Standard Operating Procedure (SOP) for Detecting Citrus Tristeza Virus (CTV) Using Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA)

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Government of Nepal
Ministry of Agriculture and Livestock Development

Secretary

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Abbreviations

+ve	Positive
CTV	Citrus tristeza virus
CTV-SY	Citrus tristeza virus - seedling yellows
DAS-ELISA	Double Antibody Sandwich Enzyme Linked Immunosorbent Assay
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene-diamine-tetra acetic acid
eg.	Example
ELISA	Enzyme Linked Immunosorbent Assay
Fig	Figure
IgG	Immunoglobulin G
KH_2PO_3	Potassium hydrogen phosphite
KCl	Potassium chloride
MoALD	Ministry of Agriculture and Livestock Development
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
$NaH_2PO_412H_2O$	Monosodium phosphate
NaOH	Sodium hydroxide
NCFD	National Centre for Fruit Development
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
pNPP	para-Nitrophenylphosphate
PVP	Polyvinyl pyrimidine
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
SOP	Standard operating procedure
UV	Ultraviolet light
-ve	Negative

Measurements

µL	Microliter
g	Gram
g/L	Gram per liter
g/mL	Gram per milliliter
h	Hours
L	Liter
M	Molar
mg	Milligram

mL	Milliliter
mm	Millimeter
nm	Nanometer
µg/mL	Microgram per milliliter
rpm	Revolutions per minute
min	Minutes
°C	Degree Celsius

1. Background

Citrus tristeza virus (CTV) a member of the genus Closterovirus within the family Closteroviridae is one of the most important viral pathogens of citrus in the world. CTV is transmitted by grafting and by several species of aphids in a semi-persistent mode. CTV is considered the most economically important virus of citrus in the world. The disease was reported to be more severe in Brazil and South Africa till 1960s when the citrus industry was established on sour orange rootstocks (DoA and FAO, 2011). The causal virus is a flexuous and thread-like rod, 2,000 nm in length and 15 nm in width. The virus particles are located in the phloem of the host plant, and disturb its transportation systems. The optimum temperatures for virus infection and multiplication are 20°C–25°C.

CTV is transmitted by some species of aphids found on citrus plants. Brown citrus aphid (*Toxoptera citricida*) is the most efficient aphid vector of CTV which is a positive single-stranded RNA virus limited to the phloem tissues; however, cotton aphid (*Aphis gossypii)* is a competent vector where *T. citricida* is absent. Similarly, black citrus aphid (*Toxoptera aurantii)* has also been shown to be potential vector of CTV in some countries, but their efficiency is very low. Citrus cultivars that flush frequently also influence epidemics as aphids feed and develop almost exclusively on young flush (Yokomi, 2019). Hence, weather, irrigation, cultivar, and horticultural practices play critical roles in the development of aphid populations. The mechanisms involved in CTV-aphid transmissibility remain largely unknown.

The virus was first reported in Nepal in 1971 from Pokhara valley in lime (Knorr and Shah, 1971). The disease was extended throughout the Nepal from east to west (Tomiyasu and Verma, 1999; Regmi et al. 1999). Large numbers of mandarin trees at Pokhara, Gorkha and Lamjung (1000 m) were affected by CTV (Tomiysu and Verma, 1999), which is accounted to the dispersion of CTV virus by non-certified citrus scions. CTV is also transmitted by parasitic plants called dodder (*Cuscuta sp.*) Since, most citrus species are propagated by grafting, the infection of CTV is also increasing world-wide.

1.1 CTV symptoms

CTV causes different symptoms on citrus plants depending on the virus strain, the variety of citrus, and the scion-rootstock combination. In Asia, various strains of CTV generally referred to as seedling yellows (CTV-SY), tristeza (CTV-T), stem pitting (CTV-SP), and a mild type, have been widespread for many years. Any of these strains may exist in a citrus plant, or they may occur together, as a complex. The fruit sizes on infected trees are small, plants are stunted and unproductive. Stunting, leaf-cupping, and malformation are the major symptoms of CTV (Oliya, 2014; Yokomi, 2019; Qin, 2023).

1. Background

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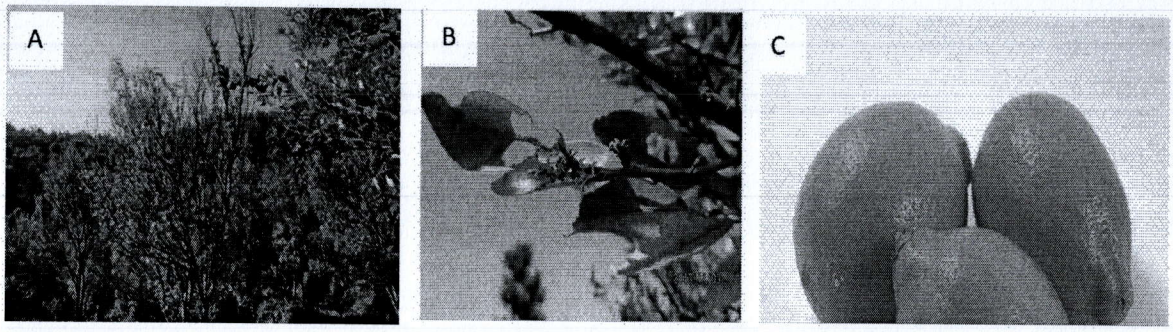

Figure 1. A: CTV infected tree in the orchard, B: Infected tree in presence of brown citrus aphid (*Toxoptera citricida)*; and C: Fruits from CTV infected tree.

1.2 Indexing of CTV

Mexican lime (*Citrus aurantifolia*), also known as West Indian lime or Key lime, is the best indicator of CTV infection. Leaves of this lime develop distinctive clear veins which become corky, followed by chlorosis and cupping of the entire leaf and stunting when they are infected with severe virus strains. Stem pitting commonly develops, varying in extent with different virus strains. Eureka lemon (*C. limon*), sour orange (*C. aurantium*) and grapefruit (*C. paradisi*) can also be used to demonstrate the presence of tristeza virus.

1.3 Control of CTV

The certification of bud-wood, and the use of resistant rootstock such as trifoliate orange (*Poncirus trifoliata* L. Raf.), are the primary counter-measures in controlling the CTV disease. The following procedure is suggested to control CTV infection-

- For CTV biocharacterization, symptom expression or phenotype of a CTV isolate remains as a focal and essential part to describe an isolate.
- Bud-stock trees should be periodically examined and indexed.
- The use of a temperature-controlled greenhouse, UC mix, fertilization, good horticulture practices, phytosanitary conditions, and zero-tolerance insect control all are essential.
- The production of virus-free trees by meristem culture and shoot-tip grafting or heat treatment is very important.
- If it is possible to keep the field permanently free of CTV, the planting of virus-free trees is practical.
- In areas where it is difficult to find a virus-free field, inoculation with a mild CTV strain (six months before propagation) protects trees against infection with a severe strain of CTV.

2. Purpose and scope

The main purpose of the standard operating procedure (SOP) is to ensure the proper diagnosis of CTV disease and make the harmonization of protocols for related laboratories for consistency and

accuracy of diagnosis. The diagnosis method is based on DAS-ELISA methods and is applicable to Citrus plants and other hosts of CTV virus. The document provides the guidance for the citrus bud wood certification program and describes the SOP for CTV testing facilities involving various steps from sample collection, laboratory requirement, laboratory test and result reporting.

3. Sampling and sample management

Sampling is a procedure in which material is collected outside a laboratory to perform a test. A sample should be representative of the material under test and should be selected based on knowledge of the distribution of the pest to be detected. Such a representative sample may not always be available, if so, this should be documented. Sampling usually involves targeting symptomatic plants or plant parts. Appropriate sampling is crucial for CTV detection and identification by biological, serological, or molecular amplification. Changes to an accepted sampling scheme could result in an effective diagnostic protocol generating false positive or false negative results.

3.1 Procedure for sample collection

- Collect the young flushes from the four directions of the tree and put it in zip lock poly bag.
- Tag the tree and the sample properly.
- Put the zip lock poly bag with sample in an ice box and transport it to the laboratory as soon as possible (as virus are more sensitive to temperature, it is very critical to transport the samples immediately to the lab for getting the precise results).

3.2 Procedure for sample management and storage in the laboratory

- Make record file with the original sample name and lab code. Keep the information of the sample properly.
- Start the test immediately after the sample is received, and store the remaining sample in the refrigerator, at 4°C before further processing. Shoots, leaf petioles, fruit peduncles and flowers can be stored at 4°C for up to seven days before processing. Fruits can be stored for one month at 4°C. Use beyond these time frames may result in lower titres and the potential for false negative results in diagnostic methods.
- Sample tagging according to plant: The sample collector should take extra caution for ensuring the appropriate sample tagging and marking the plant to avoid mismatch.
- While doing the laboratory work, the chances of mixing the sample are always there, so lab worker/analyzer should be very careful while handling the experiment.

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3.3 Records of sampling

Details of sampling should be recorded and communicated to the appropriate personnel. Records should include the following:

Table 1. Detail sampling information

Detail of the sample provider	Name: …… Office: ---------- Address: ----------	Designation: ---------- Contact No.: ---------- Other: ----------
Contact detail of the sample collector	Name: ---------- Gender: ---------- Office: ----------	Occupation: ---------- Age: ---------- Contact No.: ---------- Other: ----------
Plant history	Name of species: ---------- ----------	Propagation type: Grafted ( ) Seedling ( )
	Sample from: screen house ( ) open orchard ( )	Age of the tree : ---------- ----------
	Production status of tree: ---------- ----------	Other: ---------- ----------
Sampling location	Elevation: ----------	Latitude: ----------
	Longitude: ----------	Municipality: ----------
	Ward/tole: ----------	District: ----------
	Province: ----------	Other: ----------
Visual symptoms on plants:	Yes ( )	No ( )
If symptomatic plant	Plant canopy ( )	Die back of twig ( )
	Seedling yellows (CTV-SY) ( ) Tristeza (CTV-T) ( ) Stem pitting (CTV-SP) ( ) Fruit color: ---------- Off season blooming: yes ( ) No ( ) Leaf-cupping ( )	Fruit size: small ( ) Tree size: Normal ( ) stunted ( ) Tree unproductive ( ) Malformation ( )
	Root: (optional)	Other:----------
Sampling procedure:	Sampling Date: ----------	Sampling time: ----------
	Tagging of the sample and tree: Yes ( ) No ( )	Orientation of sample leaf: Below canopy ( )

		From all direction ( ) Matured (fully expanded) young flush ( )
	Number of leaf in a sample: ----------	Maximum number of sample in a packet: 10 ( ) 20 ( ) Other (if below 10)----------
	Surface sterilization of leaf: ( ) Removing of moisture: ( ) Removing of dust : ( )	Sample transport in ice box: Yes ( ) No ( )
	Means of sample transport: ----------	Date of sample provided to Lab: ----------
Other symptoms on plants:	Nutrient deficiency: Yes ( ) No ( ) Other: ----------	Orchard management: Very good ( ) Good ( ) No ( )
Purpose of sampling	Regular diagnosis ( )	Survey ( )
	Quarantine purpose ( )	Other: ----------
At Laboratory	Date of sample (cut pieces of mid-rib) storage in -20°C refrigerator or preferably lower temperature (-40°C or -70°C or -86°C) as per the facility available at laboratory	----------
	Date of sample processing	----------
	Positive sample code	----------
	Negative sample code	----------
	Plate lay out with sample code in record file	----------
	No. of replication and sample code of each replication	2 ( ) 3 ( ) 4 ( )
	Status of color reaction in each sample	Dark yellow (DY) ( ) Light yellow (LY) ( ) No change (NC) ( )
	Keep record of optical density (OD) at 405 nm for each sample, positive and negative control	----------
	Save the folder in computer (if possible)	----------
	Analyze the data	----------

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	Categorize the result based on the value obtained on positive, negative control	Severely positive (), Moderately positive ( ) Negative ( )
	Record the result (both in soft/hard copies)	----------
	Do not disclose the result prior to the official decision	
	Do not share the data to third party without official permission	
	Other:- ----------	

4. Procedure for the CTV test

- DAS-ELISA is used for the detection of CTV titer in collected samples. The ELISA procedure using CTV-positive specific antibody is performed as described by Clark and Adams. The absorbance value is measured at 405 nm using an ELISA reader. The experiment is repeated three times.
- The CTV test kit is available commercially in the market, so, you can use the one reliable test kit or can perform manually by following the guideline.
- The kit should contain the required number of chemicals (including IgG, conjugate, positive control, negative control, extraction buffer, conjugate buffer, substrate buffer, egg albumin, substrate (pNPP), washing buffer, urea and microtiter plate) and each chemical should have sufficient self-life, and well labeled.

4.1 Laboratory equipment and chemicals necessary for DAS-ELISA testing facility

Any biotechnology/pathology laboratory work space can be used for ELISA testing facility, however, the laboratory should include the following equipment/glassware/consumables.

Table 2. List of the laboratory equipment and chemicals necessary to conduct the ELISA testing

S.N.	Name of equipment/glassware/consumables/chemicals	Quantity (at least)
1	ELISA plate reader (with variable filters must include 405 nm and with variable plate size)	1
2	ELISA plate washer (optional)-	1
3	Weighing balance 4 digit	1
4	pH meter (desktop type)-1	1
5	Vortex	1
6	Incubator (at least of 45L)	1
7	Hot air oven	1
8	Weighing balance 4 digit	1
9	Hot plate magnetic stirrer	1
10	Centrifuge	1
11	Refrigerator-1 (normal)	1

12	Deep freezer (-20)	1
13	Clean 1000 mL container or bottle	2 pieces
14	Clean 200 to 300 mL container or bottle (glass or plastic)	2 pieces each
15	Measuring cylinder (10, 50, 100, 500 µL)	1 set of each
16	Double distilled water/ double distillation unit/laboratory water purification system	1 set
17	A marker, wax pencil to mark the samples and plates	6
18	Pasteur pipettes	2
19	A roll of masking tapes (3" wide)-1	1
20	Single channel pipettes or micropipettes (0.5-10 µL, 10-100 µL, 20-200 µL, 100-1000 µL)	1 set each
21	Multichannel micropipette (20-200 µL)	1 piece
22	Micropipette tips for each pipette sets	Depending on the sample volume
23	Micropipette tips holding box (for each set)	2 pieces
24	Boat shaped alumina ceramic boat (size (L*W*H): (115*30*20))	4 pieces
25	2 ml Polypropylene deep well plate (96 well)	4 pieces
26	Mortar and pestle	4 sets
27	Poly bags (non-zip) and zip lock (150 g, 250 g)	As per the sample volume
28	DAS-ELISA reagents/Kit/plates/buffer (see section 4)	
29	Tissue paper/paper towel	
30	Disposable laboratory gloves	
31	Disposable masks	
32	Laboratory slippers/slipper racks	
33	Laboratory aprons/apron hangers	

Note: prior to the process of purchasing of each item it is recommended to consult with the expert (of related field).

4.2 Procedure for preparation of buffer solutions and chemicals for DAS-ELISA based diagnosis of CTV

The following procedure is used to prepare buffer and chemicals for DAS-ELISA based diagnosis of CTV.

Table 3. Name of the chemical and procedure to make different buffer solutions required for DAS-ELISA test for CTV detection.

S.N.	Name of the buffer solutions	Name of the chemical	Amount required for 1L final volume	pH
1	Coating buffer	Sodium carbonate (Na_2CO_3)	1.59 g	9.6
		Sodium hydrogen carbonate ($NaHCO_3$)	2.93 g	

2.	Phosphate buffer saline (PBS)	Sodium chloride (NaCl)	8 g	7.4
		Potassium hydrogen phosphite (KH_2PO_3)	0.2 g	
		Monosodium phosphate ($NaH_2PO_412H_2O$)	2.9 g	
		Potassium chloride (KCl)	0.9 g	
3.	Washing Buffer	PBS	1 L	7.4
		Tween-20	0.5 ml	
4.	Extraction buffer	PBS	1 L	7.4
		Polyvinylpyrrolidone (PVP-40)	20 g	
5.	Conjugate buffer	PBS	1 L	7.4
		PVP-40	20 g	
		Ovalbumin (OVA)	2 g	
6.	Substrate buffer	Diethanolamine	97 mL	9.8
		Double distilled water	903 mL	
7.	Reaction stopping solution	Sodium hydroxide (NaOH)	120 g (3M)	

4.3 Procedure of CTV detection

The detail procedure for detecting CTV in citrus leaf samples is given below-

4.3.1 Coating the plate with antibody

- The antibody (CTV antisera) is mixed with the coating buffer at 1:1000 ratio.
- 0.2 mL or 200 µL of coating buffer is poured into each well of the ELISA plate with the help of a micropipette and is allowed to incubate at 37°C for 2-4 h.
- Then wash the coated plate three times with a washing buffer (PBS-Tween).

4.3.2 Sample extraction and loading the virus sample

- Macerate 0.5 g of excised tissue from test plant using mortar and pestle or the extraction bags with 5 mL of extraction buffer and proceed with the homogenization of the tissue.
- Recover at least 1 mL of homogenate, and transfer it into microcentrifuge tubes (1.5 mL). Centrifuge at low speed (2000–3000 rpm) to pellet the plant debris.
- Then, load 0.2 mL or 200 µL (pipetting from the upper part of the microcentrifuge tube) of the supernatant into at least three replicates of the ELISA plate.
- Each ELISA plate includes the following control: CTV positive, CTV negative and the PEP buffer

- Cover the loaded plate and incubate overnight or 12 h at 4°C.
- After incubation, remove the plant sap from the well and wash three times with a washing buffer (PBS-T).

4.3.3 Conjugate loading

- The conjugate antibody is mixed with the conjugate buffer at 1:1000 ratios, also called secondary CTV antiserum.
- Then, pour 200 µL of secondary CTV antiserum into each well and incubate the plate at 37°C for 2-4 h.
- Wash the plate three times with washing buffer (PBS-T).

4.3.4 Substrate loading

The final step of DAS-ELISA is to add substrate which is alkaline phosphate. The substrate solution is freshly prepared in substrate buffer at the rate of 1 mg/mL. The substrate is prepared as shown in Table 3.

- Dispense 200 µl of substrate solution into each well of ELISA plate.
- Incubate the plate for 30 min to 2 h for the color reaction to take place.
- Strong yellow color develops in the positive control and CTV positive samples.

4.3.5 Result reading

After the yellow color formation (Fig 3 B) in the positive samples, the optical density (OD) of each well is measured at 405 nm wavelength using an ELISA reader. According to OD values of the controls, plate readings are made at 30 min, 1 h, and/or 2 h. Samples with OD values 2–2.5 times higher than the average value of the negative control are considered positive for CTV. Then the degree of CTV infection is categorized as negative, mild, moderate, and severally positive based on the OD value obtained in positive samples and test samples.

4.3.6 Reaction stop

The color reaction is stopped after the plate reading. This is done by adding 5 µL of 5% NaOH solution in each well.

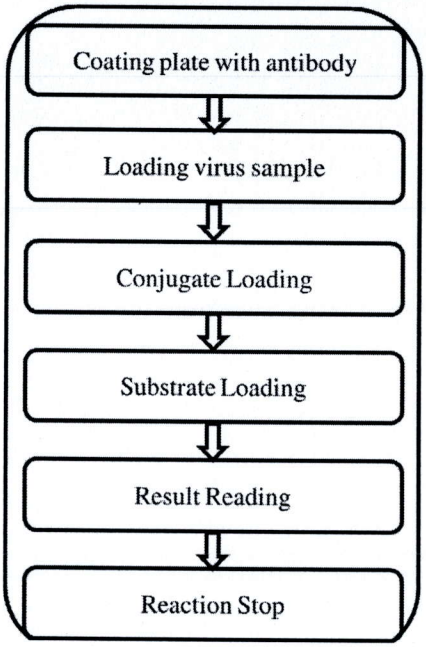

Figure 2. Schematic diagram of CTV detection using DAS-ELISA protocol

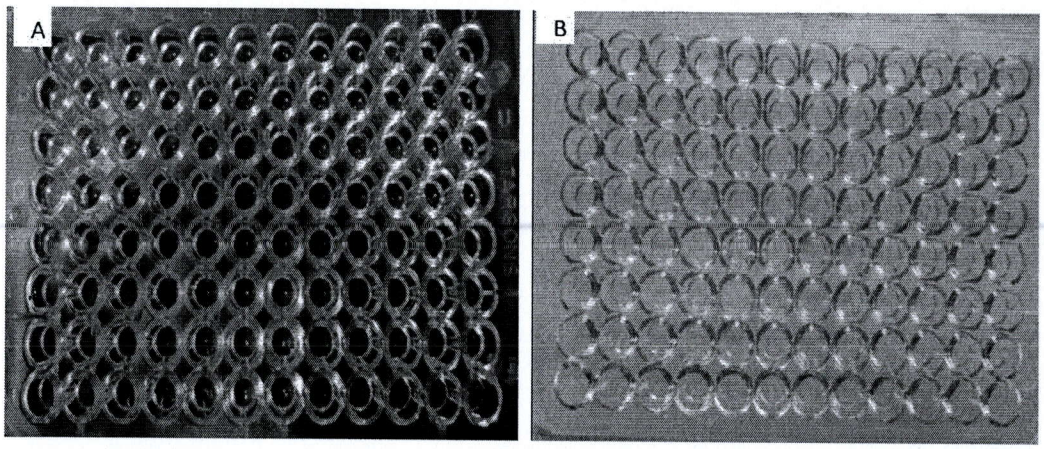

Figure 3. ELISA test, A: before addition of substrate; B: after addition of substrate, appearance of yellow color indicates positive reaction

5. Result reporting

After completion of the diagnostics work, the identification needs to be reported to the submitter within the time frame. The final report should contain the submitter information, sample information, tests done, diagnosis result, and signature of the identifiers. When content of the

report is missing or incorrect then amended report should be sent to the submitter. The laboratory shall have appropriate documentation system and procedures for the reporting of results.

6. Fate of sample

Laboratories must treat a sample once it has been fully analyzed and the final diagnostic report released. Samples may be either disposed of in a manner appropriate to their biosecurity risk or retained for future use.

6.1 Disposal of sample

Before a decision is made to dispose of a sample, the laboratory should decide whether it should be retained as evidence or it should be disposed after autoclaving or by taking appropriate means to minimize pest risk.

6.2 Sample or specimen retention

A laboratory may choose to retain samples and their related specimens for many reasons. Samples may also be retained in secured manner because of their diagnostic value as reference specimens, or for legal actions. The original sample should be kept at 2 to 8°C for up to 72 hours after collection and if a delay in testing or shipping is expected, store specimens at -70°C or lower.

7. Safety and precautions

- Sample tagging according to plant: The sample collector should take extra caution for ensuring the appropriate sample tagging and marking the plant to avoid mismatch.
- While doing the laboratory work, the chances of mixing the sample are always there, so laboratory worker/analyzer should be very careful while handling the experiment.
- Working laboratory environment and instruments should be clean, and sterile.
- Do not forget to change the micropipette tips in each sample.
- Do proper tagging of the sample in each well (you can make your note in a copy).
- Follow each step of the DAS-ELISA testing protocol very carefully.
- Instructions should be read before using any equipment or chemicals.
- All compounds shall be handled as if they are highly toxic unless it is known to the contrary.
- All laboratory work must be performed with the proper personal protective equipment (PPE), including goggles or glasses, gloves, and lab coat according to needs. Additional protection may be necessary, depending on the process.
- All experiments using highly toxic materials shall be clearly labeled as such, using a sign or tag, signed, and dated.
- The laboratory space should be well ventilated or must have safe exhaust fans.
- The laboratory must have fire extinguisher and first aid kits. Medical emergency numbers and fire brigade office numbers shall be clearly displayed.

8. Waste disposal and management

- All generators of potentially hazardous wastes must ensure segregation, accurate and complete labeling and safe storage, transport, treatment and disposal of such wastes.
- Wastes should be minimized where possible.
- Waste chemicals and solvents are stored in suitable areas whilst awaiting collection and must not be accumulated.
- Regular disposal from the laboratories must be part of the laboratory program.
- Waste should be segregated and mixing avoided where possible.
- If you are generating a large amount of one particular type of waste, have a separate residue container for it.
- Ensure the container is not leaking and there is no spillage on the exterior of the container.
- Untrained staff and students must not handle hazardous wastes and must not be given such responsibility.
- PPE should be a consideration when handling chemical waste. Reference should be made to the material safety data sheet.
- Broken glassware and solid waste obtained from sample preparation should be segregated and disposed separately.
- Plant material and/or supplies used in the examination and isolation of the suspect sample must be destroyed using a biologically monitored autoclave. The autoclave must be set at a minimum of 15 psi, 121°C for 30 min. All tools and other equipment must be sanitized and/or sterilized before re-use.

9. Distribution and storage of document

The SOP will be distributed as below:

- Ministry of Agriculture and Livestock Development (MoALD), Singhadurbar, Kathmandu
- Department of Agriculture (DoA), MoALD, Hariharbhawan, Lalitpur
- Seed Quality Control Centre (SQCC), MoALD, Lalitpur
- National Center for Fruit Development (NCFD), DoA, MoALD, Kirtipur, Kathmandu
- Central Agricultural Laboratory (CAL), DoA, MoALD, Hariharbhawan, Lalitpur
- Warm Temperate Horticulture Centre, NCFD, DoA, MoALD, Kirtipur, Kathmandu
- Citrus Development Centre, NCFD, DoA, MoALD, Tansen, Palpa
- National Plant Pathology Research Center (NPPRC), Nepal Agricultural Research Council (NARC), Khumaltar, Lalitpur
- Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur
- National Citrus Research Program (NCRP), NARC, Paripatle, Dhankuta
- Provincial Plant Protection Laboratories

The original approved SOP will be placed in the approved SOP folder and will be held by Chief, NCFD.

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