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# Detection survey protocol for *Striga angustifolia* (D. Don) C. J. Saldahna 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 *Striga angustifolia* (D. Don) C. J. Saldahna

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 noxious weeds *Striga* 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 *Striga angustifolia* 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 *Striga* Lour. (Witchweeds) comprises approximately 42 species of annual obligate root parasitic plants (Mohamed, Musselman and Riches, 2001; Mohamed and Musselman, 2019). It is mainly distributed in tropical and subtropical regions, and some species are major pests of agricultural crops in these regions. *Striga* seeds can contaminate seeds or grain by multiple pathways during transportation, storage and trade. Among several parasitic *Striga* species, *Striga angustifolia* (D.Don) C.J. Saldahna (1963), known as narrow-leaved Witchweed, is a hemiparasitic annual or biennial and grows primarily in the seasonally dry tropical biome. In 1825, the weed was first reported in Nepal as *Buchnera angustifolia* (D.Don) in Prodromus Florae Nepalensis (volume 91). It is an erect, annual or biennial herb, related to the Orobanchaceae family of root parasites. Soon after germination, it uses chemical exudates (like Stregtolactins) from the host's root to develop a special organ called haustorium that siphons nutrients from the host plant. Parker and Riches (1993) reported that *Striga* parasitism resulted in chlorosis and wilting and therefore, in drastic reduction of host-plant growth and development and even in plant death under a severe infection. The symptoms are similar to those seen from severe drought damage, nutrient deficiency and vascular disease. It parasitizes important food and forage grain grasses (*Poaceae*) and are therefore among the most agronomically destructive parasitic plants globally. Based on severity, the crop yield loss due to *Striga* infection has been estimated to range from about 10-100%. In account to the seriousness of the root parasite, implementing a thorough survey and surveillance

technique is essential to precisely tracking and managing the spread of *Striga*. 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 *Striga* survey and surveillance activities.

**1.2 Identity and taxonomy of target pest** (CABI, 2019)

**1.2.1 Identity**

**Preferred scientific name:** *Striga angustifolia* (Don) Saldanha

**Preferred common name:** Narrow-leaved Witchweed

**Other scientific names:** *Buchnera angustifolia* Benth., *Buchnera euphrasioides* Benth., *Striga euphrasioides* (Benth.) Benth., *Buchnera bifida* (Buch.) Ham., *Buchnera hirsute* (Wall.)

**Nepali local name:** Parajibi jhar

**EPPO code:** STRAN (*Striga angustifolia*)

**1.2.2 Taxonomy**

Taxonomic tree of the *Striga* is presented below (CABI, 2019)

| | |
|---|---|
| Kingdom: | Plantae |
| Phylum: | Streptophyta |
| Class: | Equisetopsida |
| Subclass: | Magnoliidae |
| Order: | Lamiales |
| Family: | Orobanchaceae |
| Genus: | *Striga* |
| Species: | *Striga angustifolia* |

**1.3 Host range**

The main host are: Maize (*Zea mays*), Sorghum (*Sorghum bicolor*), Sugarcane (*Saccharum officinarum*), Ricegrass paspalum (*Paspalum scrobiculatum*), Rice (*Oryza sativa*), Goose grass (*Eleusine indica*), Barnyard grass (*Echinochloa crus-galli*)

The *Striga* are obligate root parasitic plants of the major agricultural cereals crops, including millets, in tropical and semi-arid regions of Africa, Middle East, Asia and Australia. Consequently they cause severe to even complete losses in crop grain yield (Atera and Itoh, 2011; Kountche et al., 2016).

**1.4 Weed biology**

## Detection survey protocol for *Striga angustifolia* (D. Don) C. J. Saldahna

NPPO-Nepal, 2025

*Striga* are annual, chlorophyll-bearing, root-parasitic plants that need a host plant to complete their life cycle. The latter is complex, intimately associated with that of the host and to the climate, particularly during postripening (Hearne, 2009). *Striga* plants have a high reproductive capacity: a single plant can produce 100,000_200,000 very tiny (0.15~0.30 mm in diameter) seeds, which are easily dispersible (Parker and Riches, 1993; Gurney et al., 2006; Hearne, 2009). *Striga* seeds require a period of pretreatment, conditioning in a moist warm environment (30°C in germination bioassays) for 2-16 days before they acquire the potential to germinate (Logan and Stewart, 1991; Parker and Riches, 1993). After this phase, germination of *Striga* seeds will be initiated only upon induction by some specific chemicals, such as *strigolactones* (SLs), released by the host roots onto the rhizosphere (Bouwmeester et al., 2003; Matusova et al., 2005; Shen et al., 2006; Zwanenburg et al., 2009; Yoneyama et al., 2010; Xie et al., 2010). The concentration of such chemicals is very low and ranges from 10210 to 10215 mole m23 (Hearne, 2009). The root system in *Striga* is vestigial. Instead of a usual angiosperm root system, germinating seeds establish a sticky radical, which, in response to haustorial initiation factors derived from the host roots, develops to a haustorium. The haustorium connects the host and its parasite. Indeed, upon coming in contact with a host root, the haustorium develops a wedge-shaped group of cells that penetrates the host root cortex and endodermis to establish parasite-host xylem-xylem connections (Albrecht et al., 1999). This allows the direct uptake of water, assimilates, and nutrients from the host plant to the parasite. Following the establishment of the host-parasite connection, *Striga* depends entirely on the host before emergence from the soil. During this holoparasitic stage of development, the parasite inflicts severe damage to the host. Subsequently, the parasites grow toward the soil surface and emerge above the ground, develop chlorophyllous shoots (hemiparasitic stage), and produce flowers and seeds which remain viable in the soil for 20 years or even more (Parker and Riches, 1993; Berner et al., 1997). However, in spite of being capable of photosynthesis, *Striga* cannot survive independently of a host in the post-emergence stage. Joel et al. (2007) indicated that subsequent haustoria development, attachment and penetration, as well as further growth and development of the parasite also require signals or resource commitment from the host plant.

A schematic version of the intricate life cycle of *Striga* is provided in Figure 1.

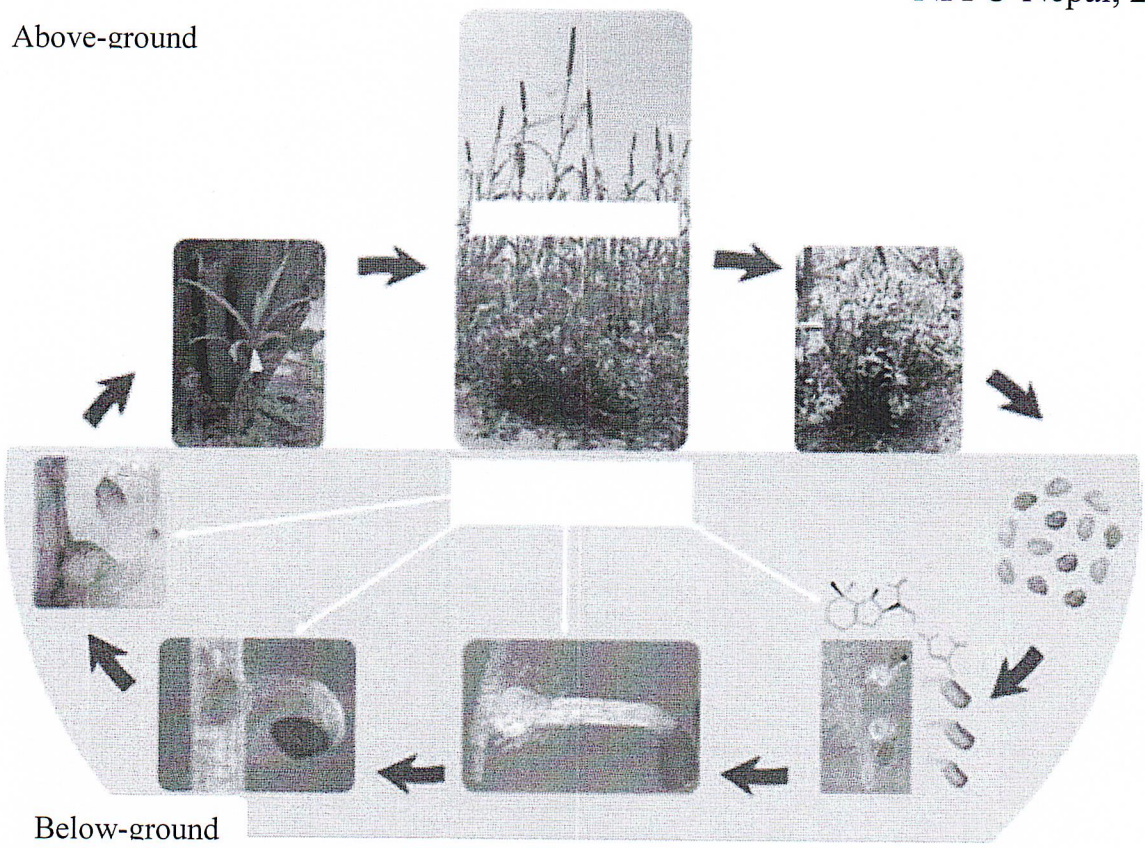


**Figure 1.** Schematic illustration of the *Striga* life cycle
(Source: Kountche et al., 2016)

### 1.5 Mode of dispersion

The primary mode of dispersion of *Striga angustifolia* occurs through seed dispersal mechanisms (CABI, 2019). Some of the modes of its dispersion are:

a) **Wind dispersal:** *Striga angustifolia* produces tiny, lightweight seeds that are easily blown by wind over long distances which allows the seeds to colonize new areas with suitable environmental conditions.
b) **Water dispersal:** Water runoff is an alternate mode of dispersal in areas with seasonal flooding or rainfall that enables the seeds to spread to low-lying agricultural fields where host plants grow.
c) **Human activities:** Seeds can adhere to farming tools, vehicles or footwear and they are often accidently disperse through the movement of infected soil or crop materials.
d) **Animal movement:** Seeds can transport externally on fur, hoofs from infected plantation to a plantation of uninfected field.
e) **Bale as a seed bank:** Hay bales may function in a similar fashion as soil seed banks to allow seeds to persist in agricultural ecosystems for prolonged periods.

## 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 the detection survey is to determine the presence or absence of *Striga angustifolia* 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 usually occurs after the host plants begin their reproductive phase. This is often during the monsoon or post-monsoon period (September-December) in Nepal. Detection surveys need to be done during the pre-planting, vegetative stage and reproductive stage of the host crop (*i.e.*, maize) for detection of *Striga angustifolia* 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 *Striga angustifolia*
- Mini glass/plastic vials for weed seeds
- Chemicals (preservatives), if necessary

### 2.5 Identification method

Classification and identification of *Striga* species depends largely on floral characters. Morphological identification of *Striga* plants (including seeds) is based on known reference specimens, literature descriptions and taxonomic keys and descriptions. Considerable data from molecular studies of *Striga* 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 *Striga* species are summarized in Table (1). Where capsules are intact, differences in capsule size can also be used for identification.

*Striga angustifolia* is developed along with the host plant (Figure 5). It is an erect, annual or biennial herb, 10-50 cm tall (Figure 4). Stems have stiffy erect, sub-quadrangular, simple, rarely apically branched, hispidulous, more or less velvet-hairy to densely covered in short rough hairs. Leaves are nearly opposite but alternate above, linear to linear-lanceolate, 1-2 cm long, 1-4 mm wide, margin entire, acute, finely pubescent. Flowers are borne alternate solitary in the axils (axillary), together sometimes, forming long, lax terminal spikes (Figure 2). Lower bracts are leaf-like, the upper liner to subulate, bracteoles. Calyx is tubular, 1.0-1.2 cm long, prominently 15-ribbed, 5-toothed, finely pubescent, teeth linear-lances shaped, 3-5 mm long, elongating in fruit. Corolla are white or cream with a greenish tube, pubescent outside, tube 1.0-1.5 cm long, abruptly curved just above the calyx teeth and inflated above. Lobes of lower-lips are obovate, 6-8 mm long and about 5 mm wide, rounded. Stamens 4, didynamous, included; anthers 1-celled. Capsule is ovoid, shorter than calyx, 4-5 mm long, ±3 mm in diameter, apiculate, valves sharply recurved after dehiscence, pointed as tip.

**Table 1.** Summary of the main morphological characteristics of mature plants of the four most economically damaging *Striga* species

| Species | Plant height (cm) | Stem | Leaf pubescence | Flower colour | Capsule (L×W in mm) |
|---|---|---|---|---|---|
| *Striga angustifolia* | 10-50 | Stiffy erect, simple branches, more or less velvet-hairy to densely covered in short rough hairs | Puberulent | White or cream with a greenish tube | 4-5 |
| *Striga asiatica* | 10-30 | Erect, square; usually branched in agricultural fields, wild | Strigose | Most commonly scarlet red, rarely yellow or white | 7×2 |

| | | | | | |
|---|---|---|---|---|---|
| | | plants often unbranched | | | |
| *Striga gesnerioides* | 11-25 | Many stems arising from a usually bulbous base; numerous adventitious roots | Puberulent | Purple, pink or yellow, depending on host | 10-20×3 |
| *Striga hermonthica* | Upto 100 | Usually sparsely branched | Strigose | Pink | 12-15×2.0-2.5 |

**Figure 2.** Flower of the *Striga angustifolia*

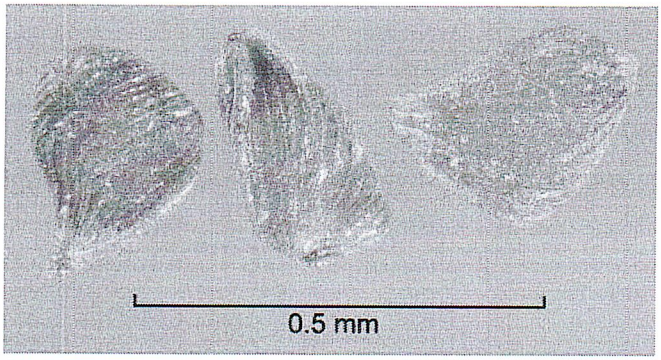


**Figure 3.** Seed of the *Striga angustifolia*

**Figure 4.** Whole plant of *Striga angustifolia*

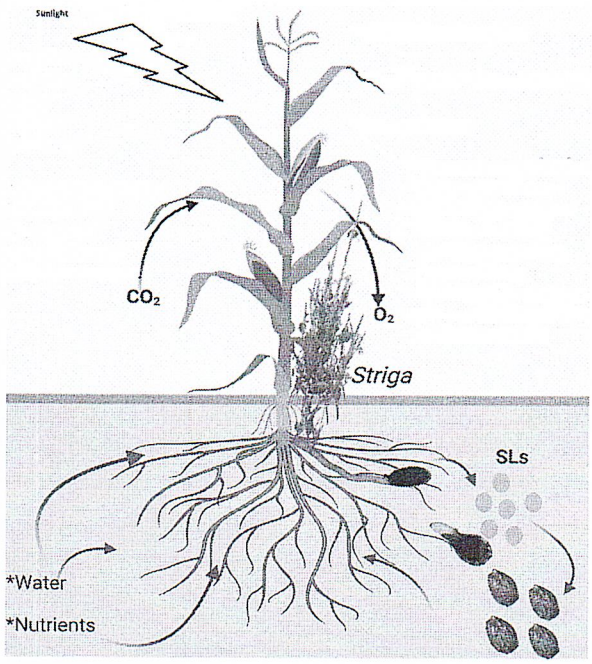


**Figure 5.** Conceptual framework of *Striga*-host interaction

(Source: CABI, 2019; https://powo.science.kew.org; Makaza et al., 2023)

**2.5.2 Identification of seed:** Seeds are oblong, cuneate, longitudinally ribbed, golden brown and minute (Figure 3).

### 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

Random selection can examined by four method:

a) **Random method:** It consist 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 10m. The sampling procedure is as follows: the $i^{th}$ sample is chosen randomly from the simulated field and its distance to each of the $(i\text{-}l)$ first samples is calculated; if any of these distances is smaller than 10m, then the sample is discarded and a new one chosen; otherwise, the $(i+l)^{th}$ sample is selected.

c) **Distance20 method:** in this method, the same procedure as Distance10 method is used but with a minimal sampling distance of 20m.

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). Systematic selection can examined by two 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 divide into $n^2$ rectangles of $l/n \times w/n$ $m^2$; the first sample is choose 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 $[x_1+(i\text{-}l)\times w/n;\ y_1+(i\text{-}l)\times l/n]$ and the co-ordinates of the $i^{th}$ sample taken on the second diagonal are $[x_1+(i\text{-}l)\times w/n;\ l\text{-}y_1\text{-}(i\text{-}l)\times l/n]$.

b) **Zig-zag method:** The samples are taken from three lines assembled vaguely as an "S". The sampling process for $N=3n\text{-}2$ 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.

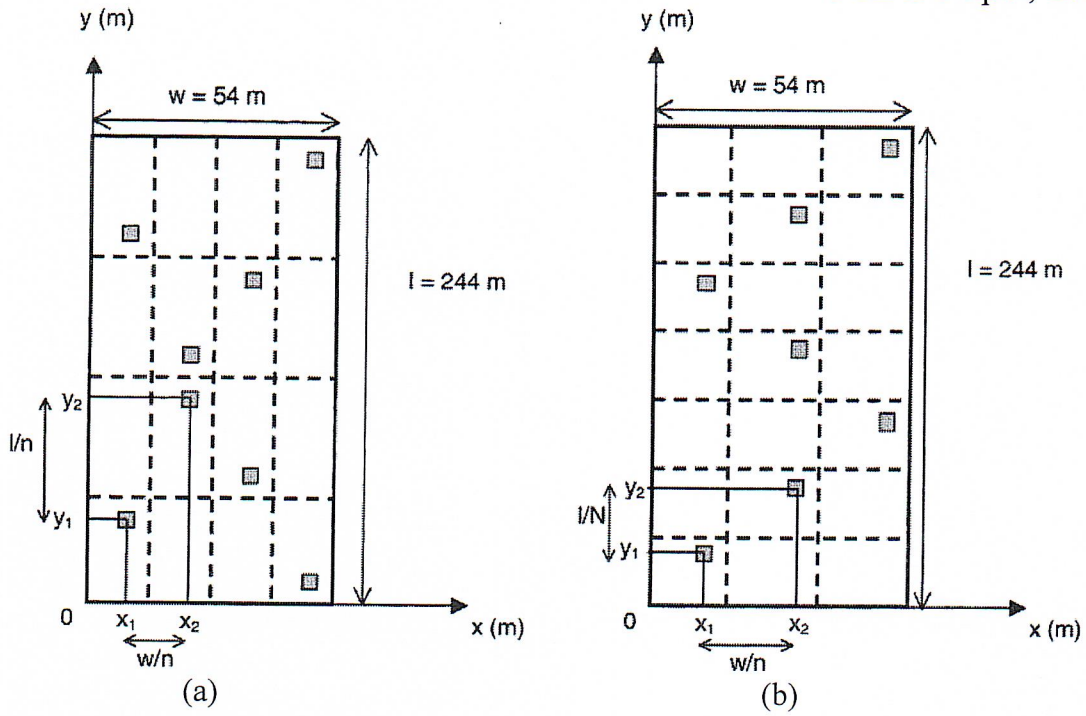


**Figure 6.** Example of a systematic sampling plan (a) *n=4* selecting eight samples (*N=2n=8*) and using diagonals, (b) *n=3* selecting seven samples (*N=3n-2=7*) and using lines assembled as a zigzag (Colbach et al, 2000)

### 2.8 Specimen collection and preservation

#### 2.8.1 Collecting technique

- Select normal looking specimens i.e. free of insect damage or disease.
- Collect plants that represent the range of variations in the population (2-3 plants in each field), not just a typical specimen.
- For young plants, collect the entire plant (tops and roots)
- For mature plants, collect stems, twigs, leaves, and flowers or fruits of plants and retain as much of the root system as possible.
- Tag 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 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 laboratory

- 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, should preserve the specimen and keep all the records safely. The documentation system should be well maintained by the NPPO-Nepal and member institutions should have easy access to it.

## 5. Molecular diagnosis of *Striga angustifolia*

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 a diverse 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.

**References**

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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