segment are slightly darkened. The larvae are found under the soil surface. The larvae can be found feeding on the underground plant parts.

Pupa: It pupate in the soil and emerge as adults in late July.

Figure A1. Corn flea beetle adult (Source: Caroline Roper, 2011)

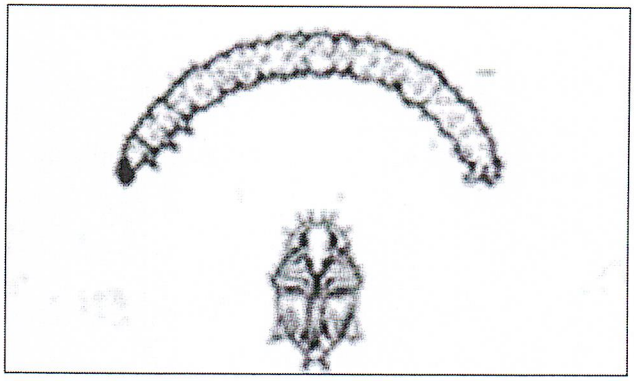

Figure A2. Corn flea beetle larvae and pupa

Figure A3. Corn flea beetle injury

Annex 4. 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.