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Detection survey protocol for *Chromolaena odorata* (L.) R.M. King & H. Rob. in Nepal

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March, 2025

22.03.2025

Approved
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March 12, 2025

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NPPO-Nepal, 2025

Endorsed by NPPO-Nepal on March 12, 2025

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 *Chromolaena* 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 *Chromolaena odorata* 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 *Chromolaena* (L.) R.M. King & H. Rob. (Siam weed) comprises approximately 165 species of perennial and shrub plants. Among several species of *Chromolaena*, *Chromolaena odorata* is a very widely distributed tropical shrub that is still expanding its range, and is considered one of the world's worst weeds. This flowering shrub is native to north and Central America, and has been introduced to parts of Asia, Africa and Australia. The weed was first reported In Nepal in the 1950s, the Philippines and Papua New Guinea in the 1960s (Henty & Pritchard, 1973) and has spread to Australia in 1994 (Muniappan, Reddy & Lai, 2005) and most of the Micronesian islands in 2000 (Waterhouse, 1994). It spreads very rapidly after disturbances like fire or clearing of land for agriculture, and suppresses the growth of native understorey species. It continues to spread due to its effective short- and long-distance dispersal. It can form pure stands where established, often in disturbed areas, grasslands, fallow areas and forestry plantations, and is highly competitive. It is a herbaceous to woody perennial that has wind dispersed seeds, that can also attach to fur, clothes, etc. and this facilitates long distance seed dispersal. Leaves release a pungent odour when crushed, and are used as traditional medicine in Indonesia, Malaysia, Thailand and Nigeria. However, it is toxic to cattle, and can cause allergies in humans. It reduces crop yield, completely smothering the crop and increasing the time spent on weeding farms and cost of control. It can also transmit pathogenic fungi (VayssiFre, 1957; Esuruoso, 1971; Oritsejafor, 1986) and act as a host for insect pest including *Zonocerus variegatus* (Modder, 1984; Chapman et al., 1986), whose nymphs feed on leaves, flowers and fruits in Africa; *Aphis citricola* and *Rhopalosiphum maidis* in India; as well as various other polyphagous insects. In

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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 *Chromolaena*. 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 *Chromolaena* survey and surveillance activities.

1.2 Identity and taxonomy of target pest (CABI, 2007)

1.2.1 Identity

Preferred scientific name: *Chromolaena odorata* (L.) R.M. King & H. Rob.

Preferred common name: Siam weed or Devil weed

Other scientific names: *Eupatorium conyzoides* Vahl, *Eupatorium odoratum* L., *Osmia odorata* (L.) Schultz-Bip.

Nepali local names: Seto Banmara

EPPO code: EUPOD (*Eupatorium odoratum*)

1.2.2 Taxonomy

Taxonomic tree of the *Chromolaena* is presented below

Kingdom:	Plantae
Phylum:	Streptophyta
Class:	Equisetopsida
Subclass:	Magnoliidae
Order:	Asterales
Family:	Asteraceae
Genus:	*Chromolaena*
Species:	*Chromolaena odorata*

1.3 Host range

The main host are: Maize (*Zea mays*), Sugarcane (*Saccharum officinarum*), Tobacco (*Nicotiana tabacum*), Rice (*Oryza sativa*), Cotton (*Gossypium hirsutum*), Tea (*Camellia sinensis*)

The *Chromolaena odorata* has become a troublesome weed in open spaces, clearing around human settlements, in early secondary succession, crop and pasture lands, and in young tree crop plantations. It is now a dominant species through the humid and sub-humid zones because of its strong heliophilic character and vigorous vegetative development. It has become a major invasive weed of natural and disturbed ecosystems, from grassland, young tree crop plantations (such as

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rubber, oil palm, coffee, cacao and fruit trees), cassava, yams, banana, plantation, forest margin, river bank to abandoned fields and disturbed forest. It is considerably less important as a weed in cereal and legume plantations. The invasion of pasture lands by it causes cattle to avoid these lands and subsequently to overgraze non-infested lands.

1.4 Weed biology

The occurrence of *Chromolaena odorata* is said to be limited to latitudes 30°N and 30°S (Muniappan & Marutani, 1988), and in altitudes around 2000m in locations where a minimum annual rainfall amounts to 1100mm and a dry season of no more than five months (Gautier, 1992). This species is heliophile, has an optimal temperature range of 20-37°C (Muniappan & Marutani, 1991). It grows on a wide range of soil types, but not on inundated sites. However, it requires a minimum length of the growing period to accumulate sufficient resources to establish and persist (Joshi, 2006). Once established, this weed grows rapidly. The seedlings emerge after four to twelve days, remain small during the first three months, and develop rapidly afterwards (Audru et al. 1988). It has an extremely fast growth rate (up to 20mm/day) and often forms a dense thicket of the height of 1.5-2.5m in open areas and up to 10m among the trees that can shade out the existing vegetation. The plant can spread through sexual and asexual means but the main spread method is by sexual propagation. Initially *C. odorata* spreads by seed, but after establishment it may also reproduce vegetatively from lateral branches as the nodes of these branches may form roots (Gautier, 1992); regrowth occurs after slashing and burning. Sexual propagation begins when the plant forms a flower at 1 year old that increases until the stand reaches 10 years old (Witkowski & Wilson, 2001).

Flowering is initiated by a combination of shortening days, decline in rainfall and drop in temperature (Gautier 1993). It flowers at the end of the rainy season and after flowering the leaves fall and the stems die back. The flower is capable of producing large quantities of seed with estimated from 93,000 (Weerakoon, 1972) to 1,600,000 (Wilson, 1995) seeds per plant. Between 33 and 66% of the seeds are viable, but only 2% germinate immediately *in situ*, the rest disperse or remain dormant (Erasmus & van Staden 1986, Yadav & Tripathi 1981). The light, narrow and hairy seeds are easily dispersed by wind over a short distance and can spread over a long distance by attaching to clothing, fur of animals, machinery etc. but the primary long distance vector responsible for its spread is human activity. In invaded areas, some of these seeds can persist for several years (Witkowski, 2002).

In general terms, germination behaviour is adapted to secondary habitats but depends widely on the population considered (Edwards, 1974), especially with respect to dormancy and the effect of shade. Humidity and temperatures above 20°C enhance germination. Green and far-red light reduce germination, whereas red or white light has a positive effect (Soerohaldoko, 1975; Ambica and Jayachandra, 1980; Erasmus and Van Staden, 1986). Seedlings often appear in very high densities (>2000 per m²) and very severe intraspecific selection occurs in the first months of growth. In heavily disturbed environments, it competes effectively with other plants and crops and may become the dominant species. An important aspect of this weed problem in agriculture is the high labor required for its control and the increased pressure on lands that are free of or only slightly infested by established bushes of these plants. A schematic version of the habit of flowering plants and inflorescence is provided in Figure 1.

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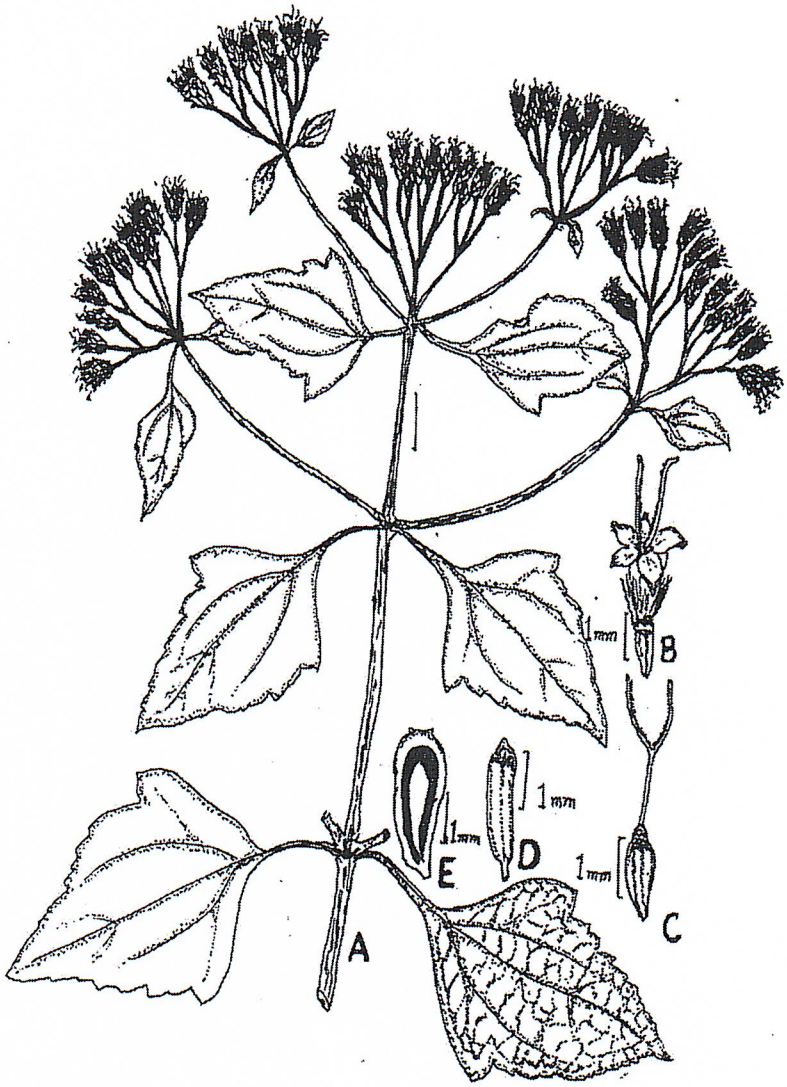

Figure 1. Schematic diagram of *Chromolaena odorata* (L) R.M. King & H. Rob. A. Twig, B. Floret, C. Pistil, D. Stamen, E. Ovary L.s.
(Source: Sankara Rao et al., 2019)

1.5 Mode of dispersion

The primary mode of dispersion of *Chromolaena odorata* occurs through seed dispersal mechanisms (CABI, 2007). Some of the modes of its dispersion are:

a) **Wind dispersal:** *Chromolaena* produces tiny, lightweight seeds (achenes) that are easily blown by wind over long distances which allows the seeds to colonize new areas with suitable environmental conditions.
b) **Human activities:** Achenes can easily adhere to clothes, farming tools, vehicles or footwear and they are often accidently dispersed through the movement of infected soil or crop materials. The contamination of planting materials is also an important mechanism for seed dispersal over large distances.

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c) **Animal movement:** Achenes can transport externally on fur and feathers thus animal dispersal is probably sometimes responsible for new foci of invasion.

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 *Chromolaena odorata* 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 post-monsoon period (November-February) 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 *Chromolaena odorata* 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

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- Field guide with image of *Chromolaena odorata*
- Mini glass/plastic vials for weed seeds
- Chemicals (preservatives), if necessary

2.5 Identification method

Classification and identification of *Chromolaena* species depends largely on floral characters. Morphological identification of *Chromolaena* plants (including seeds) is based on known reference specimens, literature descriptions and taxonomic keys and descriptions. Considerable data from molecular studies of *Chromolaena* 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

Chromolaena odorata is a herbaceous to woody perennial with a bushy habit which can be climbing 2-7m high. This many-branched plant becomes lianescent when it has the opportunity to climb on a support. Isolated individuals start to branch when they reach a height of about 120 cm. After the first year of growth, the plant develops a strong, woody underground storage organ, which can reach a diameter of 20 cm and abundantly branched. Stems are cylindrical, yellowish. Twigs are slightly striolate longitudinally, pubescent, opposite-decussate. Leaves are simple, opposite-decussate and without stipules. They are rhomboid-ovate to ovate with an acute apex and a cuneate base. The blades are trinerved a few millimetres after the base, roughly crenate-serrate beyond their maximum breadth, slightly pubescent above and pubescent with numerous small yellow dots below (a lens is needed to see this). The petiole is 1-3 cm long, and the blade 5-14 cm long and 2.5-8 cm broad (Figure 4). Leaves and twigs produce a characteristic smell when crushed (Gautier, 1992). Capitula are grouped in one, three or five convex trichotomic corymbs 5-10 cm in diameter, at the end of the twigs. The involucre is cylindrical, 8-10 mm long by 3-4 mm broad. It is made of a series of four or five oblong bracts, the external being the shorter. These bracts are obtuse, chartaceous, pale in colour with three or five nerves. The receptacle is convex, without scales. There are 15-35 florets per capitulum and flowers are hermaphrodites; ovary inferior. The corolla is 5 mm long, tubular, pubescent at apex and has five lobes. Its colour ranges from pale-lilac to white. Styles are of the same colour, exerted and flexuous (Figure 2 & 3). Cypselae are composed of a 3- to 4-mm-long fusiform blackish achene, with five beige barbelate ribs, overtopped by a pappus of about 30 barbelate beige capillary bristles which are 4-5 mm long (Gautier, 1992). Retief (2002) has found that in southern Africa the genus *Mikania* shows strong similarity to *Chromolaena odorata* in its distribution, habit, leaf blade outline, floret colour (both have white corollas), capitulum outline and structure of the achene.

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Figure 2. Flowering habit of *Chromolaena. odorata*

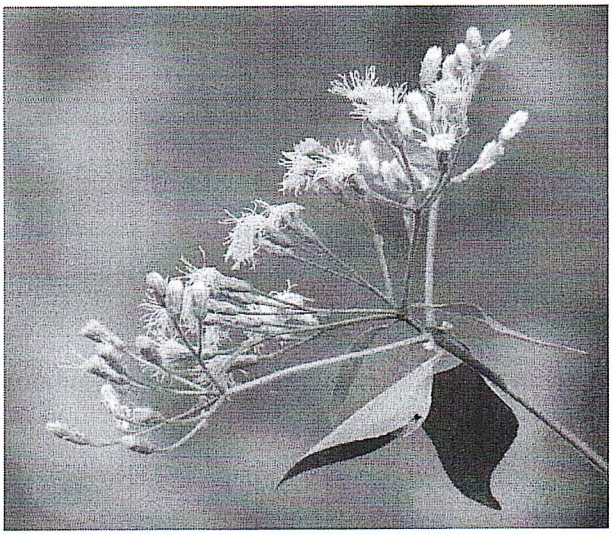

Figure 3. Flower of *Chromolaena odorata*

Figure 4. Leaves of *Chromolaena odorata*

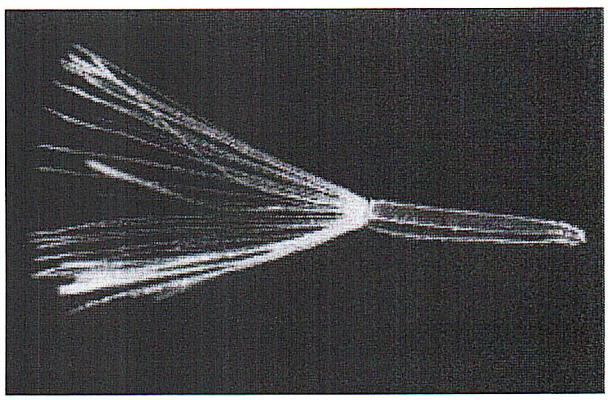

Figure 5. Seed of the *Chromolaena odorata*

(Source: CABI, 2007)

2.5.2 Identification of seed: Achenes 4mm long, linear, 5-angled, scabrous, black; pappus many, 4-7mm long, setaceous, yellowish (Figure 5).

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:

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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 use 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 $N=3n-2$ 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.

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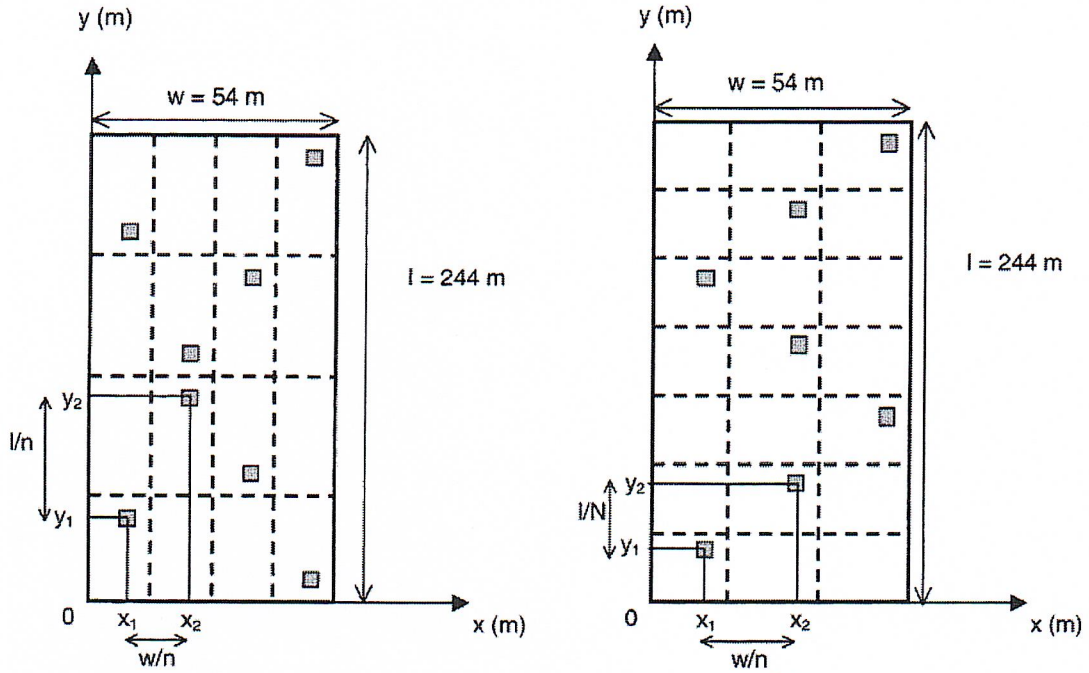

Figure 6. Example of a systematic sampling plan (a) *n=4* selecting eight samples (*N=2n=8*) and using diagonals, (b) *n=3* selecting seven samples (*N=3n-2=7*) and using lines assembled as a zigzag (Colbach et al, 2000)

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 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.

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- 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

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- 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 *Chromolaena odorata*

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.

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- 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 1. 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		

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rbcL	rbcLa-F	ATGTCACCACAAACAGAGACTAAAGC	62	Kress et al., 2009
	rbcLa-R	GTAAAATCAAGTCCACCRCG		

Table 2. 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 3. 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.....

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Appendix 2. Label attached on herbarium sheet

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

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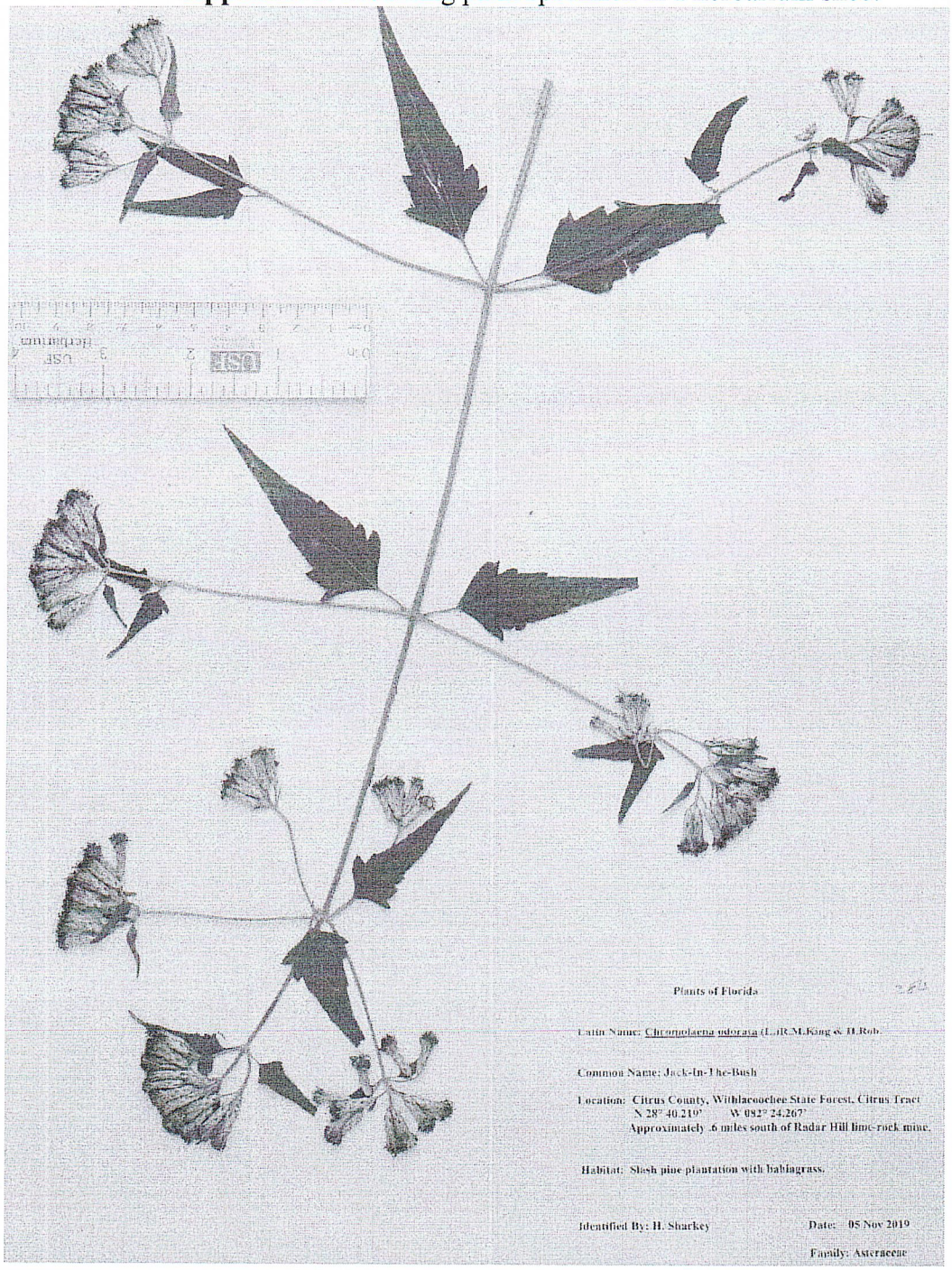
Appendix 3. Mounting plant specimen in a herbarium sheet



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