12.

Detection survey protocol for *Ageratina adenophora* (Spreng.) King & H. R. in Nepal

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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 *Ageratina* 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 *Ageratina adenophora* 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 a laboratory for diagnosis and preservation.

1.1 About the pest (weed)

The genus *Ageratina* (Spreng.) R. King & H. Rob. (Crofton weed) comprises approximately 60 species of plants belonging to the aster family. *Ageratina* species are mostly perennial herbs, but a few are annuals and many of the tropical species are shrubby or treelike. Among several species of *Ageratina*, *Ageratina adenophora* is a very widely distributed perennial herb that is still expanding its range, and is considered one of the world's worst weeds. This flowering herb is widely distributed in more than 30 countries and regions of tropical and subtropical countries, including China, New Zealand, India, Nepal, Pakistan, Thailand, Malaysia, Philippines, Singapore, eastern Australia, northern America and South Africa (Wan *et al.* 2010). In Nepal, the weed was first reported during the 1952s, and was found at 200-3200 m altitude and has probably been introduced to eastern Nepal from India. It forms a dense stand that suppresses the growth of other species through competition and allelopathic (chemical) effects due to leachates. It is toxic to livestock. A single plant can produce several thousand seeds which are easily dispersed by the wind and water. Local people use the leaf juice to stop bleeding from minor cuts. It is used to make compost, green manure, and is also used in biogas plants. The char produced from its stem is used to make pellets, briquettes and biochar. People remove plants manually from forests and agricultural lands (Adhikari et al., 2022).

This species invades and occupies farmland, competing with native crops and trees for fertilizer, water, sunlight and space, thus causing serious crop harvest losses. It is estimated to cause economic losses to animal husbandry and grassland ecosystem services of RMB 0.99 (US$ 0.14) and 2.63 (US$ 0.37) billion per year, respectively in China (Wang et al., 2017). This species

has been reported to host leaf spot-causing pathogenic fungi *Alternaria alternata* (Wan et al., 2001; Zhou et al., 2010) and *Passalora ageratinae* (Buccellato et al., 2021). It hosts a wide variety of fungal pathogens, particularly those belonging to the family Didymellaceae, found to be abundantly present in leaf spots and the healthy leaves of *Ageratina adenophora* (Chen et al., 2020). On account of the seriousness of the invasive weeds, implementing a thorough survey and surveillance technique is essential to precisely tracking and managing the spread of *Eupatorium*. The development and implementation of an effective protocol would facilitate detecting early infestation, prompt response, and the implementation 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 introductions. 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 *Eupatorium* survey and surveillance activities.

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

1.2.1 Identity

Preferred scientific name: *Ageratina adenophora* (Spreng.) R.M. King & H. Rob.

Preferred common name: Croftonweed

Other scientific names: *Eupatorium adenophorum* Spreng., *Eupatorium glandulosum* Kunth, *Eupatorium pasdadense* Parish

Nepali local names: Kalo Banmara

EPPO code: EUPAD (*Ageratina adenophora*)

1.2.2 Taxonomy

Taxonomic tree of *Ageratina* is presented below

Kingdom:	Plantae
Phylum:	Spermatophyta
Class:	Dicotyledonae
Order:	Asterales
Family:	Asteraceae
Genus:	*Ageratina*
Species:	*Ageratina adenophora*

1.3 Host range

The main host are: Banana (*Musa paradisiaca*), Avocado (*Persea americana*), Maize (*Zea mays*), Sugarcane (*Saccharum officinarum*), Tobacco (*Nicotiana tabacum*), upland rice (*Oryza sativa*), Cotton (*Gossypium hirsutum*), Tea (*Camellia sinensis*), Soyabean (*Glycine max*)

The principal hosts of *Ageratina adenophora* are pasture species. This species is a regulated weed in some parts of the world, notably Australia (New South Wales and Queensland) and the USA (Florida, Hawaii) and others. It is a declared noxious weed or Class A noxious plant in a number of US states where it is not yet present (USDA-ARS, 2004), indicating its potential risks and impacts, and a desire for it not to be introduced. Risks of introduction are high, as seeds may be a contaminant in a variety of traded products, including cereal, stockfeeds, forage seeds, soil, sand and gravel, and may also be imported in mud attached to vehicles, agricultural machinery and livestock themselves.

1.4 Weed biology

Ageratina adenophora is a multi-stem perennial herb with a woody rootstock, growing up to a height of 2 m. Individuals from introduced ranges have higher plant height, stem diameter, leaf length, leaf breadth and leaf area in comparison with individuals from native ranges (Feng et al., 2009). Longer vegetative growth phase, higher biomass accumulation, higher above/below ground ratio and higher coverage than native species enable this weed to establish monoculture stands (Gao et al., 2013). This species primarily reproduces by seeds which are produced apomictically without fertilization (Parsons & Cuthbertson, 2001). It is likely that there is failure of meiosis due to the auto-triploid nature (with 51 chromosomes) of the plant, highly abnormal meiosis and high pollen sterility (Khonglam & Singh, 1980; Lu et al., 2008; Bala & Gupta, 2014). The seed length, breadth and mass vary from 1.37 to 1.69 mm, 0.29 to 0.51 mm, and 28.4 to 52.4 l g, respectively, and seed breadth and mass increase with increasing elevation between 640 and 2430 masl (Li & Feng, 2009). The plant also reproduces vegetatively by stem fragments (Parsons & Cuthbertson, 2001). The relative importance of different reproductive modes appears to depend on environmental conditions. Under low light, it reproduces primarily by seeds, whereas under high-intensity light, it reproduces primarily by vegetative clones (Yu & Ma, 2006). Under favourable conditions, the weed can produce 7000–10000 seeds per plant, of which 70–85% of seeds are viable (Parsons & Cuthbertson, 2001). When the viable seeds are buried in the soil, due to unfavourable environmental conditions, ca. 70% of them enter enforced dormancy and the remaining proportion into secondary dormancy, thereby increasing the longevity of seeds (Yadav & Tripathi, 1982). The seeds on the soil surface survive for only one germination season (about 5 months), whereas at soil depths of 5–10 cm they remain viable for 4 years (Shen et al., 2011). Seeds of *Ageratina adenophora* are considered moderately photoblastic with only 17% germination in the dark (Lu et al., 2006). This species invades and occupies farmland, competing with native crops and trees for fertilizer, water, sunlight and space, thus causing serious crop harvest losses. The schematic diagram of *Ageratina adenophora* is presented in Figure 1.

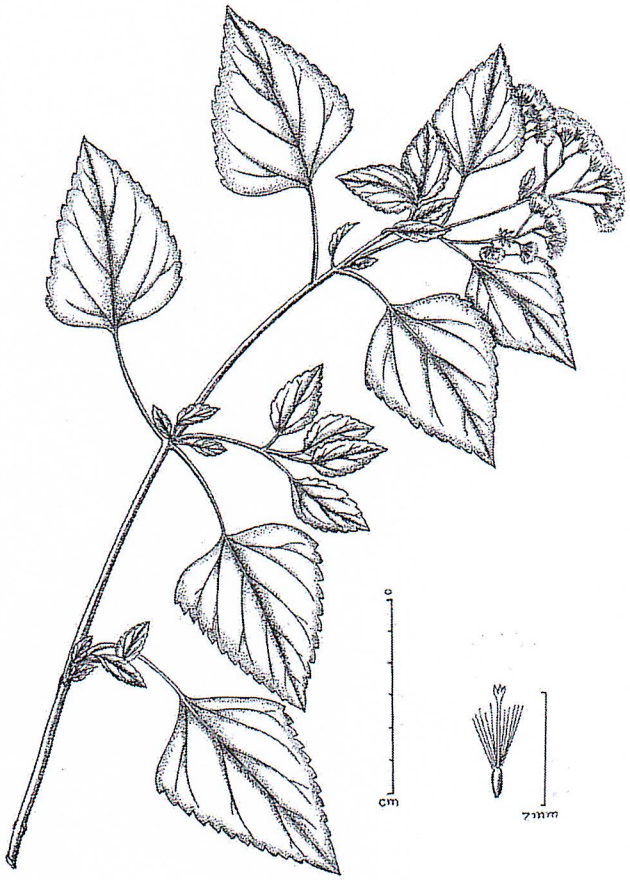


Figure 1. Schematic diagram of *Ageratina adenophora*
(Source: Tripathi et al., 2006)

1.5 Mode of dispersion

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

a) **Natural dispersal:** Natural dispersal of *Ageratina adenophora* is principally by seed, which is adapted for dispersal by both wind and water due to the feathery hairs of its pappus.
b) **Human activities:** Seeds 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. Seeds may also contaminate stockfeed.
c) **Animal movement:** Seeds can transport externally on skin, fur and feathers with mud thus animal dispersal is probably sometimes responsible for new foci of invasion.
d) **Agricultural practices:** Local extension of colonies and an increase in plant density can occur when bent and broken stems take root where they contact the soil, or when pieces of root have a portion of the crown attached. These can be easily moved during cultivation.
e) **Accidental introduction:** An important means of spread of *Ageratina adenophora* is movement as an impurity in agricultural produce, mainly cereals, forage and other seeds, also in sand and gravel used for road making, soil and in mud sticking to animals, machinery and other vehicles, as well as to footwear and clothing.

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 *Ageratina adenophora* 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 (September) 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 *Ageratina adenophora* 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 *Ageratina adenophora*

- Mini glass/plastic vials for weed seeds
- Chemicals (preservatives), if necessary

2.5 Identification method

Classification and identification of *Ageratina* species depends largely on floral characters. Morphological identification of *Ageratina* plants (including seeds) is based on known reference specimens, literature descriptions and taxonomic keys and descriptions. Considerable data from molecular studies of *Ageratina* 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 *Ageratina* species are summarized in Table 1. *Ageratina adenophora* is an upright herbaceous shrub, with branched stem that may heighten up to 2 m tall, reproduced by seed and vegetatively from a short, pale, yellow rootstock. Stems are purplish, numerous, erect, smooth, cylindrical; shortly branched towards the apex, 1-2 m long, occasionally longer; glandular, hairy at first but becoming woody with age and rooting at the nodes, if damaged. Stems arise from a short, thick, pale-yellow rootstock with a carrot-like odour when broken, giving rise to numerous branching secondary roots extending laterally to a radius of 1 m and downwards to 40 cm; adventitious roots may form on the first 3 cm of stem above the ground. Leaves dark green; opposite, broadly trowel-shaped, 5-8 cm long, 2.5-7.5 cm wide, with serrated edges, tapering towards the apex and narrowing abruptly at the base into a slender stalk 2-4 cm long; 3-nerved, glabrous or slightly pubescent, toothed along the apical margins. Petioles are brown (Figure 4). Flowers comprise 50 to 70 white, tubular florets about 3.5 mm long; grouped into heads 5-6 mm diameter within a row of green bracts and arranged in flat clusters up to 10 cm across at the end of the branches (Figure 2 &3) (Parsons & Cuthbertson, 1992).

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

Species	Plant height (cm)	Plant appearance	Leaf	Flower
Ageratina adenophora	Up to 200	Branched; stem is purplish, numerous, erect, smooth, cylindrical; shortly branched towards the apex	Dark green; opposite, broadly trowel-shaped; serrated edges, tapering towards the apex	Creamy white
Ageratina riparia	Up to 30	Prostrate shrub, reddish and purplish stem, sparsely pubescent	Narrow, prominently veined and elongated glabrous leaves	White

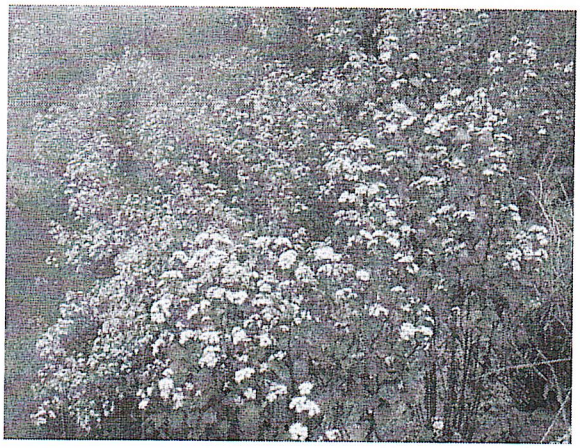


Figure 2. Flowering habit of *Ageratina adenophora*

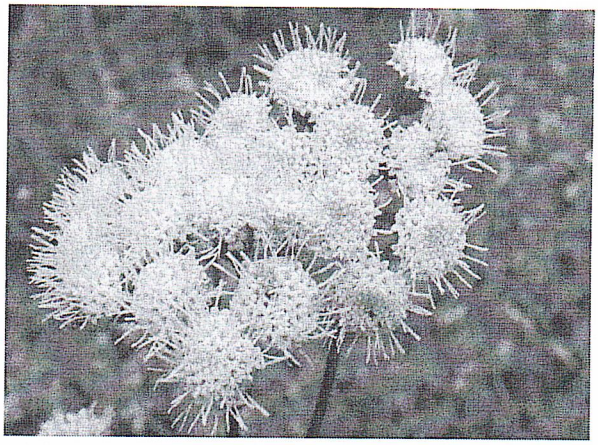


Figure 3. Flower of *Ageratina adenophora*

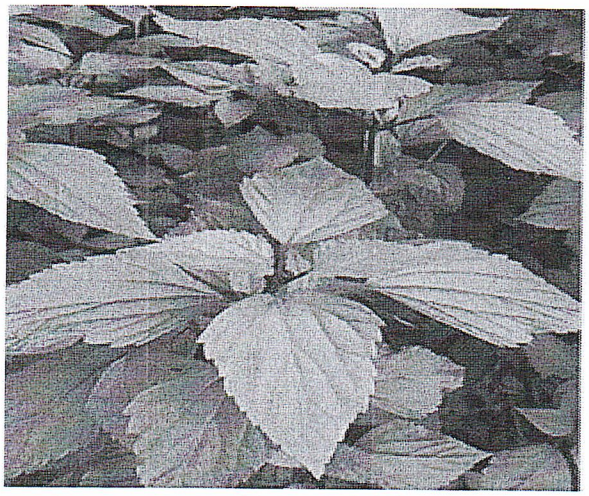


Figure 4. Leaves of *Ageratina adenophora*

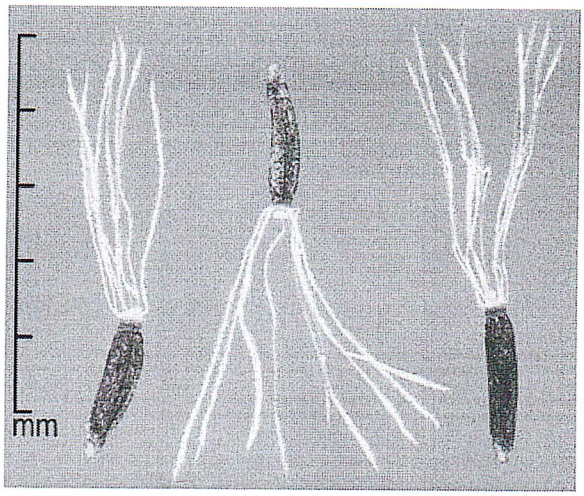


Figure 5. Seed of the *Ageratina adenophora* (Source: https://keyserver.lucidcentral.org)

2.5.2 Identification of seed: Seeds are dark brown to black, slender, angular, 0.3-0.5 mm width, 1.5-2 mm long; topped by a pappus of 5 to 10 fine white hairs approximately 4 mm long (Figure 5).

2.6 Number of specimens 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 $(i\text{-}1)$ 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 $(i+1)^{th}$ 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.

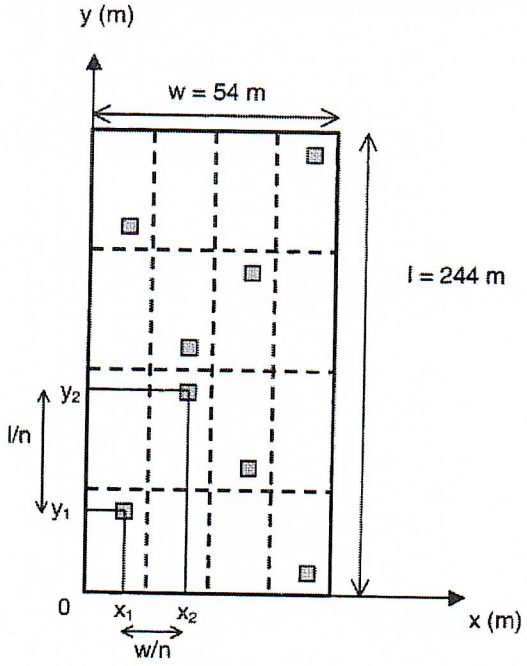
d) **Stratified method:** The field is divided into five equal parts and then, a fifth of the required samples is selected randomly in each of these parts.

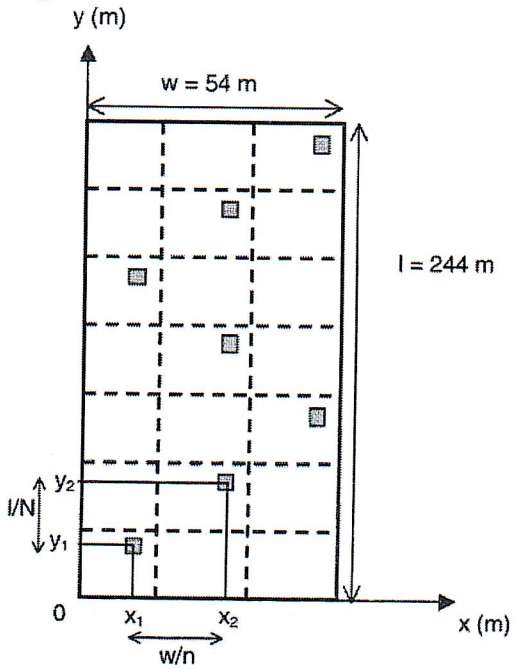
2.7.2 Methods based on systematic selection

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 $[x_1+(i\text{-}1)\times w/n;\ y_1+(i\text{-}1)\times l/n]$ and the co-ordinates of the i^{th} sample taken on the second diagonal are $[x_1+(i\text{-}1)\times w/n;\ l\text{-}y_1\text{-}(i\text{-}1)\times l/n]$.

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.





(a) (b)

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 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 *Ageratina adenophora*

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

Adhikari, B., B.B. Shrestha, M.F. Watson, L.N. Sharma, S. Bhattarai, C.A. Pendry, E. Paudel & K. Sharma (Dhakal). (2022). *Invasive Alien Plants of Nepal: A field guide to 27 problematic species*. Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal.

Antil, S., J.S. Abraham, S. Sripoorna, S. Maurya, J. Dagar, S. Makhija, P. Bhagat, R. Gupta, U. Sood, R. Lal & R. Toteja. (2023). DNA barcoding, an effective tool for species identification: a review. *Mol. Biol. Rep. 50* (1), 761–775. https://doi.org/ 10.1007/s11033-022-08015-7.

Bala, S & R.C. Gupta. (2014). Meiotic behavior in two triploids *Erigeron annus* and *Eupatorium adenophorum*. *Biolife* 2, 679–686.

Buccellato, L., J. T. Fisher, E. T. F. Witkowski, & M. J. Byrne. (2021). The effects of a stem gall fly and a leaf pathogen on the reproductive output of Crofton weed, *Ageratina adenophora* (Asteraceae), in greenhouse and field trials. *Biol. Control* 152:104453. doi: 10.1016/j.biocontrol.2020.104453

CABI. (2019). *Ageratina adenophora* (Crofton weed). CABI Compendium. https://doi.org/10.1079/cabicompendium.23243

Chen, L., J. Zhou, T. Zeng, Y.-F. Miao, L. Mei, G.-B. Yao, et al. (2020). Quantifying the sharing of foliar fungal pathogens by the invasive plant *Ageratina adenophora* and its neighbours. *New Phytol.* 227, 1493–1504. doi: 10.1111/nph.16624

Chen, S., H. Yao, J. Han, C. Liu, J. Song, L. Shi & C. Leon. (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PloS One, 5*(1), e8613. doi:10.1371/journal.pone.0008613.

Colbach, N., F. Dessaint & F. Forcella. (2000). Evaluating field-scale sampling methods for the estimation of mean plant densities of weeds. Weed Research, 40, 411-430.

Costion, C., A. Ford, H. Cross, D. Crayn, M. Harrington & A. Lowe. (2011). Plant DNA barcodes can accurately estimate species richness in poorly known floras. *PLoS ONE* **6**(11): e26841. DOI: 10.1371/journal.pone.0026841

Doyle, J. J. & J. L. Doyle. (1990). Isolation of plant DNA from fresh tissue. *Focus, 12*(1), 13-15.

Feng, Y.L., Y.B. Lei, R.F. Wang, et al. (2009). Evolutionary trade-offs for nitrogen allocation to photosynthesis versus cell walls in an invasive plant. Proceedings of the National Academy of Sciences of the United States of America 106, 1853–1856.

Gao, X., Y. Zhao, X. Yang & S. Sun. (2013). Linking trait differences to community dynamics: evidence from *Eupatorium adenophorum* and co-occurring native species during a three-year succession. *PLoS One* 8, e50247.

Khonglam A. & A. Singh. (1980). Cytogenetic studies on the weed species of *Eupatorium* found in Meghalaya, India. Proceedings: Plant Sciences 89, 237–241.

Kress, W. J., D. L. Erickson, F. A. Jones, N. G. Swenson, R. Perez, O. Sanjur, & E. Bermingham. (2009). Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot

in Panama. *Proceedings of the National Academy of Sciences of the United States of America, 106*(44), 18621-18626.

Li, Y.P. & Y.L. Feng. (2009). Differences in seed morphometric and germination traits of Crofton weed (*Eupatorium adenophorum*) from different elevations. *Weed Science* 57, 26–30.

Lu, H., J. Shen, W. Sang, X. Zhang & J. Lin. (2008). Pollen viability, pollination, seed set and seed germination of Crofton weed (*Eupatorium adenophorum*) in China. *Weed Science* 56, 42–51.

Lu, P., W. Sang & K. Ma. (2006). Effects of environmental factors on germination and emergence of Crofton weed (*Eupatorium adenophorum*). *Weed Science* 54, 452–457

Parsons, W.T. & E.G. Cuthbertson. (1992). Noxious Weeds of Australia. Melbourne, Australia: Inkata Press, 692 pp.

Parsons, W.T. & E.G. Cuthbertson. (2001). Noxious Weeds of Australia, 2nd eds, 239–242. CSIRO Publishing, Collingwood, Vic., Australia.

PPD/NPPO-Nepal. (2071 BS). Technical Guidelines for Detection Survey of Plant Pests in Nepal. Plant Protection Directorate / Executive Secretariat of National Plant Protection Organization – Nepal, Hariharbhawan, Lalitpur, Nepal.

Scherrer, B. (1983) Techniques de sondage en ecologie. In: StrateÂgies D'eÂchantillonnage En EÂcologie (ed. S Frontier), 63±162. Maison de Presses de l'UniversiteLaval-Quebec PUL, Canada.

Shen, Y.X., C.Y. Zhao & W.Y. Liu. (2011). Seed vigour and plant competitiveness resulting from seeds of *Eupatorium adenophorum* in persistent soil seed bank. *Flora* 206, 935–942.

Tiwari, K.L., S.K. Jadhav & S. Gupta. (2012). Modified CTAB technique for isolation of DNA from some medicinal plants. *Research Journal of Medicinal Plants*, 6: 65-73.

Tripathi, R.S., S.P.S. Kushwaha & A.S. Yadav. (2006). Ecology of three invasive species of Eupatorium: A review. International Journal of Ecology and Environmental Sciences, 32(4): 301-326

USDA-ARS. (2004). Germplasm Resources Information Network (GRIN). Online Database. Beltsville, Maryland, USA: National Germplasm Resources Laboratory. https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysearch.aspx

Wan, Z. X., J. J. Zhu, & S. Qiang. (2001). The pathogenic mechanism of toxin of *Alternaria alternata* (Fr.) Keissler to *Eupatorium adenophorum. J. Plant Resour. Environ.* 10, 47–50 (In Chinese).

Wan, F., W. Liu, J. Guo, S. Qiang, B. Li, J. Wang, *et al.* (2010). Invasive mechanism and control strategy of *Ageratina adenophora* (Sprengel). Science China Life Sciences, 53, pp. 1291-1298

Wang, C., H. L. Lin, Q. S. Feng, C. Y. Jin, A. C. Cao, & L. He. (2017). A new strategy for the prevention and control of *Eupatorium adenophorum* under climate change in China. *Sustainability* 9:11. doi: 10.3390/su9112037

Yadav, A.S. & R.S. Tripathi. (1982). A study on seed population dynamics of three weedy species of Eupatorium. *Weed Research* 22, 69–76.

Yu, X.J. & K.P. Ma. (2006). Variation in reproductive characteristics of *Eupatorium adenophorum* populations in different habitats. *Weed Research* 46, 319–326.

Zhou, Z. X., H. Jiang, C. Yang, M. Z. Yang, & H. B. Zhang. (2010). Microbial community on healthy and diseased leaves of an invasive plant *Eupatorium adenophorum* in Southwest China. *J. Microbiol.* 48, 139–145. doi: 10.1007/s12275-010-9185-y

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. :	Family:
bb	cc
Latin name	Common name
dd	ee
Local name	Location
ff	gg
Date of collection	Collected by
hh	ii
Identified by	Verified by
Remarks:	

Appendix 3. Sample of mounting plant specimen in a herbarium sheet