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# Detection survey protocol for *Solanum carolinense* L. 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 for *Solanum carolinense* L.

**NPPO-Nepal, 2025**

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## 1. Background information

With entry in the WTO, Nepal gets 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. The 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 threat to the related industries within the country. Nepal should provide adequate description of the health status of plant based industries, while negotiating access to foreign trades. Prospective importers of Nepalese agriculture related commodities assess 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 negotiate with importing countries on a fair trading system. This document gives detailed guidelines for detection surveys of the invasive weeds *Solanum* in the agriculture field. 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 Plant Quarantine and Protection Act, 2064, article 6(2), survey and surveillance function and responsibility is designated to NPPO-Nepal as per the sub clause (i) "To perform such other function as prescribed". This technical guideline to undertake pest detection survey of *Solanum carolinense* has been prepared with a view to guide the survey activity. This guideline is prepared for researchers, plant protectionists, teachers, and other concerned professionals. This document will be a guide to submit specimens to the laboratory for diagnosis and preservation.

### 1.1 About the pest (weed)

The genus *Solanum* (L.) (nightshade) comprises approximately 1500 species of plants belonging to the solanaceae family. Among several species of *Solanum*, *Solanum carolinense* is a very widely distributed perennial herb that is still expanding its range, and is considered one of the world's worst weeds. This species invades and occupies farmland, competing with native crops and trees for fertilizer, water, sunlight and space, thus causing serious crop harvest losses. This species has a high capacity for spatial dispersal by natural means and different human-mediated pathways. Thus, it is likely that the plant will expand its range and infest cultivated land, particularly around existing naturalised populations. The extent of yield loss depends largely on the density of *Solanum carolinense* but also on the crop type, and low-growing crops seem to be more affected. Frank (1990) reported that *Solanum carolinense* grown for 3 years and 1 year prior to planting snap beans (*Phaseolus vulgaris*), reduced yield by 48-65% and 18-20%, respectively. This species contains solanin and is poisonous to cattle, horses and sheep when ingested (Kingsbury, 1964). It is also an important alternate host for insect pests of crop plants such as the Colorado potato beetle (*Leptinotarsa decemlineata*) (McIndoo, 1935) and the pepper maggot (*Zonosemata electa*) (Foott, 1963). It is also host for the potato psyllid (*Paratrioza cockerelli* [*Bactericera cockerelli*]), which transmits psyllid yellow disease to potatoes and tomatoes (Wallis, 1951), tomato leaf spot fungus (*Septoria lycopersici*) (Pritchard and Porte, 1921) and several viruses (Weinbaum and Milbrath, 1976; Ramsdell and Myers, 1978). In account to the seriousness of the invasive weeds, implementing a thorough survey and surveillance technique is essential to

precisely tracking and managing the spread of *Solanum*. The development and implementation of an effective protocol would facilitate detecting early infestation, prompt response, and the implement of control measures – all of which are essential for ensuring sustainable exports of haylage from Nepal to China on the basis of Pest Risk Analysis (PRA) of the World Trade Organization Agreement on the application of sanitary and phytosanitary measures (WTO SPS). The spread of seeds mostly occurs due to increasing trade, travel and other accidental introduction. Because the probability of introducing the weed from infested to other non-infested countries is high, a vigilant approach to surveillance and quarantine is required. This protocol provides guidelines for planning and conducting *Solanum* survey and surveillance activities.

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

**1.2.1 Identity**

**Preferred scientific name:** *Solanum carolinense* (L.)

**Preferred common name:** Horsenettle or Ball nettle

**Nepali local names:** Kanthakari

**EPPO code:** SOLCA (*Solanum carolinense*)

**1.2.2 Taxonomy**

Taxonomic tree of the *Solanum* is presented below (CABI, 2021)

| | |
|---|---|
| Kingdom: | Plantae |
| Phylum: | Spermatophyta |
| Class: | Dicotyledonae |
| Order: | Solanales |
| Family: | Solanaceae |
| Genus: | *Solanum* |
| Species: | *Solanum carolinense* |

**1.3 Host range**

The main host are: Maize (*Zea mays*), Alfalfa (*Medicago sativa*), Potato (*Solanum tuberosum*), Tomato (*Solanum lycopersicum*), Soyabean (*Glycine max*), Groundnut (*Arachis hypogea*), Wheat (*Triticum aestivum*), Cotton (*Gossypium hirsutum*), Beetroot (*Beta vulgaris*)

This species grows as weed in grains and vegetables fields, orchards, pastures, gardens, nurseries, riverbanks, roadsides and disturbed areas. It grows in a wide range of soil types, but thrives in sandy or gravelly soils. The plant grows rapidly during hot weather and tolerates drought (Bradbury and Aldrich, 1957).

**1.4 Weed biology**

The distribution of *Solanum carolinense* is limited in cool environments by intense frost, and the length of the growing season (Bassett & Munro, 1986). Stems are frost sensitive and tops usually die following frost in autumn. It needs warm temperatures for germination, sprouting and

growth. The plant grows rapidly during hot weather (Ilnicki & Fertig, 1962). Freshly harvested seeds are highly dormant, and alternating temperatures from 20 to 30°C increase germination. Nishida et al. (2000) pointed out that germination of *Solanum carolinense* does not occur at temperatures below 14°C under field conditions. *Solanum carolinense* is a prolific seed producer. It can produce ca. 40–170 seeds per fruit, with a single plant producing up to ca. 5000 seeds (Bassett & Munro, 1986; Ilnicki & Fertig, 1962). Seeds can germinate and plants emerge from depths of 10 cm (Ilnicki & Fertig, 1962). Seeds retain viability for at least 3 years when buried at depths of 8–12 cm according to Brown and Porter (1942). Solomon (1983) remarked that seeds remained viable for at least 7 years when stored under laboratory conditions. *Solanum carolinense is* pollinated by a variety of generalist insects. In North America, non-specialist bees (*Lasioglossum* spp., *Bombus* spp. *Xylocopa* spp.) are described as the main pollinators of this species (Quesada-Aguilar et al., 2008; Wahlert et al., 2015). The poricidal anthers must be vibrated by pollinators to release pollen (i.e., buzz pollination; Hardin et al., 1972). *Solanum carolinense* is an andromonoecious species (i.e. plants bear either hermaphrodite flowers or male flowers or both) with a system of gametophytic self-incompatibility (GSI; Travers et al., 2004), which is quite uncommon among other weed species. The schematic diagram of *Solanum carolinense* is shown in Figure 1.

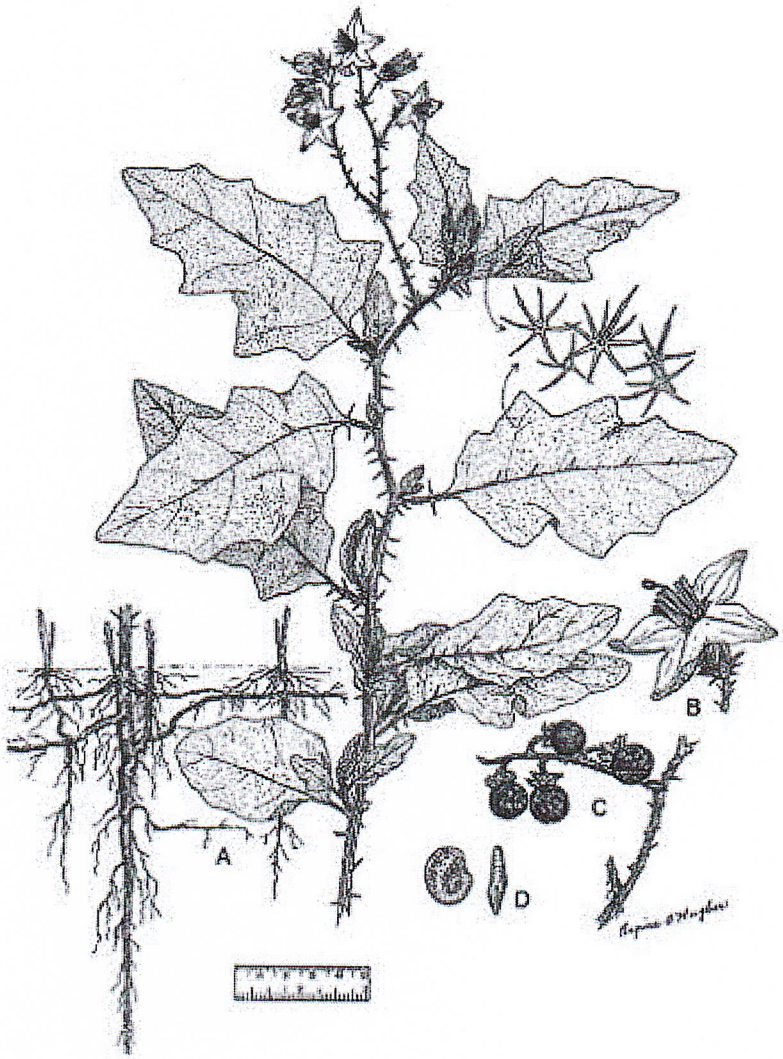


**Figure 1.** Schematic diagram of *Solanum carolinense* L. A. habit, B. flower, C. berries, D. seeds (Source: http://www.namethatplant.net)

### 1.5 Mode of dispersion

The *Solanum carolinense* propagates by creeping roots and seeds (CABI, 2021). Some of the modes of its dispersion are:

a) **Natural dispersal:** Natural dispersal of *Solanum carolinense* is principally by seeds, roots and root cutting. Natural dispersal on a small scale could occur through seed dissemination. Horizontal roots can extend several metres from the taproot and contribute to small-scale dissemination.

b) **Human activities:** The movements of root fragments through agricultural machinery is strongly suspected to be the main dispersal vector from field-to-field. .

c) **Animal dropping:** The seeds can maintain viability after passing through the digestive tract of cattle, horses, pigs or sheep. The berries may be eaten by farm animals and the seeds subsequently scattered over large areas in animal droppings.

d) **Agricultural practices:** Tillage of fields infested with *Solanum carolinense* promotes the dissemination of the plant by cutting the roots and dragging them elsewhere provided that other favourable conditions such as good weather conditions, less competitive crops plants and ineffective weed control are available. Harvesting operations may transport mature berries to other places, which also encourages dissemination.

e) **Movement in trade:** Seeds of *Solanum carolinense* may be a contaminant in grain imported for (i) animal feed mixtures and (ii) human consumption, including for processing. The grain imported for human consumption is likely to be less contaminated than that for animal consumption as regulations are stricter. This species can infest many crops, particularly maize and soybean and these crops are harvested at a period when seeds of *Solanum carolinense* are present, thus, the seed lots can be infested by seeds of this species. The long distance spread of *Solanum carolinense* has been speculated to be due to the inter-state movement of hay in the USA.

## 2. Detection survey

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 methodologies based on statistical sampling, which are 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 detection survey is to determine the presence or absence of *Solanum carolinense* 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 timing of survey should be coincided with the flowering period as the plant species is generally identified by morphology and flower characteristics. Flowering often occurs during the monsoon period (July-August) in Nepal. Detection surveys need to be done during pre-planting, vegetative stage and reproductive stage of the host crop (*i.e.*, maize) for detection of *Solanum carolinense* 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

- Field press bag
- Herbarium press (45×30 cm)
- Secateurs and scissor
- Plant digger (hand hoe or shovel)

- Gloves, scales and ropes
- Zip locks plastic bags (18×41 cm)
- Newspaper and Blotting sheet
- Topographic maps
- Altimeter
- GPS or mobile apps with geotagging
- Magnification glass
- Forceps, needle, brush and glue
- Field notebook, logbooks
- Permanent ink pens and tags
- High pixel digital camera
- Field guide with image of *Solanum carolinense*
- Mini glass/plastic vials for weed seeds
- Chemicals (preservatives), if necessary

### 2.5 Identification method

Classification and identification of *Solanum* species depends largely on floral characters. Morphological identification of *Solanum* plants (including seeds) is based on known reference specimens, literature descriptions and taxonomic keys and descriptions. Considerable data from molecular studies of *Solanum* are available and can be helpful for species determination, but until methods can be simplified and made more uniform they are of limited value for phytosanitary purposes.

#### 2.5.1 Identification of mature plants

Morphological characteristics of mature plants of the *Solanum* species are summarized in Table (1). *Solanum carolinense* is a perennial herb, up to 1.2 m tall, unbranched or branched near the base, with both vertical and horizontal roots, the latter spreading horizontally up to 5 m. Stems are armed with slender yellowish spines (prickles) up to 6 mm long. Leaves are also sparsely to moderately armed with prickles up to 6.5 mm long on the major veins abaxially and adaxially. Leave blades 2–15 × 2–10 cm in size, margins lobed with 1–4 lobes per side, sometimes very deeply lobed almost to the midrib, apex is acute to obtuse, and the petioles are 0.4–4 cm in size (Figure 4). Inflorescences consist of 1–20 flowers. They are white, lilac, or purple and star-shaped with five yellow poricidal anthers (Figure 2 & 3). Fruits are 1–2 × 1–1.8 cm in size, light green with darker green mottling or pale greenish-white when immature, bright yellow at maturity and glabrous (Figure 5) (Bassett and Munro, 1986; Wahlert et al., 2015).

**Table 1.** Summary of the main morphological characteristics of mature plants of the *Solanum* species

| Species | Plant height (cm) | Plant appearance | Leaf | Flower |
|---|---|---|---|---|
| *Solanum carolinense* | 120 | Branched stem armed with slender yellowish spines (prickles) | Undersides of leaves defensively armed with needle-like hairs | Usually white, rarely blue to lavender |

| | | | | |
|---|---|---|---|---|
| *Solanum elaeagnifolium* | 90 | Erect bushy-branched plant | Entire plant except for the upper surface of the leaves, covered by tiny, dense, canescent, tomentum stellate hairs which gives the stems and leaves a silvery-green appearance | Light blue to violet-purple; prickles are found on the pedicles |
| *Solanum dimidiatum* | 60-90 | Stems are covered in stellate hairs but plant is still dark green; not velvety appearance | Stout prickles are distributed along the stems and on the main veins on the underside of the leaves | Blue, violet-purple or white |

**Figure 2.** Flowering habit of *Solanum carolinense*

**Figure 3.** Flower of *Solanum carolinense*

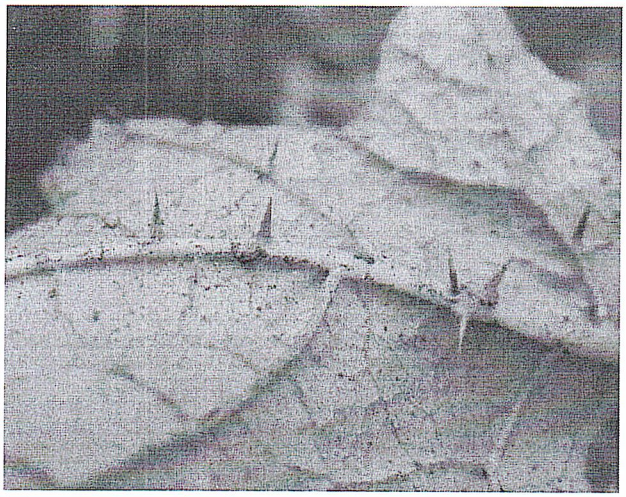

**Figure 4.** Underside of leaves of *Solanum carolinense*

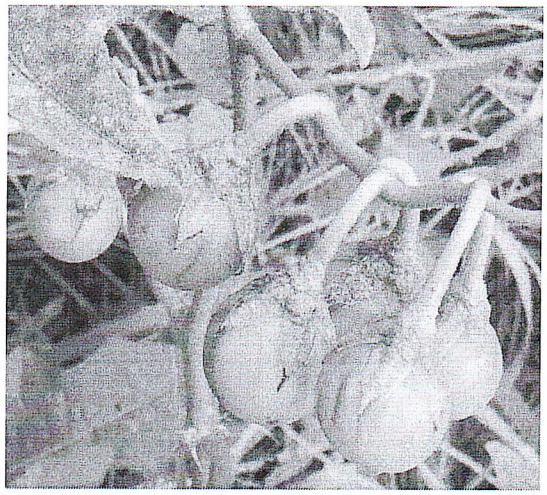

**Figure 5.** Berries of the *Solanum carolinense*

(Source: CABI, 2021)

**2.5.2 Identification of seed:** Seeds are 1.7–2.4 × 1.6–1.8 mm in size, flattened-reniform, lenticular, yellow, and the surface is finely foveolate.

**2.6 Number of specimen sampled for identification**

All the specific host fields should be monitored. At least 2-3 specimens in each field should be collected for identification.

**2.7 Sampling methods**

The weed sampling procedures can be followed as suggested by Colbach et al. (2000). They are described below and choose one of them, depending on field situations.

**2.7.1 Methods based on random selection**

There are four random methods:

a) **Random method:** It consists of choosing samples entirely randomly from the field and is often used in weed research.
b) **Distance10 method:** Samples are required to be separated by at least 10 m. The sampling procedure is as follows: the $i^{th}$ sample is chosen randomly from the simulated field and its distance to each of the ($i$-$l$) first samples is calculated; if any of these distances is smaller than 10 m, then the sample is discarded and a new one chosen; otherwise, the $(i+l)^{th}$ sample is selected.
c) **Distance20 method:** The procedure is the same as in the Distance10 method, but with a minimal sampling distance of 20 m.
d) **Stratified method:** The field is divided into five equal parts and then, a fifth of the required samples is selected randomly in each of these parts.

**2.7.2 Methods based on systematic selection**

The systematic positioning of samples is often used to ensure that samples are placed independently of the experimenter, avoiding or choosing unknowingly certain field areas while increasing sampled field area (Scherrer, 1983). There are two systematic selection methods:

a) **Diagonal method:** The samples are selected on the two diagonals of the field. The sampling process for $N=2n$ samples is as follows: the field (of length $l$ and width $w$) is divided into $n^2$ rectangles of $l/n \times w/n$ m$^2$; the first sample is chosen randomly in the rectangle located on the field edge; if its coordinates are $(x_1, y_1)$; then the coordinates of the $i^{th}$ sample taken on the same diagonal are $[x_1+(i-1)\times w/n;\ y_1+(i-1)\times l/n]$ and the co-ordinates of the $i^{th}$ sample taken on the second diagonal are $[x_1+(i-1)\times w/n;\ l-y_1-(i-1)\times l/n]$.

b) **Zig-zag method:** The samples are taken from three lines assembled vaguely as an "S". The sampling process for $N=3n-2$ samples consisted of dividing the field into $n \times N$ rectangles of $w/n \times l/N$ m$^2$. The first sample of co-ordinates $(x_1, y_1)$ is again chosen randomly in the rectangle located on the field edge and the subsequent samples are chosen according to a protocol similar to that for the diagonal method.

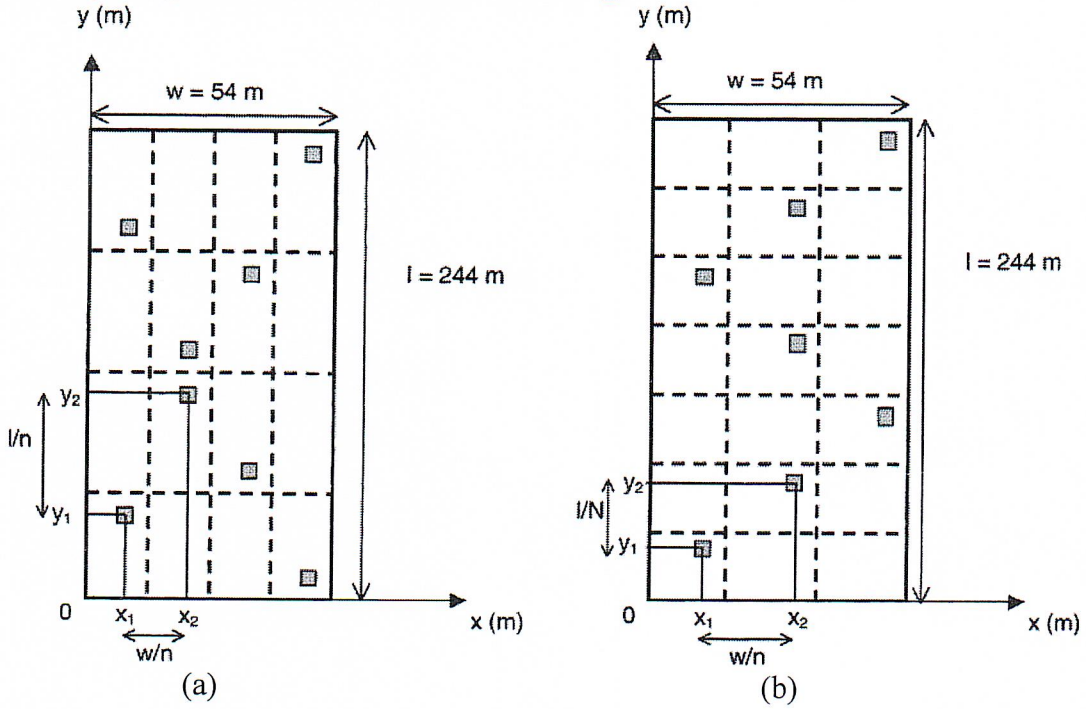


**Figure 6.** Example of a systematic sampling plan - (a) $n=4$ selecting eight samples ($N=2n=8$) and using diagonals, (b) $n=3$ selecting seven samples ($N=3n-2=7$) and using lines assembled as a zigzag (Colbach et al, 2000)

## 2.8 Specimen collection and preservation

### 2.8.1 Collecting technique

- Select specimen in good condition, free of insect damage or disease
- Collect plants that represent the range of variation in the population (2-3 plants in each field), not just atypical specimens
- For seedling plants, collect the entire plants (tops and roots)

- For mature plants, collect stems, twigs, leaves, and flowers or fruits of plants and retain as much the root system as possible
- Properly tagged with collection number

### 2.8.2 Take photographs of the specimen in the field

- Take pictures of the entire plant in its natural environment (remove any other vegetation around it).
- Make sure to capture leaves, stems, roots and flowers as much as possible.
- In many cases, one can remove the plant from the soil, brush or wash off the soil from the roots and lay it on a white paper background.
- Save the picture in a JPEG format with a clear resolution that allows one to see plant detail.

### 2.8.3 Precautions to be taken in the field

- Place the sample in a sealed plastic bag with moist paper towels (water may be sprinkled inside the bag, if needed) and keep cool or place it under a shade till transport.
- Press specimens in the field while collecting and whenever possible because delay causes a significant difference in the final shape and appearance (discoloration, structure etc.).

### 2.8.4 Pressing and drying

- Fold sufficient newsprint sheets (ca. 10) in half to form 45×30 cm folders and place plants to be pressed on the right half of the folder.
- Arrange plants carefully with a minimum overlap and flowers open showing both top and underside; leaves showing both upper and underside; bend or cut stem to accommodate the right half of the sheet.
- Turn the left half sheets over the specimens on the right half and press them using a plant press frame (45×30 cm) or by keeping under uniform weight covering the whole sheet area
- Loose seeds can be collected in separate packets and sealed.
- While pressing, prevent plant parts from curling or wrinkling during the drying process.
- Change the newspaper sheets in between, if they get moistened during drying. Drying can be done by placing the plant press frame with the specimens 15 cm above the home light bulb (60 or 100 watts) for ca. 24 hr.

### 2.8.5 Mounting technique

- Keep ready herbarium sheet (mounting paper) (45×30 cm)
- Glue the label to the bottom right corner of the herbarium sheet.
- Run a bead of glue only along the top edge of the label.
- Arrange and glue the dried specimens on the herbarium sheet.
- Arrange the specimens on the sheet in such a manner that there will not be a balancing problem in storage.
- Attach the specimen to the mounting paper with thin ribbons of glue running from the paper across the plant part to the paper.
- A dot of glue beneath the flower head may be needed if the head is large and cannot be held down with a strap of glue on the petiole.
- If only one flower is placed on the sheet, protect it by gluing a transparent flexible covering over it.

- Use line straps or white gummed mounting tape to the size required to attach thick stems.

**2.9 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

**2.10 Diagnostic laboratories**

- National Agronomy Research Centre, Nepal Agricultural Research Council, Khumaltar, Lalitpur
- Central Agricultural Laboratory, Department of Agriculture, Hariharbhawan, Lalitpur
- Department of Agronomy, Agriculture and Forestry University, Rampur, Chitwan
- Department of Agronomy, Institute of Agriculture and Animal Science, Tribhuvan University, Kirtipur, Kathmandu
- Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu
- National Herbarium and Plant Laboratories, Department of Plant Resources, Godawari, Lalitpur
- Natural History Museum, Swayambhu, Kathmandu

## 3. 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 weed. The reports should include infestation maps, photographs and specimen vouchers. If specimens cannot be identified morphologically, they should be identified by molecular methods.

## 4. 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.

## 5. Molecular diagnosis of *Solanum carolinense*

A number of methods are available for molecular diagnosis of plant species. They involve different steps, starting from genomic DNA extraction to their sequencing. One of the most commonly used methods is described below. However, the method is not necessarily mandatory to follow. Any other established/adopted methods may be used alternatively.

### 5.1 Sample preparation and genomic DNA extraction by CTAB method

The CTAB DNA extraction method is cheap, effective and applicable for a wide range of applications including DNA barcoding, shotgun sequencing and log-read sequences. The protocol utilizes organic solvents and the harmful nature of some of the solvents along with relatively long time period consumption to complete the protocol can, therefore, be a limitation of using this method.

The CTAB method given by Doyle and Doyle (1990) was modified by Tiwari et al. (2012), and it can be used for genomic DNA extraction from plants.

- Take 1 g of plant parts (leaves, nodal region and meristematic region) each separately from the selected plants.
- Wash properly and air dry them to remove any trace of water present.
- Add 2 mL of CTAB extraction buffer to them, and grind them properly using mortar and pestle.
- Transfer the paste to Eppendorf tubes and keep them in a water bath at 65°C for 45 min.
- Take the tubes out of the water bath and keep at room temperature for 5 min, and then centrifuge it at 12,000 rpm for 15 min.
- Take 1 mL supernatant from each tube, transfer them to another tube with a wide bore pipette, add 500 µL of Phenol (P):Chloroform (C):Isoamyl alcohol (I) to each tube, shake for 15 min in a shaker, and centrifuge at 12,000 rpm for 12 min.
- Take supernatant again in another Eppendorf tube, add equal volume of P:C:I, shake well, and centrifuge at 12,000 rpm for 10 min.
- Transfer supernatant to a new Eppendorf tube, add equal volume of C:I, shake well, and centrifuge at 10,000 rpm for 10 min.
- Transfer the supernatant (containing DNA) to a new Eppendorf tube and add pre-chilled isopropanol (2/3 amount of the supernatant), keep at -20°C for 2 hr, then centrifuge at 8,000 rpm for 8 min.
- Discard the supernatant from tubes, add 300 µL 70% ethanol to the pellet, mix well, centrifuge at 8,000 rpm for 10 min; discard the ethanol, dry the pellet by incubating it at 37°C in a dry bath for ca. 1 min, dissolve the pellet in 100 µL of TAE buffer and store at -20°C in a deep freezer.

### 5.2 Quantification of extracted genomic DNA

By using a spectrophotometer (like NanoDrop 1000, Thermo-Fisher, USA), quantitative analysis of DNA (ng/µL) can be assessed through comparison of the absorption ratio for A260/A280. The reagent contamination can also be assessed using the NanoDrop by comparing the absorption ratio of A260/A230. DNA integrity and fragment size can be assessed qualitatively using 1.5% agarose gel electrophoresis stained with ethidium bromide.

### 5.3 Polymerase chain reaction and sequencing

The marker that demonstrates sequence variations that offer sufficient discriminatory power to distinguish closely related species is ideal for species identification in adiverse family. The Consortium for Barcode of life (CBOL) has established various working groups dedicated to identifying universal barcode genes for different taxonomic groups, including cytochrome oxidase subunit I (*COI*) in metazoans, maturase K (*matK*), ribulose-1,5-bisphosphate carboxylase (*rbcL*), internal transcribed spacer (*ITS*) in plants (Antil et al., 2023). These primers produced specific

amplicon sizes (i.e., 507bp of *ITS1*; 550bp of *rbcL* & 850bp of *matK*) while running PCR reactions consisting of approximately 50 ng per 1 µL of template DNA. The amplified products can then be sent to DNA sequencing laboratories (like Apical Scientific Sdn. Bhd., Malaysia) for bidirectional sequencing. Sequencing can be performed using the same primers employed for amplification through capillary electrophoresis on the Sanger DNA Sequencer. The resultant sequences after editing using the software like BioEdit V7.0.9.0 and publicly available sequences in NCBI portal for the *matK* gene, *rbcL* gene and *ITS* gene should be included in the analysis procedure using different methods for detection of species.

**Table 2.** List of potential primers that can be used for PCR and their sequences

| Region | Primer | Sequence 5'-3' | Tm (°C) | References |
|---|---|---|---|---|
| *ITS1* | 5a fwd | CCTTATCATTTAGAGGAAGGAG | 50 | Chen et al., 2010 |
| | 4 rev | TCCTCCGCTTATTGATATGC | | |
| *ITS2* | S2F | ATGCGATACTTGGTGTGAAT | 56 | Chen et al., 2010 |
| | S3R | GACGCTTCTCCAGACTACAAT | | |
| *matK* | 3FKIM | CGTACAGTACTTTTGTGTTTACGAG | 52 | Costion et al., 2011 |
| | 1RKIM | ACCCAGTCCATCTGGAAATCTTGGTTC | | |
| *rbcL* | rbcLa-F | ATGTCACCACAAACAGAGACTAAAGC | 62 | Kress et al., 2009 |
| | rbcLa-R | GTAAAATCAAGTCCACCRCG | | |

**Table 3.** PCR reaction condition for ITS region (Chen et al., 2010)

| Reagent | Volume (µL) | Final concentration |
|---|---|---|
| PCR master mix | 12.5 | 1X |
| Forward primer (10 µM) | 1.25 | 0.5 µM |
| Reverse primer (10 µM) | 1.25 | 0.5 µM |
| Diluted template DNA | 1.0 | 50 ng/µL |
| Nuclease free water | 9.0 | n/a |
| Total volume | 25 | |

**Table 4.** Steps for PCR amplification of ITS region (Chen et al., 2010)

| Stages | Temperature | Time | Size range (bp) |
|---|---|---|---|
| Initial denaturation | 94°C | 5 min | 707 bp for ITS1 |
| Final denaturation | 94°C | 1 min | 571-1153 bp for ITS2 |
| Annealing | 50°C | 1 min | |
| Elongation | 72°C | 1.5 min | |

| | | |
|---|---|---|
| Final extension | 72°C | 7 min |
| Holding | 4°C | ∞ |

### 5.4 Electrophoresis

PCR products are subjected to an agarose gel (1.5-2%) electrophoresis.

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**Appendix 1.** Sample of a page of field book

Flora of ..... State.....

Serial ..... Date.....

Name.....

Family.....

Local Name.....

Habit.....Height.....Fl..... Fr.....

Locality..... Alt.....

Soil.....

Vegetation type.....

Associated plants.....

Distribution..... Abundance.....

Uses.....

Significant notes.....

.....

.....

.....

Photograph..... Collector.....

Identified by.....

**Appendix 2.** Label attached on herbarium sheet

| HERBARIUM | |
|---|---|
| 11 | aa |
| Serial no. :<br>bb | Family:<br>cc |
| Latin name<br>dd | Common name<br>ee |
| Local name<br>ff | Location<br>gg |
| Date of collection<br>hh | Collected by<br>ii |
| Identified by | Verified by |
| Remarks: | |

**Appendix 3.** Mounting plant specimen in a herbarium sheet