13.

Detection Survey Protocol for *Peronosclerospora philippinensis* (W. Weston) C. G. Shaw in Nepal

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

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Detection Survey Protocol of *Peronosclerospora philippinensis* (W. Weston) C. G. Shaw
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 *Peronosclerospora philippinensis* 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 to undertaking a pest detection survey of *Peronosclerospora philippinensis* 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)

Peronosclerospora philippinensis is an oomycete pathogen responsible for causing Philippine downy mildew in maize (CABI, 2013). It belongs to the family Peronosporaceae and is one of the most destructive downy mildew pathogens affecting cereal crops, particularly in tropical and subtropical regions. *P. philippinensis* is considered the most virulent of the downy mildew pathogens affecting maize, causing substantial losses to crop production (Murray, 2009). Under normal conditions, a 40-60% yield reduction is observed; however, under favourable conditions the losses reach up to 80-100% (Exconde & Raymundo, 1974). This pathogen thrives under warm, humid conditions and spreads through airborne conidia and oospores, which can survive in plant debris or soil.

1.2 Identity and taxonomy of the target pest

1.2.1 Identity

Preferred scientific name: *Peronosclerospora philippinensis* (W. Weston) C.G. Shaw, 1978
Preferred common name: Philippine downy mildew of maize

Other scientific names: *Sclerospora maydis* Reinking
Sclerospora philippinensis W. Weston

Nepali name: सेते रोग
EPPO code: PRSCPH

1.2.2 Taxonomic tree of the pathogen is presented below (CABI, 2023)

Domain: Eukaryota
Kingdom: Chromista
Phylum: Oomycota
Class: Oomycetes
Order: Peronosporales
Family: Peronosporaceae
Genus: *Peronosclerospora*
Species: *Peronosclerospora philippinensis*

1.3 Host range

The host range of *Peronosclerospora philippinensis*, a destructive oomycete pathogen causing downy mildew, primarily includes species within the Poaceae (grass) family. Its hosts are predominantly cereal crops and other grasses. Maize (*Zea mays*), sorghum (*Sorghum bicolor*), sugarcane (*Saccharum officinarum*), Johnson grass (*Sorghum halepense*), oats (*Avena sativa*), pearl millet (*Pennisetum glaucum*) etc. are the important hosts of the pathogen (CABI, 2024). Among them, maize and sorghum are the primary hosts.

1.4 Disease symptoms

The symptoms caused by *P. philippinensis* primarily on maize and sorghum, are due to systemic infection of the pathogen. Various types of symptoms can be visualized, such as:

a) **Chlorosis** - Infected plants exhibit pale green to yellow streaks or patches on the leaves. Chlorosis typically starts as irregular streaks along the leaf veins and progresses, leading to widespread discoloration (Fig. 1). These symptoms often appear within a few days of infection (Singh et al., 2020).

b) **White downy growth** -Thick, white, wooly growth of conidia and conidiophores can be observed underneath infected leaves (Fig. 2), particularly under high humidity and in the early morning. This growth is a diagnostic feature (Gupta and Paul, 2002; Magill et al., 2006). These symptoms may appear as early as 3 days after infection.

c) **Stunting, necrosis and drying** - As the disease progresses, leaves become narrow and abnormally erect, and the plant remains stunted (Fig. 3). Over time, chlorotic areas turn necrotic, resulting in leaf desiccation and premature drying of leaves (Singh et al., 2020).

d) **Tassel Malformation (Phyllody), sterility and poor grain formation** - In maize, tassels may be malformed or completely replaced by leafy structures. This symptom is known as "tassel phyllody" and is associated with systemic infections. Infected plants often fail to produce viable ear formation. Grain formation is severely reduced, leading

to significant yield losses (Magill et al., 2006). Severely infected plants may die prematurely before reaching the reproductive stage, especially under conducive environmental conditions.

Systemic symptoms such as stunting, tassel malformation, and sterility are prominent in maize, while leaf symptoms and stunting and floral sterility are more commonly observed in sorghum (*Sorghum bicolor*).

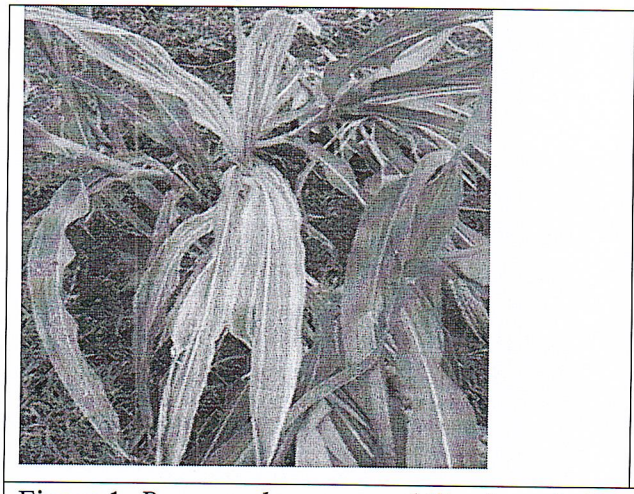

Figure 1. *Peronosclerospora philippinensis* (downy mildew of maize); field symptoms on maize (*Zea mays*) (Source: CABI, 2020)

Figure 2. Sign (downy growth) of *P. philippinensis* on the underside of a leaf (Source: CABI, 2020)

Figure 3. Symptoms, showing stunted and leaf streaks in maize plant (Source: CABI, 2020)

1.5 Epidemiology

Over 20,000 conidia per sq. cm. of leaf can be formed on leaves, which were continuously wet for 12 hr. The optimum temperatures for conidial formation range from 16 to 28°C. Germination is considerably enhanced in dew water and in plant whorl exudates. Disease incidence can be observed higher with rainfall >13 cm; mean temperatures of 26.3-27.5°C and relative humidity of 88% (Payak, 1975).

1.6 Mode of dispersal / Pathway

a) **Airborne conidia:** Philippine maize downy mildew pathogen is commonly disseminated by air currents and rain splash. This results in localized spread among neighboring hosts of the pathogen (Weston, 1923; Singh et al., 2020).
b) **Infected plant materials:** Plant materials, including crop residues from infested fields, can harbor the pathogen. When infected material is transported across regions or countries, the pathogen can be introduced into previously uninfected areas, especially if quarantine measures are not enforced (Irwin et al., 1999; Singh et al., 2020).
c) **Contaminated seeds:** The disease could also be spread through seeds, especially if they have not been properly dried and have a moisture content of more than 30% (Magill et al., 2013; Murray, 2009; USDA, 2013; Purdue University, undated).
d) **Soil-borne oospores:** Oospores, the sexual spores of *P. philippinensis*, can persist in the soil for extended periods, serving as a source of primary inoculum in subsequent growing seasons (Singh et al., 2020). However, long-distance transmission through infected host planting material is unlikely because oospores are rarely reported (Acedo & Exconde 1967).

1.7 Disease vector

Not known.

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 *Peronosclerospora philippinensis* 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

Detection surveys need to be done during the early seedling (5-15 days after emergence), vegetative stage (15–45 days after emergence), tasseling to silking stages (45–75 days after emergence) and reproductive stage (75 days onward) of the host crop (i.e., maize) for detection of *Peronosclerospora philippinensis* in the field.

2.3 Selection of survey area

Field plots of maize and concerned crops in the target areas.

2.4 Materials required for survey

Paper bags (envelops), blotter papers or stack of newspaper, plant press, scissor, hand lens, gloves, face mask, forceps, tags, permanent markers, GPS, camera, and data sheets.

2.5 Identification method

To identify *Peronosclerospora philippinensis,* a combination of diagnostic methods is typically used. Morphological identification of *P. philippinensis* is based on known reference specimens, literature descriptions and taxonomic keys and descriptions.

2.5.1 Morphological features of *Peronosclerospora philippinensis*

Mycelium is hyaline and coenocytic (non-septate), grows intercellularly in host tissues and produces haustoria that penetrate host cells to absorb nutrients. The mycelia are branched, slender (8 µm in diameter) and irregularly constricted (Fig. 4). The conidiophores are dichotomously branched, measuring 15-26 x 150-400 µm, erect, emerging from the stomata and terminal branches bear conidia. The conidia are hyaline, ovoid to round, cylindrical, slightly rounded at the apex, 17-21 x 27-39 µm. The haustoria are simple, hyaline and vesiculiform to subdigitate (Crouch et al., 2022). Oospores, which are rarely produced, are spherical, smooth-walled and approximately 22 µm in diameter.

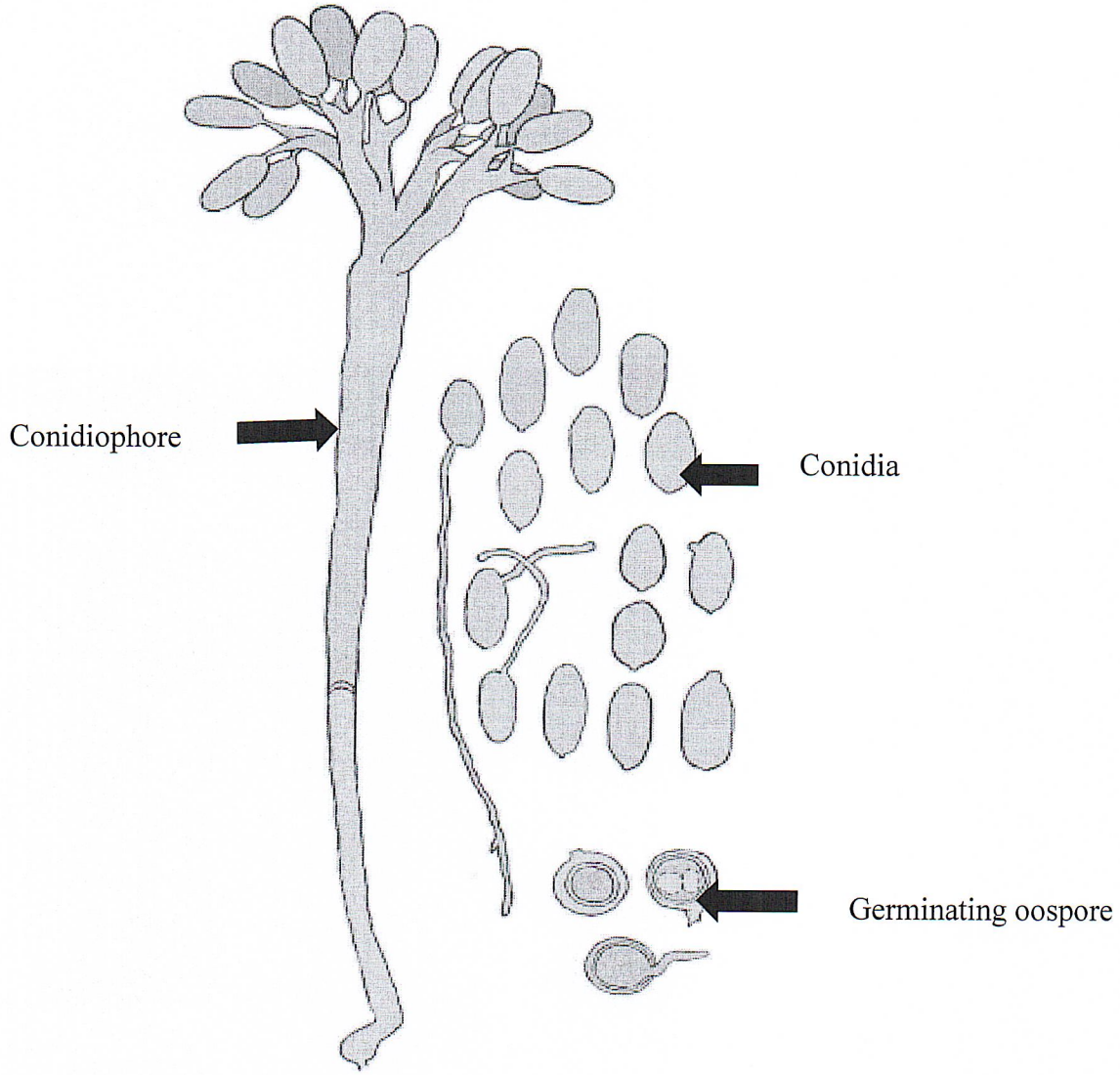

Figure 4. Morphology of *Peronosclerospora philippinensis* (Crouch et al., 2022)

Table 1. Key differences between various *Peronosclerospora* species

Pathogen	Host range	Optimum temp. for sporangia production	Conidiophores / Sporangiophores	Conidia / Sporangia	Oospores
P. philippinensis (Philippine downy mildew)	Oats, teosinte, cultivated and wild sugarcane, cultivated and wild sorghum	21-26°C	Erect and dichotomously branched two to four times, 150 to 400µm in length and emerge from stomata	Ovoid to cyclindrincal (17-21µm x 27-38µm), slightly rounded at apex	Rare, spherical (25 to 27µm in diameter and smooth-walled

P. sacchari (Sugarcane downy mildew)	Sugarcane, teosinte, sorghum and wild grasses	20-25°C	160 to 170µm in length erect and arise singly or in pairs from stomata.	Elliptical, oblong (15-23µm x 25-41µm) with round apex	40 to 50µm in diameter, globular, yellow
P. sorghi (Sorghum downy mildew)	Cultivated and wild sorghum, Johnson grass, teosinte, wild grasses (*Panicum, Pennisetum, Andropogon* species)	17-29°C	Erect, dichotomously branched, 180 to 300µm in length. Emerge singly or in groups from stomata.	Oval (14.4-27.3 × 15-28.9µm), borne on sterigmata (about 13µm long	Spherical (36µm diameter average), light yellow or brown in color
Sclerophthora macrospora (crazy top)	Oats, wheat, sorghum, rice, finger millet, various grasses	24-28°C	Very short (14µm on average).	Lemon-shaped (30-65 x 60-100µm), operculate	Pale yellow, circular (45-75µm)
S. rayssiae var. *zeae* (Brown stripe downy mildew)	*Digitaria* species	22-25°C	Short determinate and produced from hyphae in the substomatal cavity	Oval to cyclindrical (18-26 x 29-67µm)	Spherical (29-37µm in diameter), brown in color

2.6 Number of plants to be sampled

The number of maize plants to sample for the survey and surveillance of *Peronosclerospora philippinensis* depends on the purpose of the survey, such as detection, delimitation, or monitoring; the field size; and the required confidence level for disease detection (Fletcher et al., 2010).

a) Based on the area of the field (Aggarwal et al., 2022)

- Small fields (up to 1 hectare): Sample at least 100 plants randomly across the field.
- Large fields (>1 hectare): Use a systematic approach such as a transect or grid pattern to sample 1-2% of the total plants in the field.

b) Initial detection

- For initial disease detection, sample 10 plants for 100 m^2 for inspection. (FAO, 2023).

2.7 Sampling method

Several sampling methods are available to assess crops for plant disease. A common technique is to sample plants at random or uniform intervals along a path of a predetermined design. This technique is used to obtain samples from a field within a reasonable time. Conventional sampling includes the diagonal, W, V and X (Basu et al., 1977; Lin et al., 1979), which cover an entire field or are restricted to subdivisions of a field. These are referred to as whole-field and partial-field designs, respectively. The entire-field X and W designs are equivalent

to one another, and are the most precise; the diagonal design is intermediate and the partial field designs are the least precise (Lin et al., 1979).

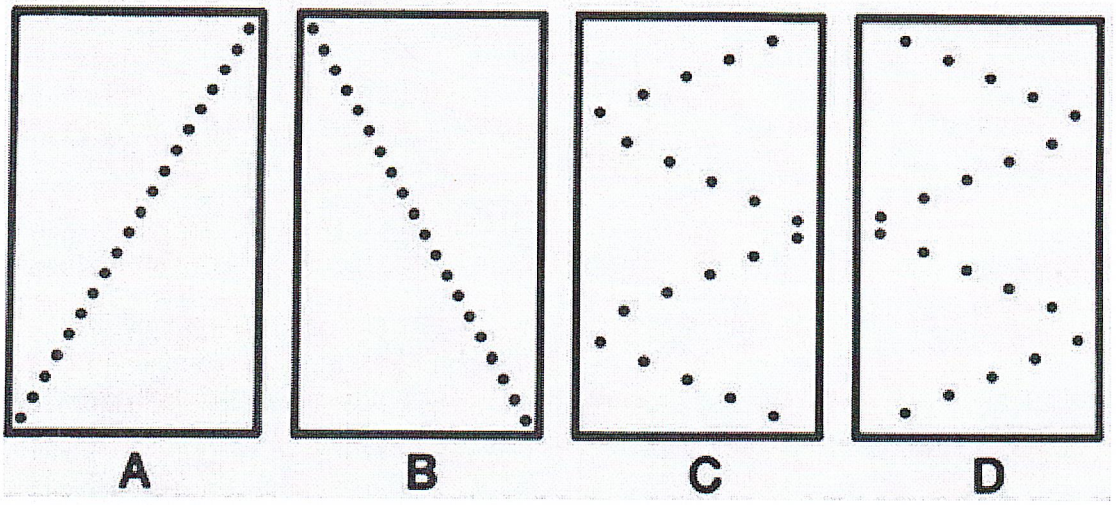

Figure 5. Sampling designs. Points represent sample sites. A. right diagonal, B. left diagonal, C. right W, D. left W (Source: Delp et al., 1986)

2.8 Plant parts to be observed

When surveying or monitoring maize for *P. philippinensis*, the following plant parts should be carefully observed for symptoms and pathogen structures:

a) **Leaves:** Look for chlorotic streaks or patches on the upper leaf surface and white, downy fungal growth on the lower leaf surface (especially during early mornings).
b) **Tassels:** Look for malformation or phyllody in tassels.
c) **Stem:** Look for stunted growth and deformities in the stem.
d) **Young seedlings:** Look for chlorotic streaks and uneven or stunted growth

2.9 Specimen collection and preparation

For the proper collection and preparation of samples:

- Identify plants showing typical symptoms of the disease.
- Collect samples from various parts of the fields, especially from high-risk zones like field edges, low-lying areas, and regions near water sources.
- Collect leaves with visible symptoms and, if available, sample both early-stage and advanced-stage symptoms.
- Collect malformed or sterile tassels if symptoms are present.
- If seedlings are affected, uproot the entire plant carefully, ensuring the roots and above-ground parts remain intact.
- Place each sample in a separate paper envelope (do not use plastic bags) 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.

- Assign a voucher number for future reference.
- Transport samples to the diagnostic lab as soon as possible.

2.10 Herbarium preparation

For the proper herbarium preparation of samples:

- Select maize leaves with clear downy mildew symptoms.
- Collect multiple leaves from different plants for variation.
- Preferably collect in the early morning, when sporulation is visible.
- If whole leaves are too large, cut representative sections (~15–20 cm).
- Place the diseased leaves between sheets of newspaper or blotting paper.
- Arrange them flat and spread out to avoid overlap.
- Stack the papered samples in a plant press and apply even pressure.
- Change the blotting paper every 2-3 days to prevent fungal growth.
- Use herbarium sheets for mounting.
- Attach the dried leaves using glue or adhesive tape.
- Arrange to show key symptoms (both upper and lower leaf surfaces).
- Label the herbarium sheet with:
  - ✓ Scientific name: *Zea mays* (Maize)
  - ✓ Disease name: Downy mildew (*Peronosclerospora* sp.)
  - ✓ Collection details (date, location, collector's name, and specimen number)
  - ✓ Symptoms observed
  - ✓ Host and pathogen identification details

2.11 Preparation for diagnosis

2.11.1. Surface sterilization: Surface sterilize small leaf sections showing symptoms with 1% sodium hypochlorite for 30 seconds, followed by rinsing with sterile water.

2.11.2. Preparation of moist chamber: Place two layers of filter paper at the bottom of the 9 cmPetri dish. Moisten the material with sterile distilled water. Ensure it is damp but not waterlogged to prevent leaf tissues from rotting.

2.11.3. Incubation: Place surface sterilized leaf sections onto the moistened blotter with the underside of the leaf facing upward. Place the Petri dish containing leaf sections in an incubator at 24°C for 24 hours. If sporulation is not observed, extend incubation up to 48-72 hours.

2.11.4. Microscopic examination: Examine the leaf tissue under a stereomicroscope to confirm the presence of conidiophores and conidia. For confirmation, slide preparation should be carried out (Cardwell et al., 1997; Janruang & Unartngam, 2018). To prepare slides, the following steps should be taken:

- Gently scrape the sporangia from the leaf surface using a fine brush or needle.
- Place the spores into a small drop of lactophenol on a clean glass slide.
- Carefully place a cover slip over the drop to avoid air bubbles.

- Gently press the cover slip to spread the sample evenly.
- Examine the slide under a compound microscope.
- Identify the sporangia
- Capture photographs for record-keeping and comparison.
- If needed, preserve slides by sealing the edges with nail polish for long-term storage.

Since *P. philippinensis* is an obligate biotroph, culturing cannot be done in an artificial medium.

2.12 Data recording and mapping

- Data should be recorded in several aspects like
  - ✓ Date of collection
  - ✓ Collection number
  - ✓ Locality
  - ✓ GPS coordinates
  - ✓ Elevation
  - ✓ Host plant type and infestation severity
  - ✓ Plant growth stage
  - ✓ Local name(s)
  - ✓ Habit and habitat
- Use mapping tools like GIS to create infestation distribution maps

2.13 Diagnostic laboratories

- 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
- Private laboratories – Center for Molecular Dynamics Nepal (CMDN), Thapathali, Kathmandu Nepal Plant Disease and Agro Associates (NPDA), Balaju, Kathmandu, and others, identified if any.

2.14 Reporting

The responsible or concerned organizations (diagnostic laboratory) or an independent surveyor, after analysis and identification, should submit a report to the NPPO-Nepal for the reporting/declaration of the pathogen. The reports should include infestation maps, photographs and specimen vouchers.

2.15 Record keeping

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

2.16 Molecular identification of *Peronosclerospora philippinensis*

There are several molecular techniques described for molecular detection of the fungi. One of them is given below, which is not necessarily a mandatory method to follow. Any other established/adopted methods may be used alternatively.

2.16.1 DNA extraction

Infected corn should be incubated as indicated above to get the fresh conidiophores and conidia produced on the upper and lower corn leaves. Conidiophores and conidia should be isolated using sterilized small syringes under a stereo-microscope and transferred to 50 ml of 5% (w/v) Chelex 100 buffer using sterilized distilled water in 1.5 ml Eppendorf tubes. Then, the solution should be boiled in a water bath for 8 min and mixed by vortexing three times and internal transcribed spacer 1 using PCR.

2.16.2 PCR reaction and sequencing

The DNA should be amplified in the regions of Domain 1- Domain 2 (D1/D2) of 28S rDNA using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 primers (5'-GGTCCGTGTTTCAAGACGG-3'). PCR reactions should be carried out in 50 µl containing 5 µl of genomic DNA, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM dNTP mixed, 20 pmol for forward and reverse primers and 1U Taq polymerase. Perform PCR Thermocycler with follows steps: initial denaturation at 96°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 sec., 848 annealing at 60°C for 30 sec. and 72°C for 30 sec. with a final extension of 4 min at 72°C. Conduct the amplification of the internal transcribed spacer1 (ITS1) region coupled with the 28S rDNA region using ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS2 primers (5'- GCTGCGTTCTTCATCGATGC-3'). Perform PCR reactions in 40 µl containing 5 µl of genomic DNA, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl), 2.5 mM MgCl2, 0.2 mM dNTP mixed, 10 pmol for forward and reverse primers and 1U Taq polymerase. Perform PCR in a Thermocycler (Biometra) as follows: initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 sec., annealing at 55°C for 1 min and 72°C for 1 min with a final extension of 10 min at 72°C. After amplification, 5 µl of all PCR products should be electrophoresed in a 1% (W/V) agarose gel and purified using a GeneJET PCR purification kit (Thermo Scientific, Lithuania) and sent for sequencing (Janruang & Unartngam, 2018).

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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.