Survey Protocols for the Citrus Pests

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

Survey Protocols for the Citrus Pests

Government of Nepal
Ministry of Agriculture and Livestock Development
Plant Quarantine and Pesticide Management Centre
Hariharbhawan, Lalitpur

Table of Contents

Survey Protocol for Fruit Flies

NPPO Nepal endorsement on 6th May, 2013
First amendment on 17th May, 2023

1. Background information

A phytosanitary trade agreement for an export of citrus fruit from Nepal to Tibet, China signed in 2012 and 2019 where China declared *Bactrocera dorsalis, Bactrocera cucurbitae, Bactrocera zonata, Bactrocera correcta* and *Bactrocera tsuneonis* being the regulated quarantine citrus pests in China. Presently in Nepal, *Bactrocera tsuneonis* is taxonomically renamed authentically in *Bactrocera minax* (Enderlein) (Paudyal *et al.*, 2016; Adhikari *et al*., 2022). It is observed in the agreement that Nepal needs to export pest free citrus fruits to China with respect to the above mentioned 5 fruit flies. Detection survey in the pest surveillance activity is a major tool to know the status of citrus production sites on above pests. Thus, fruit fly detection survey protocol is developed to commence a national planned survey in citrus orchards. This protocol has been endorsed by National Plant Protection Organization, Nepal (NPPO-Nepal).

2. Target pests

Fruit flies belong to Diptera: Tephritidae and are major pest of fruit and fruit vegetables. Among different fruit fly species reported by different authors, *Bactrocera dorsalis, B. zonata, Zeugodacus cucurbitae, Z. tau, Z. scutellaris* and *B. minax* are predominantly occurring species in the horticultural ecosystem of Nepal. Among different reported fruit fly species of Nepal, Chinese citrus fly, *Bactrocera minax* (Enderlein), is one of the most destructive insect pests of citrus in China, Bhutan, India and Nepal.

- *Bactrocera dorsalis* (Hendel, 1912) (Oriental fruit fly)
- *Bactrocera cucurbitae* (Coquilett, 1899) (Melon fruit fly)
- *Bactrocera zonata* (Saunders, 1841) (Peach fruit fly)
- *Bactrocera correcta* (Bezzi, 1913) (Guava fruit fly)
- *Bactrocera minax* (Enderlein, 1920) (Chinese citrus fly)

Taxonomic tree of insect:

Domain: Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Uniramia
Class: Insecta
Order: Diptera
Family: Tephritidae
Genus: *Bactrocera*

Species: *dorsalis, zonata, correcta, minax*
Genus: *Zeugodacus*
Species: *cucurbitae*

3. Surveillance purpose

As per the phytosanitary trade agreement for the export of citrus fruits from Nepal to Tibet, China in 2012 and 2019, surveillance of fruit fly is proposed to detect and monitor the fruit fly species in the citrus orchards of Nepal. It will generate the data base of the fruit fly species for the stakeholders to facilitate the citrus export to China.

4. Scope

For the purpose of performing detection and management of these fruit flies, the survey surveillance programme of fruit fly has great scope in Nepal with respect to the global citrus trade.

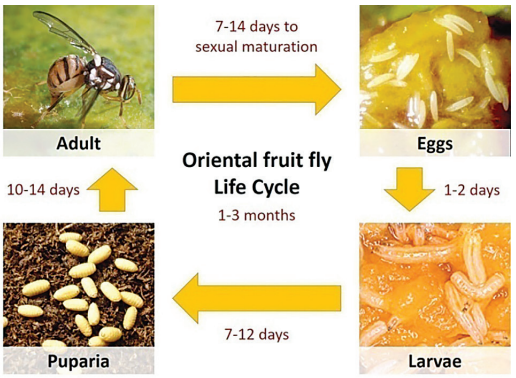
5. Justification for surveillance

According to the phytosanitary trade agreement that was signed between China and Nepal in 2012 and 2019 for the export of citrus fruit from Nepal to Tibet both signatory countries declared the following quarantine fruit fly species; *Bactrocera dorsalis*, *Bactrocera cucurbitae*, *Bactrocera zonata*, *Bactrocera correcta*, and *Bactrocera tsuneosis* (previously, Bractocera minax was misidentified as *Bactrocera tsuneonis* during the trade agreement phases). The surveillance program is crucial for performing detection and management of these fruit flies.

6. Insect biology

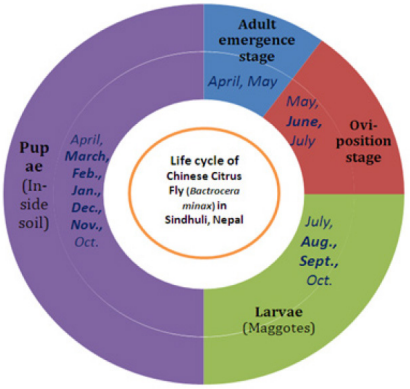
6.1 Life cycle and field identification

Oriental fruit fly, melon fruit fly, peach fruit fly and guava fruit fly breed throughout the year in tropical environment (Figure 1) with a range of generations, respectively, 4-5 generations of *B. dorsalis* (Li, Yang, Wang, Wang, & Wei, 2019), 8-10 generations of *Z. cucurbitae* (Dhillon, Singh, Naresh, & Sharma, 2005), 7-9 generations of *B. zonata* (Zingore *et al*., 2020), and 8 generations of *B. correcta* (Liu & Ye, 2009). However, Chinese citrus fly (*Bactrocera minax*) is a univoltine fruit fly species (Adhikari, Thapa, Joshi, Du, & Tiwari, 2022) (Figure 2). Field identification of fruit fly species could be taken from the authorized literatures and publications.



(Source: Li, 2019)

Figure 1. Life cycle of *Bactrocera dorsalis*



(Source: Adhikari et al., 2020)

Figure 2. Life cycle of *Bactrocera minax*

6.2 Mode of dispersal

According to Fletcher (1989), adult flight and the transport of infested fruits are the main means of movement and dispersal from one place to other places. Many *Bactrocera* spp. can fly 50-100 km.

6.3 Host range

S N	Fruit fly species	Hosts	References
1	*Bactrocera dorsalis* (Hendel, 1912) (Oriental fruit fly)	The oriental fruit fly attacks over 300 cultivated and wild fruits including Annona, avocado, banana, bittermelon, citrus, coffee, guava, macadamia, mango, papaya, passion fruit, peppers, persimmon, and tomato.	Mau & Matin, 2007a
2	*Bactrocera cucurbitae* (Coquilett, 1899) (Melon fruit fly)	Melon flies have more than 80 hosts. They are major pests of beans, bittermelon, Chinese wax gourd, cucumbers, edible gourds, eggplant, green beans, hyotan, luffa, melons, peppers, pumpkins, squashes, togan, tomatoes, watermelon, and zucchini.	Mau & Matin, 2007b
3	*Bactrocera zonata* (Saunders, 1841) (Peach fruit fly)	They feed on more than 50 commercial and wild host plants; including peach, guava, mango, apricot, citrus, prickly pear and fig.	Allwood *et al*., 1999, White & Elson-Harris, 1992

S N	Fruit fly species	Hosts	References
4	*Bactrocera correcta* (Bezzi, 1913) (Guava fruit fly)	These feed on cashew, carambola, mango, sapodilla, spanish cherry, Jamaican cherry, guava, water apple and common jujube.	Plant Health Australia, 2018
5	*Bactrocera minax* (Enderlein, 1920) (Chinese citrus fly)	This is an oligophagus pest species attack only on citrus fruits (Rutaceae) sweet orange, mandarin, lemon etc.	Xia *et al.,* 2018, Dorji *et al.*, 2006, Chang *et al.*, 2018

7. Fruit infestation symptoms and insect rearing

Adult female flies lay eggs just beneath the skin of the fruits. Oviposition spot in the fruit rind could be observed. Small holes where the maggots penetrated the fruits' surface, a brown, oozing fluid pouring from the egg laid portions are the symptom of infestation. The punctures with some sticky gum should be observed. As a result of their feeding activity by the maggots, the fruits turn prematurely yellow around the feeding site and eventually drop. When the maggots are mature they leave the fruit by making an exit hole, and enter into the soil to pupate. The fallen fruits in orchard and maggots inside an opened fruit should be observed. Infected fruits are lighter in weight and have holes in the outer rind of the fruit.

Laboratory rearing of maggots from infested fruits can be performed in the insect rearing container to acquire pupae and observe adult emergence. Maggots infested fruits should be collected, sliced to open with a knife, and maggots may exit out from the fruits. Place those maggots in rearing container filled with garden soil (20.7% moisture, loamy soil) (Adhikari, Thapa, Joshi, Du, & GC, 2021). Maintain the rearing media's moisture content for B. minax. Examine the pupae (after 30-50 days for B. minax and after 3-5 days for other species) and count by gently stirring the soil and placed in the same container. Record adult flies' emergence as per life cycle of the fruit fly species and proceed for the identification. Besides, adult emergence can be assessed in the tree canopy placing net in the ground where maggots infested fruits dropped.

8. Design of survey program

Design of survey program of fruit fly includes detailed methodology of trapping, monitoring and detection using parapheromone lures and protein bait.

8.1 Observation season

Observation should be performed throughout the citrus tree phenology in citrus orchards. Fruit fly observation can be done throughout the year by means of different

traps male lures (parapheromones) and protein bait. Trap density: should be 1 trap per ha in each trapping mode depending upon the field topography.

8.1.1 Male lures (Parapheromones):

Use parapheromones namely; Methyl Eugenol (ME) and Cue lure (CL) in ready to use dispenser; Stainer trap, apply 5 drops ME or CL each with 10 drops of Malathion 50 EC to knock down the trapped flies; Hang Steiner traps in tree branches at a height of 2 m above the ground, with two traps (ME and CL) in a set each separated by at least 5 m from each other, and 100 m distance between each set of traps. Replace lure in every fortnight days; fortnight collection interval; handle ME and CL separately; dispose used lures by burrowing in soil.

Fruit fly species concerned:

Fruit fly species	**Lure**
Bactrocera dorsalis	ME
Zeugodacus cucurbitae	CL
Bactrocera zonata	ME
Bactrocera correcta	ME

8.1.2 Protein bait: MCPhail trap; Protein bait (25% Protein hydrolysate with 0.1% Abamectin) 1 part mixed with 2 part water bait solution up to 1 cm at the bottom of trap. Hang traps in tree branches at a height of 2 m above the ground, with other trap (ME and CL) in a set each separated by at least 5 m from each other, and 100 m distance between each set of traps. Replace bait in every fortnight; fortnight collection interval; attention should be made not to spill bait solution in the ground.

Fruit fly species concerned

- *Bactrocera dorsalis* (Hendel, 1912) - Oriental fruit fly
- *Bactrocera cucurbitae* (Coquilett, 1899) - Melon fruit fly
- *Bactrocera zonata* (Saunders, 1841) - Peach fruit fly
- *Bactrocera correcta* (Bezzi, 1913) - Guava fruit fly
- *Bactrocera minax* (Enderlein, 1920) - Chinese citrus fly

McPhail trap with mesh covering above the height of the protein bait solution should be adopted to prevent attracted fruit flies dip in the bait solution. Place Malathion 10 drops in cotton for knock down of attracted flies.

Protein bait trap is especially focused to the para-pheromone non- attracting species of fruit fly – Chinese citrus fly, *Bactrocera minax* along with other species.

The life cycle of Chinese citrus fly, *Bactrocera minax* is univoltine (long diapause period) and adult emergence begin with spring season. So, install the protein bait trap from February 1 till July 30 (Magh 15 till Sawan 15) to monitor the Chinese citrus fly, *Bactrocera minax.*

8.2 Sampling

7.3.1 Sample tree selection for observation: Fruit bearing trees during fruit maturity stage at the orchard should be selected.

7.3.2 Number of sample trees for observation: Randomly select ten fruits in each of the two fruit bearing trees in 10 orchards from each district; maintain fortnightly observation interval, collect maggot infested suspected fruits to rear in laboratory for adult emergence.

Apply a technique to collect soil emerged fruit flies under the tree canopy using a net of dimension, 2.5 m length x 2.0 m breadth x 1.5 m height) and / or use protein bait trap (McPhail trap).

8.3 Plant parts used for observation

Observe fruits for eggs. For *Bactrocera minax*- after eating protein, early stage/immature fruits and for other species- mature fruit. Observe fruit infestation symptoms.

9. Fruit fly collection and preservation

9.1 Ways to collect fruit fly specimens

As mentioned above collect the fruit fly specimen from parapheromone trap and Protein bait trap.

9.2 Preservation of fruit fly specimens

- **Dry preservation of fruit fly adults:** Fruit fly adults should be stored properly and put in a sealed vial or container. The vial should contain a label with the collection information, including the collection location, date of collection, collector, host etc. Some of the collected fruit flies will be pinned, spread and displayed in a display-box for their taxonomic identification in the future. Adults may be frozen until dead for an hour and then dried out and preserved in a vial with tissue to safeguard the specimen.
- **Wet preservation of maggots with special warm water treatment:** In most cases, adult flies are required to identify flies accurately to the species level; but, in other cases, larvae may need to be preserved for morphological analysis to at least

identify flies to the family or genus level. Larvae are kept in these conditions by first submerging them for 2-4 minutes in hot (about 65°C) water, following which they are removed and allowed to cool to room temperature. When the larvae are at room temperature, immerse them in 50% ethanol for 15–30 minutes before moving them to 70% ethanol for storage, making sure to include any necessary collection information with the sample.

- **Sample preparation for molecular identification:** Fruit flies should either be maintained dry or stored in ethanol as close to 100% as possible for molecular analysis.

9.3 Ways to packing fruit fly specimens

Collect fruit fly in inflated plastic bottle (size depending on the number of fruit fly specimens) and knock down them in killing jar/ ethyl acetate/ CTC/ chloroform fume; pack the fruit fly specimens in plastic/ tin box along with collection data (collector's name, host name, location and date of collection, source of specimens as pheromone/ protein bait/ trap-net with respective code numbers).

9.4 Insect preparation:

- Insect separation based on insect taxonomy (eg. Diptera, Tephritidae, *Bactrocera*,);
- Record of local insect museum code (eg. ED, Dip, Tephri, KTM, No.,).

9.5 Insect packing based on nature of insect preservation:

9.5.1 For dry preserved insects: dry preserved insects should be placed inside the stout cardboard box with thermocol platform with deep placement of insect pin inside the thermocol; minute pouch filed with silica gel should be placed in one corner of the box without disturbing insect specimen; packing box cover must have sender and receiver's addresses; the box should be wrapped with suitable and durable material until it reaches to addressee; for scientific communication with legal approach, the insect packing box should go along with official permission paper from country of destination.

9.5.2 For wet preserved insects: wet preserved insects should be placed inside the screw capped 70-100 % alcohol contained vials with collection data printed with pencil; wrap each vials with tissue paper to ensure their physical damage during transportation; for ensuring extra safety, the left over internal space inside the box should be filled up with thermocol specks; packing box cover must have sender and receiver's addresses; the box should be wrapped with suitable and durable material until it reaches to addressee; for scientific communication with legal approach, the insect packing box should go along with official permission paper from country of destination.

10. Collection and Preservation Materials

i. Traps (Steiner trap, McPhail trap)
ii. Male lures (Methyl eugenol, Cu-Lure)
iii. Protein bait
iv. Malathion insecticide
v. Cotton role
vi. Insect collection kit:
Collection bottles (plastics), glass vials with tight cap, killing jar, killing agent (ethyl acetate), forceps, scalpel, camel hair brush, led pencil
vii. Insect sweeping net
viii. Insect pins (no.3)
ix. Insect pinning block
x. Insect spreading board
xi. Entomological needle and camel hair brush
xii. Magnifier (10X-20X lens)
xiii. Microscope (dissecting)
xiv. Absolute alcohol (ethanol)
xv. Relaxing jar
xvi. Formaldehyde
xvii. Scissors
xviii. Data observation sheet
xix. White paper sheet
xx. Gel pen
xxi. Labels (acid free card stock)
xxii. Permanent black ink pen
xxiii. Insect rearing cage
xxiv. Insect rearing net-cage
xxv. Sterilized soil media
xxvi. Color fruit fly species photo sheets
xxvii. Insect display box, container etc.

11. Place and person for specimen diagnosis

National:

Plant Quarantine and Pesticide Management Centre, Hariharbhawan, Lalitpur
National Entomology Research Centre, NARI, NARC, Khumaltar, Lalitpur
Natural History Musuem, Tribhuvan University, Swayambhu, Kathmandu
Central Agriculture Laboratory, Hariharbhawan, Lalitpur
Department of Entomology, AFU, Rampur, Chitwan
Department of Entomology, TU/IAAS, Kirtipur

International:
British Museum (Natural History), London, UK;
CSIRO, Canberra, Australia;
East West University, Hawaii, USA;
ICAR-NBAIR, Bangaluru
Department of Entomology, Indian Agriculture Research Institute, New Delhi;
Indian Institute of Horticulture Research, Hassergatta, Banglore, India;
Central Institute for Sub-Tropical Horticulture Research, Lucknow;
Molecular diagnosis: ICAR-NBAIR, Bangaluru, India

12. Literature cited:

Adhikari, D. & Joshi, S. L. (2018). Occurrences and field identities of different species of Fruit Flies in Sweet Orange (*Citrus sinensis*) Orchards in Sindhuli, Nepal. *Journal of Natural History Museum, Nepal.* Vol. 30. 2016-18. pp. 47-54.

Adhikari, D. & GC. Y. D. (2020). Opportunity to Export Citrus Fruit from Nepal to China: Activities Accomplished on Plant Quarantine Concerned. *International Journal of Agriculture Innovations and Research.* Volume 8, Issue 5, ISSN (Online) 2319-1473.

Adhikari, D., Tiwari, D. B., & Joshi, S. L. (2018). Population dynamics of Fruit Flies in Sweet Orange (*Citrus sinensis*) Orchards in Sindhuli, Nepal. *The Journal of Agriculture and Environment.* Vol. 19, June, 2018. pp. 9-16.

Adhikari, D., Thapa, R. B., Joshi, S. L., & Du., J. J. (2021). Area-Wide Control Program in management of Chinese citrus fly, *Bactrocera minax* (Enderlein) (Diptera: Tephritidae), in citrus orchards, Sindhuli, Nepal. *The Journal of Agriculture and Environment.* Vol: 22, June, 2021. pp. 41-50.

Adhikari, D., Thapa, R. B., Joshi, S. L., Du, J. J., & Achrya, U. K. (2020). Receded Sweet Orange Losses from Chinese Citrus Fly, *Bactrocera minax* (Enderlein) in Sindhuli Citrus Orchards: Lesson from Area Wide Control Program. *Proceeding of National Horticulture Seminar Kirtipur, Kathmandu.* February 6-7, 2020.

Adhikari, D., Thapa, R. B., Joshi, S. L., Liang, X. H., & Du, J. J. (2020). Area-Wide Control Program of Chinese Citrus Fly *Bactrocera minax* (Enderlein) in Sindhuli, Nepal. *American Journal of Agricultural and Biological Sciences.* Vol. 15. 2020. pp. 1-7. DOI: 10.3844/ajabssp.2020.1.7

Adhikari, D., Joshi, S. L., Thapa, R. B., Pandit, V., & Sharma, D. R. (2020). Fruit Fly Management in Nepal: A case from plant. *Journal of Biological Control,* 34(1): 8-14, 2020, DOI: 10.18311/jbc/2020/22833

Adhikari, D., Thapa, R. B., Joshi, S. L., & Du, J. J., (2022). Frugivorous fruit flies (Diptera: Tephritidae: Dacini) with an emphasis on an invasive *Bactrocera minax* in Nepal. Presented in National Plant Protection Workshop 2022 on May 31-June 1, 2022 at Kathmandu, Nepal. Organized by PQPMC and PPS Nepal.

Adhikari, D., Thapa, R. B., Joshi, S. L., Du, J. J., & Tiwari, S. (2022). Biology and management of Chinese citrus fly, *Bactrocera minax* (Enderlein) (Diptera: Tephritidae). *Journal of Agriculture and Forestry University*, 5(1), 1–13. https://doi.org/10.3126/jafu.v5i1.48432

Adhikari, D., Thapa, R. B., Joshi, S. L., Du., J. J., & Acharya, U. K. (2020). Receded Sweet Orange Losses from Chinese Citrus Fly, Bactrocera minax (Enderlein) in Sindhuli Citrus Orchards: Lesson from Area Wide Control Program. *Proceeding of National Horticulture Seminar*, Kirtipur, Kathmandu. February 6-7, 2020.

Adhikari, D., Thapa, R. B., Joshi, S. L., Du., J. J., & GC, Y. D. (2021). Diapause intensity of Chinese citrus fly, *Bactrocera minax* (Enderlein) in Sindhuli, Nepal. *International Journal of Entomology Research,* vol.: 6 (5) pp. 37-41.

Allwood, A.J., Chinajariyawong, R.A.I., Drew, E.L., Hamacek, D.L., Hancock, H.C., Jipanin J.C., Jirasurat, M., Kong Krong, C., Kritsaeneepaiboon, S., Leong, C.T.S., Vijaysegaran, S. (1999). Host Plant records for fruit flies (Diptera: Tephritidae) in South East Asia. *Raffles Bull Zool Suppl*. 7: 1–92.

Bhandari, K., A. R. Ansari, S. L. Joshi, H. P. Subedi and M. K. Thakur. 2017. Fruit Fly (Diptera: Tephritidae) diversity in citrus fruits in eastern hills of Nepal. *Proceedings of the Ninth National Horticulture Workshop.* May 31 - June 1, 2017.

Chang, C.L., Liquido, N.J., Nakamichi, K.A.A. & Ching, A.J. (2018). Host plant records of the Chinese citrus fruit fly, *Bactrocera minax* (Enderlein) (Diptera: Tephritidae). Version 1.0 February 06, 2018. United States Department of Agriculture, 22pp. Available online at https://coffhi.cphst.org/ (12/04/2020)

Dhillon, M. K., Singh, R., Naresh, J. S., & Sharma, H. C. (2005). The melon fruit fly, Bactrocera cucurbitae: a review of its biology and management. *Journal of insect science (Online)*, *5*, 40. https://doi.org/10.1093/jis/5.1.40

Dorji, C., Clarke, A.R., Drew, R.A.I., Fletcher, B.S., Loday, P., Mahat, K., Raghu, S., & Romig, M.C. (2006). Seasonal phenology of *Bactrocera minax* (Diptera: Tephritidae) in western Bhutan. *Bulletin of Entomological Research,* 96(5), 531–538.

Drew, R.A.I., Dorji, C., Romig, M.C., & Loday, P. (2013). Attractiveness of various combinations of colors and shapes to females and males of *Bactrocera minax* (Diptera: Tephritidae) in a commercial mandarin grove in Bhutan [2006]. *Journal of Economic Entomology* 99(5):1651-1656. https://doi.org/10.1603/0022-0493-99.5.1651

Ecoman Biotech. (2014). About the Fruit Fly: Species & Biology https://www.en.ecomanbiotech.com/about-the-fruit-fly-species--biology

Fletcher, B.S. (1989). Ecology; movements of tephritid fruit flies. In: World Crop Pests 3(B). Fruit Flies; Their Biology, Natural Enemies and Control (Ed. by Robinson, A.S.; Hooper, G.), Elsevier, Amsterdam, Netherlands. pp. 209-219.

Gautam, E., Srivastava, A., Singh, L. K., Karki, S.,Adhikari, D., & Acharya, U. K. (2020). Survey and Monitoring of Chinese citrus fly (*Bactrocera minax* Enderlein) in Sweet orange orchards of Sindhuli, Nepal. *Nepalese Horticulture*, 14 (1), 56-62.

Li, A. (2019). *Bactrocera dorsalis* - Oriental fruit fly. Created by Li Anqi, last modified by Lim Yi Lin on 10 Oct 2019. Assessed from https://wiki.nus.edu.sg/display/TAX/Bactrocera+dorsalis+-+Oriental+fruit+fly on 17th May, 2023

Li, X., Yang, H., Wang, T., Wang, J., & Wei, H. (2019). Life history and adult dynamics of *Bactrocera dorsalis* in the citrus orchard of Nanchang, a subtropical area from China: implications for a control timeline, *ScienceAsia* 45 (2019): 212–220. http://www.scienceasia.org/2019.45.n3/scias45_212.pdf

Liu X. & Ye H. (2009). Effect of temperature on development and survival of *Bactrocera correcta* (Diptera: Tephritidae). *Sci. Res. Essays* 4: 467–472

Mau, R. F.L. & Matin, J. L. (2007a). Bactrocera dorsalis (Hendel). http://www.extento.hawaii.edu/Kbase/Crop/Type/bactro_d.htm

Mau, R. F.L. & Matin, J. L. (2007b). Bactrocera cucurbitae (Coquillett). http://www.extento.hawaii.edu/kbase/crop/Type/bactro_c.htm#:~:text=Melon%20flies%20have%20more%20than,tomatoes%2C%20watermelon%2C%20and%20zucchini.

Nepal China Agreement. (2012). The agreement between People's Republican China, General Administration on Quality Supervision, Monitoring and Quarantine and Government of Nepal. Department of Agriculture for Phytosanitary Protocol to export Nepalese citrus fruits from Nepal to China on 2012.

Nepal China Agreement. (2019). Protocol of Phytosanitary Requirements for the Export of Citrus Fruit From Nepal to China between Ministry of Agriculture and Livestock Development of the Government of Nepal and General Administration of Customs of the People's Republic of China.

Plant Health Australia. (2018). The Australian Handbook for the Identification of Fruit Flies. Version 3.1. Plant Health Australia. Canberra, ACT.

https://www.planthealthaustralia.com.au/wp-content/uploads/2018/10/The-Australian-Handbook-for-the-Identification-of-Fruit-Flies-v3.1.pdf

https://www.fruitflyidentification.org.au/sample-handling/#:~:text=Adults%20can%20be%20killed%20by,an%20appropriately%20accredited%20quarantine%20facility.

Paudyal, K.P., Shrestha, T.N. & Regmi, C. (2016). Citrus Research and Development in Nepal, Six Decades of Horticulture Development in Nepal. Silver Jubilee Special. Nepal Horticulture Society, Lalitpur, Nepal. pp.113-144.

Sharma, D. R., Adhikari, D., & Tiwari, D. B. (2015). Fruit Fly Surveillance in Nepal. *Agricultural and Biological Sciences Journal.* Vol. 1, No. 3, 2015, pp. 121-125.

White I.M., Elson-Harris M.M. (1992). *Fruit flies of economic significance: their identification and Bionomics*. CAB International.

Xia, Y., Ma, X.L., Hou, B.H., & Ouyang, G.C. (2018). A review of *Bactrocera minax* (Diptera: Tephritidae) in China for the purpose of safeguarding. Advances in Entomology, 6, 35-61.

Zingore, K. M., Sithole, G., Abdel-Rahman, E. M., Mohamed, S. A., Ekesi, S. Tanga, C. M., & Mahmoud, M. E. E. (2020). Global risk of invasion by Bactrocera zonata: Implications on horticultural crop production under changing climatic conditions. PLOS ONE 15(12): e0243047. https://doi.org/10.1371/journal.pone.0243047

ANNEXES

Annex 1: Field record sheet for fruit flies

Name of Fruit fly Monitoring Area:				Week/Month: From:		To:	
Trap No.	Location of trap	Date Insp.	Host	Lure Type	No of flies/ trap	Species identified	Date recharge

Annex 2: General pest record

Reference Number	
Scientific name of pest ■ Common Name ■ Species Name: ■ Family ■ Order:	
Life stage of pest	[ ] Egg; [ ] Larvae(maggot); [ ] Pupae; [ ] Adult
Scientific Name of host ■ Variety ■ Common Name: ■ Species Name ■ Family	
Plant parts affected:	[ ] Leaves; [ ] Stem; [ ] Roots; [ ]; Buds/Flowers; [ ] Fruits; [ ] Seed ; and [ ] Whole plant
Stage of crop:	[ ] Seedling stage; [ ] Vegetative growth stage; [ ] Flowering stage; and [ ] Fruiting stage
Locality ■ District ■ Municipality, ward no. and village	
Date of Collection	
Name of the Collector	
Date of Identification	
Name of the Identifier	
Date of Verification	
Name of the Verifier	

Annex 3: Specimen identification report

To,
……
……
……
(Name & Address of Field Inspector)

Trap	Location	Name of fruit fly species identified	No of flies

Signature & Date:
Name of Reference Entomologist
Address for Communication:
Tel.:
Fax:
E-mail:

Annex 4: Fruit fly surveillance & monitoring report

1. Area involved:
2. Period of reporting:
3. No of traps inspected:
4. No of traps recorded with fruit flies:
5. Details of fruit flies detected:

S.N.	Location	Fruit fly species detected	No of flies detected	Life stage detected

6. Action taken on detection of fruit flies

7. Signature & Date

8. Name

9. Designation of Officer

Annex 5: Fruit fly collection record

District:
Crop:
Address of the orchard:
Name of the fruit fly collector:

Date of collection:

Trap no.	Name of the orchard owner and GPS location	Number of fruit flies trapped			Remarks
		ME	CL	PB	

Annex 6: Fruit fly identification record

District: .....
Crop: .....
Lure: ME/CL/PB
Date:

Trap No.	Total fruit fly trapped	Species					
		species 1	species 2	species 3	species 4	species 5	species 6
1							
2							
3							
4							
5							
Total							

Name and Signature/date of Identifier:
Name and Signature/date of Verifier:

Annex 7: Fruit fly collection traps

Steiner trap

McPhail trap

Survey Protocol for Citrus Canker

NPPO Nepal endorsement on 6th May, 2013
First amendment on 17th May, 2023

1. Background information

Asiatic citrus canker is a bacterial disease affecting most citrus varieties, with grapefruit most susceptible. The disease causes scab or crater-like lesions on the rind of the fruit, which reduces marketability. The causal agent is the bacterium *Xanthomonas citri* subsp. *citri*, (reclassified from "A" pathotype *Xanthomonas axonopodis* pv. *citri*, Schaad *et al*., 2006).Citrus canker symptoms include brown spots on leaves, often with an oily or water-soaked appearance. The spots (technically called lesions) are usually surrounded by a yellow halo, and they can be seen on both the upper and lower sides of the leaf. Typical citrus canker lesions are erumpent, with a necrotic center, often surrounded by a chlorotic halo (Schuber *et al*., 2001). Similar symptoms can appear on fruit and stems. The disease is endemic in India, Japan and other south-East Asian countries, from where it has spread to all other citrus producing continents except Europe (Dhakal, Regmi & Basnyat, 2009).

Citrus canker (Source: Plantix, 2023; Gottwald, Graham, & Schubert, 2002)

2. Primary host range of citrus canker:

Aegle marmelos (bael fruit), Citrus, *Citrus aurantiifolia* (lime), *Citrus aurantium* (sour orange), *Citrus maxima* (pummelo), *Citrus hystrix*, *Citrus junos* (yuzu), *Citrus limon* (lemon), *Citrus limetta* (sweet lemon tree), *Citrus madurensis* (calamondin), *Citrus medica* (citron), *Citrus natsudaidai* (natsudaidai), *Citrus x paradisi* (grapefruit), *Citrus sunki* (sour mandarin), *Citrus reticulata* (mandarin), *Citrus reshni* (cleopatra mandarin), *Citrus sinensis* (navel orange), *Citrus tankan, Citrus unshiu* (satsuma), *Citrus reticulata* x *Poncirus trifoliata* (citrumelo), *Casimiroa edulis* (casimiroa), *Eremocitrus glauca* (Australian desert lime), *Limonia acidissima* (elephant apple), and *Poncirus trifoliata* (Trifoliate orange).

3. General Information

3.1 Location detail

District:

Municipality :	Ward:	Tole:
Elevation:	Longitude:	Latitude:

Orchard owner:

3.2 Crop information

- Age of Orchard:
- Crop (sweet orange, mandarin, or lime)
- Varieties:
- Crop stage: (fruiting or non-fruiting)
- Planting materials: 1. Sapling 2. Seedling
- Source of planting materials: 1. Private nursery 2. Self-raised 3. Government farm 4. Others
- Farm management
  1. Traditional (manuring, irrigation, pest management
  2. Improved: manuring, irrigation, pest management, all
  3. Others (e.g. organic, GAP, IPM) please specify
- No. of tree/orchard: sweet orange: …… mandarin: …….. Lime: …….
- Orchard area:

4. Time of survey

No. of survey: Four times per year

- Flowering stage (February- March):
- Fruiting stage: (May - June):
- Harvesting Stage: (October- November):
- Post-harvest stage: At the time of grading of fruits
- The major symptom of citrus canker infection is the corky lesions that develop on the leaves, stems, shoots and fruit between 7-10 days after infection.
- In severe cases the disease also leads to shoot dieback, defoliation and fruit drop.

5. Appearance of lesions

- The appearance of canker lesions can vary depending on the citrus variety, plant part affected and the age of the lesions.
- Lesions can be irregular in shape and appear atypical if found in association with a wound site or citrus leafminer (*Phyllocnistis citrella*) feeding.
- Lesions can also appear atypical if trees are water stressed through drought or reduced irrigation.

6. Plant part to be observed

- Leaf
- Twigs/branches
- Fruits
- Collateral/alternate hosts: *Sichuan pepper* (timur), *Aegle marmelos* (bael fruit), other citrus species

7. Survey method

- Serpentine/Zig-Zag Path way
- No. of survey orchard: All suspected orchards based on visual symptoms if any. Random 5 % selection of non-suspected orchards/trees.
- No. of tree/orchard : 5% tree of orchard
- In nursery field: 1% saplings/seedling per nursery

8. Sampling method

Leaf - Suspected/infected 5-10 leaves/tree from low to mid parts/strata of the tree, at least 5 leaves per quadrate from quadrate

Twigs - Suspected/infected 5-10 twigs/tree from low to mid parts/strata of the tree.

Fruit- Suspected/infected 5-10 fruits/tree from low to mid parts/strata of the tree.

Post-harvest: Suspected/infected 5-10 fruits/per 5% of lot

Testing method

- Visual symptom (as indicated above)

Dispatch of samples

- Use mailing or A4 size Nepali/paper envelope to dispatch
- Collected samples to be reached in diagnosis laboratory as soon as possible but not later than 3 days
- Leaves should be fresh and transported in cool box

9. Disease diagnostic protocol in the laboratory

Best practice in diagnostics requires that results be obtained by more than one method. The minimum diagnostic process required is:

1. Observation of symptoms consistent with canker
2. Positive reaction of leaf lesions in multiplex PCR (possibly also other reactions) ideally, the same result should be obtained from two different laboratories, for each sample.
3. Other methods also that can also be used to confirm diagnosis are:
   i) Isolation of bacteria with morphological and molecular characteristics consistent

with *Xanthomonas citri* subsp. *citri*

- Examine visual observation in all the collected samples.
- Selection of suspected samples subjected toisolation.
- Examine the presence of bacterial streaming under microscope. Samples which are positive bacterial streaming tests should be proceed for pathogen isolation and identification.

Procedure for bacterial streaming test and isolation of bacterial from field samples of for citrus canker

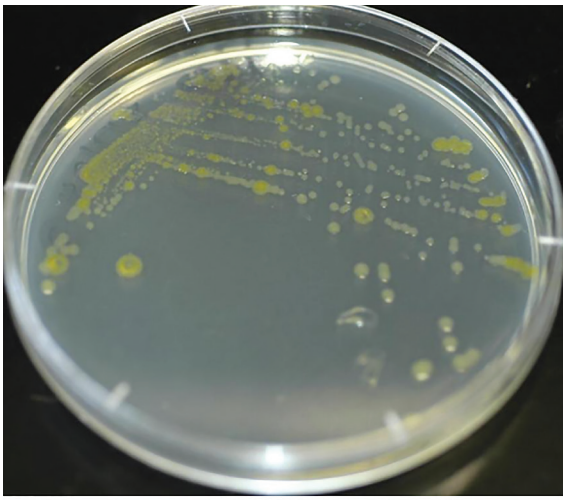
1. Surface sterilize selected material (leaf, stem etc)
   a. Place clean absorbent cloth (tissue, Kimwipe or similar) on bench
   b. Hold plant material above cloth, spray with copious quantities of 70% ethanol, both sides of material, collecting ethanol on cloth
   c. Use cloth to physically wipe the selected lesion dry
   d. Place plant material on sterile slide or in sterile Petri dish
2. Excise selected lesion using sterile scalpel blade (minimize quantity of non-lesion excised, particularly important if sampling fruit).
3. Carefully use sterile scalpel blade to halve and then quarter the excised lesion.
4. Add a drop of sterile 0.85% saline or sterile water using a sterile transfer pipette, place cover slip over top.
5. Examine for bacterial ooze within two minutes of preparing the slide, however, if the lesion is very dry a longer exudation period, up to five minutes, may be necessary.
6. Label agar plate (YDC or NA) with sample number and date.
7. Allow two to five minutes for maximum efflux of bacteria into saline.
8. Use sterile disposable loop to collect small volume of exudate, inoculate agar plate.
9. Use each of four clean sterile disposable loops to serially streak the inoculum on the plate.
10. Incubate plate at 25-28 º C.
11. Check plates for bacterial growth after 5 days and as required thereafter

Storage of bacteria for further study

For short term bacteria can be stored on nutrient agar medium, long-term storage can be done on 10-20% glycerol stock on ultra-low temperature refrigerators

Biochemical tests:

Yellow colonies/ mucoid in YDC: Colonies of *X. citri* subsp. *citri* appear lemony yellow and very mucoid on YDC/NA and have a "sticky" texture when touched with loops etc. Colonies are slow growing (rarely visible before 4 days, more usually 5 or more) and so can be overgrown by other organisms.



Source: https://www.plantbiosecuritydiagnostics.net.au/app/uploads/2018/11/NDP-9-Asiatic-citrus-canker-Xanthomonas-V1.2.pdf

Potassium hydroxide (KOH) test (Grahm test): *Xanthomonas citri* subsp. *Citir* is Gram negative bacteria, a 3% solution of KOH disrupts the outer membrane of Gram-negative bacteria resulting in the cell lysis or bursting indicated by a viscous solution. Gram-positive bacteria lack an outer membrane and therefore the cells remain intact and no viscous solution is produced.

Procedure:

1. Place two drops of 3% KOH on a glass microscope slide.
2. Using a sterile toothpick, pipette tip, or inoculating loop, select a single colony from a culture in the log phase of growth.
3. Gently touch the colony to the KOH and raise and lower the colony.

A positive reaction (viscosity) should be observed after about 30 sec. Conduct test for both controls and your unknown culture.

Gram-negative reaction: The mixture becomes gummy and can be drawn into a string between the loop and the slide.

Gram-positive reaction: The mixture does not become gummy.

Hypersensitive Reaction (HR) Test: Most plant pathogenic bacteria induce a hypersensitive reaction (rapid collapse of cells) in the leaves of *Nicotiana tabacum* (tobacco) plants into which they have been infiltrated intercellularly. However, virulent bacterial pathogens of tobacco do not induce HR in tobacco, and some species do not induce HR in tobacco if grown on nutrient rich medium (*Pantoea stewartii, Pectobacterium carotovorum* and *P. agglomerans pvs. gypsophilae and betae*). The HR test can be used to distinguish

phytopathogenic bacteria from saprophytic bacteria since saprophytes cannot usually induce HR (in tobacco or four o'clock). The HR test, followed by pathogenicity tests, is used when an accurate confidence level is required

Laboratory Procedure:

1. Make a suspension (OD_{600}=0.1 to 0.2) of each bacterial culture. Bacterial cultures should be in their log phase (exponential phase) of growth (24-48 hours old for most genera). DO NOT exceed an $OD6_{00}$=0.2.
2. On a fully emerged upper leaf (or a leaf that has full exposure to light) label your samples using a permanent marker. Label two spots per sample.
3. Fill a 10 cc syringe with inoculum or water and remove any air bubbles.
4. Place the syringe on the underside of the labeled leaf and with the tip pressed against the lower surface of the leaf and your thumb on the upper surface, gently press down on the plunger to infiltrate the inoculum or water. Infiltrate to the size of a dime. The instructor will demonstrate this technique. Before infiltrating the inoculum practice with water.
5. Inoculated plants should receive at least 8 hours of light after inoculation and should be maintained at a temperature between 75-80 ºF with a 12-hour photoperiod.
6. Observe the plants at 24, 48 and 72 hours and record the results at each time interval.
7. At 72 hours, select a positive and negative HR and check for bacterial streaming. Positive reaction: necrosis of host tissue in the infiltrated area within 72 hours. No bacterial streaming.

Negative reaction: green tissue (no response) indicates a non-pathogenic microorganism. Yellow tissue with bacterial streaming indicates the bacterium may be a pathogen of tobacco or 4 o' clock plants.

PCR using bacterial universal primers

Recently, Cubero &Graham (2002) developed improved methods that target a gene involved in virulence (of all citrus canker strains) and a region that is specific for identification of pathotype A strains. These methods are recommended for use in this diagnostic standard in parallel with an "amplification" control that identifies extracts recalcitrant to reaction because of the presence of inhibitors. This eliminates the possibility of "false negative" results. The method reported here has been validated against a wide selection of reference isolates, including the more "atypical" strains (A* and Aw).

DNA can be directly extracted from leaves using phenol/chloroform method or from bacterial colonies using "Soakmethod" or Pick and Swizzle" method or using commercial kits.

Table 1: Primer sets used for the detection of Xanthomonas citri subsp. citri

Target gene	Primer Name	Sequence (5'-3')	Reference
pth-A	DLH 1	TTGGTGTCGTCGCTTGTAT	Hartung et al. (1993)
	DLH 2	CACGGGTGCAAAAAATCT	
16S rDNA	FD1	AGAGTTTGATCCTGGCTCAG	Weisburg et al. (1991)
	rP2	ACGGCTACCTTGTTACGACTT	
pth-A	J-pth 1	CTTCAACTCAAACGCCGGAC	Cubero & Graham, (2002)
	J-pth 2	CATCGCGCTGTTCGGGAG	
Conserved BOX regions	BOX A1R	CTACGGCAAGGCGACGCTGACG	Koeuth et al. (1995)
pth-A	VM3	GCATTTGATGACGCCATGAC	Mavrodieva et al. (2004)
	VM4	TCCCTGATGCCTGGAGGATA	

Procedure

Master Mix for J-pth1&2 primers in a multiplex reaction with 16 SrDNA primers (FD1 and rP2) to screen DNA extracted from leaf lesions

Reagent (Initial concentration)	Volume in each PCR tube (µl)
Forward primer J-pth 1 (10 µM)	0.25
Reverse primer J-pth 2 (10 µM)	0.25
Forward primer FD1 (10 µM)	0.125
Reverse primer rP2 (10 µM)	0.125
Master mix	8.5
DNA template	1
Gene releaser	10
Nuclease free water	6
Total volume	25

PCR conditions

Target PCR	Initial denaturation (1 cycle only)	Denaturation	Annealing	Extension	Number of cycle	Final Extension (1 cycle only)	Fragment length
Multiplex	94/5 min	94/30"	64-60/30" Touchdown	72/45"	5	72/5 min	16 s rDNA 1500 bp, pthA 197 bp
		Then 94/30"	60/30"	72/45"	35		
J-pth	94/5 min	94/30"	58/30"	72/ 1 min	30	72/5 mins	pthA 197bp

Additional information on survey format

- Scientific name of host
- Common name of host (Include detail of cultivar/variety if known)
- Suspected pathogen fungi/bacteria/virus/nematodes
- Description of symptom observed in the field
- Local/common name of disease
- Date of survey
- Name of surveyor
- Designation
- Name of verifier
- Designation
- Signature

Literature cited:

Cubero, J., & Graham, J. H. (2002). Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Applied and environmental microbiology*,*68*(3), 1257-1264.

Dhakal, D., Regmi, C., & Basnyat, S. R. (2009). Etiology and control of citrus canker disease in Kavre. *Nepal Journal of Science and Technology*, *10*, 57-61.

Gottwald, T. R., Graham, J. H., and Schubert, T. S. (2002). Citrus canker: The pathogen and its impact. Online. Plant Health Progress doi:10.1094/PHP-2002-0812-01-RV. https://www.apsnet.org/edcenter/apsnetfeatures/Pages/CitrusCanker.aspx

https://www.plantbiosecuritydiagnostics.net.au/app/uploads/2018/11/NDP-9-Asiatic-citrus-canker-Xanthomonas-V1.2.pdf

Plantix. (2023). Citrus canker. https://plantix.net/en/library/plant-diseases/300020/citrus-canker

Schaad, N. W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I. V., Stromberg, P. E., & Vidaver, A. M. (2006). Emended classification of xanthomonad pathogens on citrus. *Papers in Plant Pathology*, 96.

Schubert, T. S., Rizvi, S. A., Sun, X., Gottwald, T. R., Graham, J. H., & Dixon, W. N. (2001). Meeting the challenge of eradicating citrus canker in Florida—again. *Plant disease*, *85*(4), 340-356.

Annexes

Annex 1. Data Collection Form

Field Datasheet		
Orchard General Information		
A Name of Field/Site visited:		
B. Citrus pocket		
C. Age of the orchard		
D. Kind of orchard :	Seedling / Grafted	
E. Relief of the orchard:	Valley / Terrace / Slope	
F. Aspect/Facing of orchard	North / South / East / West	
G. Total number of trees planted in the orchard :		
H. Total number of trees in the orchard present		
Trees died in last five years		
J. Saplings supplied by:	DAO / NGO / Local market / produced by own	
K. Management practices applied by the farmers :		
Pruning and cutting of dry or diseased branches :	Yes / No	
II. Application of plant protection chemicals :	Yes / No *If yes: What:…… Dose:…….. Time:……*	
III. Use of manures and chemical fertilizers:	Yes / No *If yes, FYM or compost:……Urea: ……. / tree, DAP: ……./ tree, Potash :……/ tree*	
IV. Intercrop grown :	Maize / Millet / vegetables / Other crops like……/ no intercropping	
V. Mulching :	Practiced / Not practiced	
VI. Irrigation :	Applied / Not applied	

Field Datasheet		
L. General appearance of the orchard and tree health :		
Number of plants showing declined stage :	(Early stage of decline : <25 % , Medium decline: 25-50 %, Severe decline : > 50 %	
No of plants that are in bearing stage		
III. Fruit yield / tree :		
2. Date/Time of Visit:		
3. GPS Reference Point	Latitude:	
	Longitude:	
	Altitude:	
4. Locality:	Village & ward:	
	VDC:	
	District:	
5. Climate Data of Locality:	Average Min. temp (in °C):	
	Average Max. temp (in °C):	
	Rainfall (in mm)	
6. Survey/Field plot No.		
7. Host Plant species/Variety inspected		
7.1 Description of habitat (e.g., vegetation, soil type and depth	Sandy.... to .....clay, 1 meter / 1-3 M / more than 3 M	
7.2 Alternate Host Plant species/Variety inspected		
8. Phenological Stage of the plant	Flushing, fruiting, flowering	
8.1 Main host		
8.2 Alternate host including vectors		
9. Sampling method	Full Sampling	
10. Contact detail of local people involved in the survey	11. Details of pest recorded	
Plant parts affected:	[ ] Leaves; [ ] Buds, [ ] Fruits; [ ] Seed ; and [ ] Whole plant	
Stage of plant:	[ ] Seedling stage; [ ] Vegetative Growth stage; [ ] Flowering stage; and [ ] Fruiting stage	

Field Datasheet	
Locality Village VDC District Province/State:	

Annex 2. Information to be filled by surveyor/ collector

Scientific Name	Common Name	Taxonomic position	Time	Plant parts affected	Symptom & Sign)	Intensity		
						Severity	Intensity	Prevalence
Xanthomonascitri subsp. *citri*	Asiatic citrus canker, citrus canker, citrus bacterial canker, asiatic canker	Bacteria, Proteobacteria, Gammaproteobacteria, Xanthomonadales, Xanthomonadaceae						

Annex 3. Format for specimen forwardal

Specimen Forwarded for identification by Diagnostic/Referral Laboratory	
1. Collection Number:	2. Date of Collection:
3. Submitting Organization:	
4. Name/Address of the Sender:	
5. Locality of Collection (District/VDC/ village):	
6. Reasons for identification:	
7. Name of the host plant species (Common/ Scientific)/variety and or/ commodity:	
8. Origin of host/commodity (where applicable):	
9. Plant Parts affected:	leaves; [ ] fruits; [ ] seeds [ ] others (_____) *tick out in appropriate box
10. Category of pest specimen/organism submitted	bacteria; [ ] others (_____)
12. Type of pest specimen/organism submitted	*[ ] preserved specimen; [ ] dry specimen with host; [ ] culture; [ ] disease specimen (fresh); [ ] disease specimen (partially dry); [ ] slide mount; [ ] others (_____)
14. Number of specimens submitted per each collection:	
15. Signature/stamp/office seal of the Sender with date: _____	
For Identifier Use	
16. Name & Address of Diagnostic/Referral Laboratory:	
17. Remarks of identifier (condition of receipt of specimens)	

18. Pest Identification (Common/Scientific Name/Taxon):
19. Description Notes, if any:
Place: _____ Date: _____ _____ (Signature/Name/Designation of Identifier)
Note: This form should be prepared in duplicate by the sender and forwarded to the identifier/referral laboratory along with each collection of specimen. The identifier should return the original copy after entering the particulars of the pest identified along with description notes and remarks if the identifier will retain any to the sender of the specimen and duplicate copy.

Annex 4. Pest record datasheet

Pest Record	
Reference Number	Name of laboratory, address, catalogue reference number of the specimen in the pest library etc.
Scientific name of pest	
Common Name:	
Growth habit:	
Habitat characters:	
Species Name:	
Family	
Order:	
Life stage of pest	Actively dividing or dormant spore, etc
Scientific Name of host Variety Common Name: Species Name Family	

Survey Protocol for Citrus greening (HLB)

NPPO Nepal endorsement on 6th May, 2013
First amendment on 17th May, 2023

1. Background Information

Citrus greening disease is one of the prominent reasons of citrus decline in Nepal (Budhathoki & Pradhanag, 1990). Citrus greening is mainly associated with three species of gramnegative, unculturable, and phloem-restricted bacteria: *Candidatus Liberibacter asiaticus, Candidatus Liberibacter africanus*, and *Candidatus Liberibacter americanus*. Among which *Candidatus Liberibacter asiaticus*is the most common species distributed all over Asia including Nepal (Paudyal, 2015). The main vector of this diseases is Asian citrus Psyllid, *Diaphorina citri*. This hemipteran insect damages the plant by sucking the sap from the foliage and excreting sugary liquid that covers the leaf with honeydew which then gets covered by sooty mold. The symptoms of the diseases can be seen at leaves and shoot, roots and symptoms on fruits and its juice quality. Mottling of leaves, corky branches and yellow shoots are pre-dominantly seen symptoms. Along with these premature fruit drop, deformed fruit sized and off flavored juice are other important symptoms to look after (McCollum & Baldwin, 2017).The pathogen colonizes in roots before showing symptoms infecting leaves (Johnson *et al*., 2014).As there is no remedial therapy available for the HLB disease, it is important to reduce the transmission of the disease by controlling its vector (Yan *et al*., 2015), using safe planning materials and removal of infected trees (Bove, 2006). Taxonomic tree of the pathogen is presented below (CABI, 2022).

Domain: Bacteria
Phylum: Proteobacteria
Class: Alphaproteobacteria
Order: Rhizobiales
Family: Phyllobacteriaceae
Genus: *Candidatus Liberibacter*
Species: *Candidatus Liberibacter asiaticus*

1.2 Primary host range of citrus greening:

Aegle marmelos (bael fruit), Citrus, *Citrus aurantiifolia* (lime), *Citrus aurantium* (sour orange), *Citrus maxima* (pummelo), *Citrus hystrix*, *Citrus* junos (yuzu), *Citrus* limon (lemon), *Citrus* limetta (sweet lemon tree), *Citrus madurensis* (calamondin), *Citrus medica* (citron), *Citrus natsudaidai* (natsudaidai), *Citrus x paradisi* (grapefruit), *Citrus sunki* (sour mandarin), *Citrus reticulata* (mandarin), *Citrus reshni* (cleopatra mandarin), *Citrus sinensis* (navel orange), *Citrus tankan*, *Citrus unshiu* (satsuma), *Citrus reticulata*

x *Poncirus trifoliate* (citrumelo), *Casimiroa edulis* (casimiroa), *Eremocitrus glauca* (Australian desert lime), *Limonia acidissima* (elephant apple), and *Poncirus trifoliata* (Trifoliate orange).

3. Mode of dispersion

In the 1960s, citrus HLB was shown to be transmitted by two insects: the African citrus Psyllid, *Trioza erytreae*, in Africa (Mcclean & Oberholzer, 1965) and the Asian citrus Psyllid, *Diaphorina citri*, in Asia (Capoor *et al*., 1967). Experimentally, both species of Psyllid have been shown to transmit both '*Ca. L. asiaticus*' and '*Ca. L. africanus*' (Massonie *et al*., 1976; Lallemand *et al*., 1986). The bacteria are transmitted by Psyllid as they feed. Psyllid nymphs are more efficient in '*Ca. L. asiaticus*' acquisition than adults, and adult D. citri acquiring '*Ca. L. asiaticus*' as nymphs are more efficient in transmission than adults that acquired '*Ca. L. asiaticus*' as adults (Pelz-Stelinski *et al*., 2010). Vertical transmission (transovarial) allows '*Ca. L. asiaticus*' to be transmitted from female *D. citri* to their offspring in low amounts (Kelley &Pelz-Stelinski, 2019). In addition, '*Ca. L. asiaticus*' can be sexually transmitted from infected male *D. citri* to uninfected females at a low rate (<4%) during mating

4. General aspect of the tree

At the beginning leaf mottling (yellowing) is seen on single branch which gradually spreads out to other branches. Thus, slightly infected tree becomes severely affected with symptoms like open growth, stunting, twig die back, sparse foliage and severe leaf and fruit drop

The whole orchard declines with 2-3 years

Symptoms on Leaves

- Leaves are reduced in shape and size
- Leaf mottling and specific mosaic symptoms are common
- Matured leaves often show irregular patches between the main veins
- Sometimes vein corking is also observed
- The veins are often prominent and yellow
- Excessive leaf drop and unseasonal flushing with very small but erect type of leaves are developed

Symptoms on fruits

- Fruits are underdeveloped and reduced in size with lopsided shape
- Most of them remain green or poorly colored even after maturity specially at the rind part
- The side exposed to sun light shows normal color and the remaining part shows dull olive color (This symptom is rare in Nepal)

5. Time of Survey: October-December (Two times)

6. Selection of Survey Area

As per the requirement of NPPO
(To begin with -Citrus pockets of Syanja and Sindhuli)

7. Number of Trees for Observation

All the trees of the orchard to find out the suspected symptoms of CGD (HLB).Blitz survey or full sampling survey

8. Parts to be Observed-

- Leaves
- twigs
- fruits

9. Sample Collection Procedure and Preservation

- Identify moderately or severely declined trees
- Tag the tree with the serial number of your sample and write the same number on the standard sample collection poly bag.
- Look at the part of the tree with leaves having HLB Symptoms (mottling, asymmetric mosaic)
- Collect 15-20 leaves with HLB symptoms but without insect damage from the 5-6 month old twigs. (collectatleast 5 leaves per quadrate)
- Put all the leaves in the plastic bag , be sure that there is no wet leaf, if there are wet leaves dry them with tissue paper
- Arrange all the leaves inside the poly bag in such a way that leaves do not overlap to each other
- Close the bag properly
- Put the bag with sample in ice box immediately, if possible
- If it is not possible put them in shaded and cool place
- Prepare packs of 20 such bags

- Prepare the list of samples with sample number, date of sample collection, name and address of owner, citrus species, age of the tree and type of origin (grafted or seedling) and any special feature of the tree and other details specified by the laboratory
- Prepare a covering letter to the laboratory requesting for HLB testing the list of samples prepared as above
- Dispatch the packs of samples to the laboratory by faster means of transport so that it reaches the laboratory within the seven days of sample collection
- Maintain your diary writing all these operations with dates and all other details
- Mark the trees with HLB positive immediately after receiving the test results.
- Zip lock bag with QR code might be helpful.

10. Diagnostic Laboratory

The standard laboratory procedures should be followed.

11. Sampling Procedure Flow Chart for HLB detection

SN	Sample type	Sample size and methods
1	Suspected prominent visual symptoms on tree	■ All samples 100% Iodine Scratch test (IST) ■ ALL 100% IST positive samples for PCR testing ■ 5 % negative samples of IST test for PCR testing
2	Suspected but less visual symptoms of HLB	■ All samples for IST ■ All 100 IST positive samples to PCR test ■ 2% of negative samples for PCR test
3	No visual symptoms	■ 1% of tree IST test ■ All positive samples for PCR test ■ 0.01% of negative samples for PCR test

Literature cited:

Bové, J. M. (2006). Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of plant pathology*, 7-37.

Budathoki, K., & Pradhanang, P. M. (1990). Production constraints of mandarin in western hills of Nepal. *Recent Advances in Horticultural Science in the Tropics 292*, 51-60.

CABI. (2022). *Candidatus Liberibacter asiaticus* (citrus greening). Retrieved from: https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.16565

Capoor, S. P., Rao, D. G., & Viswanath, S. M. (1967). *DiaphorinacitriKuwait*., a vector of the greening disease of citrus in India. Indian J. Agric. Sci, 37(572.576), 1382-1382.

Johnson, E.G., Wu, J., Bright, D.B. & Graham, J.H. (2014). Association of '*CandidatusLiberibacterasiaticus*' root infection, but not phloem plugging with root loss on huanglongbing- affected trees prior to appearance of foliar symptoms. Plant pathology 63: 290-298. DOI: 10.1111/ppa.12109

Massonie, G., Garnier, M., &Bové, J. M. (1976). Transmission of Indian citrus decline by *Triozaerytreae* (Del Guercio), the vector of South African greening. In *International Organization of Citrus Virologists Conference Proceedings (1957-2010)* (Vol. 7, No. 7).

Mann, R. S., Pelz-Stelinski, K., Hermann, S. L., Tiwari, S., & Stelinski, L. L. (2011). Sexual transmission of a plant pathogenic bacterium, *CandidatusLiberibacterasiaticus*, between conspecific insect vectors during mating. *PLoS One*, *6*(12), e29197.

McClean, APD & Oberholzer, P. C. J. (1965). Citrus psylla, a vector of the greening disease of sweet orange-research note. *South African Journal of Agricultural Science*, *8*(1), 297-298.

McCollum, G. & Baldwin, E. (2017). Huanglongbing: devastating disease of citrus. Horticultural Reviews 44: 315-361. DOI: 10.1002/9781119281269.ch7

Paudyal KP (2015) Techological advances in Huanglongbing (HLB) or citrus greening disease management. Journal of Nepal Agricultural Research Council 1: 41-50. DOI:10.3126/jnarc.v1i0.15735

Pelz-Stelinski, K. S., Brlansky, R. H., Ebert, T. A., & Rogers, M. E. (2010). Transmission parameters for *CandidatusLiberibacterasiaticus* by Asian citrus Psyllid (Hemiptera: Psyllidae). *Journal of economic entomology*, *103*(5), 1531-1541.

Timilsina, K. (2019). Citrus greening: Nepal's groves under threat. The Himalayan Times. https://thehimalayantimes.com/opinion/citrus-greening-nepals-groves-under-threat

Yan H., Zeng, J. & Zhong, G. (2015). The push–pull strategy for citrus Psyllid control. Pest Management Science 71 : 893- 896. DOI: 10.1002/ps.391

Annexes

Annex 1. Data collection form

Field Datasheet		
Orchard General Information		
A Name of Field/Site visited:		
B. Citrus pocket		
C. Age of the orchard		
D.Kind of orchard :	Seedling / Grafted	
E.Relief of the orchard:	Valley / Terrace / Slope	
F. Aspect/Facing of orchard	North / South / East / West	
G. Total number of trees planted in the orchard :		
H. Total number of trees in the orchard present		
Trees died in last five years		
J. Saplings supplied by:	DAO / NGO / Local market / produced by own	
K. Management practices applied by the farmers :		
Pruning and cutting of dry or diseased branches :	Yes / No	
II. Application of plant protection chemicals :	Yes / No If yes: What:…… Dose:…….. Time:…….	
III. Use of manures and chemical fertilizers:	Yes / No If yes, FYM or compost:……Urea: ……. / tree, DAP: ……./ tree, Potash :……/ tree	
IV. Intercrop grown :	Maize / Millet / vegetables / Other crops like……/ no intercropping	
V. Mulching:	Practiced / Not practiced	

Field Datasheet		
VI.Irrigation :	Applied / Not applied	
L. General appearance of the orchard and tree health :		
Number of plants showing declined stage :	(Early stage of decline : <25 % , Medium decline: 25-50 %, Severe decline : > 50 %	
No of plants that are in bearing stage		
III. Fruit yield / tree :		
2. Date/Time of Visit:		
3. GPS Reference Point	Latitude:	
	Longitude:	
	Altitude:	
4. Locality:	Village & ward:	
	VDC:	
	District:	
5. Climate Data of Locality:	Average Min. temp (in °C):	
	Average Max. temp (in °C):	
	Rainfall (in mm)	
6. Survey/Field plot No.		
7. Host Plant species/Variety inspected		
7.1 Description of habitat (e.g., vegetation, soil type and depth	Sandy.... to .....clay, 1 meter / 1-3 M / more than 3 M	
7.2 Alternate Host Plant species/Variety inspected		
8. Phenological Stage of the plant	Flushing, fruiting, flowering	
8.1 Main host		
8.2 Alternate host including vectors		
9. Sampling method	Full Sampling	
10. Contact detail of local people involved in the survey	11. Details of pest recorded	

S N	Scientific Name	Common Name	Category	Order	Family	Life Stages	Time	Plant parts affected	Symptom & Sign)	Behavioural notes	Intensity
1	CLA	HLB	Bacteria	..	...	...					
10. Any additional information (including collection of specimens for investigation):											
11. Name/Signature of surveyor with Date:											

Annex 2. Format for specimen forward

Specimen Forwarded for identification by Diagnostic/Referral Laboratory	
1. Collection Number:	2. Date of Collection:
3. Submitting Organization:	
4. Name/Address of the Sender:	
5. Locality of Collection (District/VDC/village):	
6. Reasons for identification:	
7. Name of the host plant species (Common/ Scientific)/variety and or/ commodity:	
8. Origin of host/commodity (where applicable):	
9. Plant Parts affected:	leaves; [ ] fruits; [ ] seeds [ ] others (_____) *tick out in appropriate box
10. Category of pest specimen/organism submitted	bacteria; [ ] others (_____)
12. Type of pest specimen/organism submitted	*[ ] preserved specimen; [ ] dry specimen with host; [ ] culture; [ ] disease specimen (fresh); [ ] disease specimen (partially dry); [ ] slide mount; [ ] others (_____)

14. Number of specimens submitted per each collection:	
15. Signature/stamp/office seal of the Sender with date:	
For Identifier Use	
16. Name & Address of Diagnostic/Referral Laboratory:	
17. Remarks of identifier (condition of receipt of specimens)	
18. Pest Identification (Common/Scientific Name/Taxon):	
19. Description Notes, if any:	
Place: _____ Date: _____ _____ (Signature/Name/Designation of Identifier)	
Note: This form should be prepared in duplicate by the sender and forwarded to the identifier/referral laboratory along with each collection of specimen. The identifier should return the original copy after entering the particulars of the pest identified along with description notes and remarks if the identifier will retain any to the sender of the specimen and duplicate copy.	

Annex 3. Pest record datasheet. (to forward from PQPMC)

Pest Record	
Reference Number	Name of laboratory, address, catalogue reference number of the specimen in the pest library etc.
Scientific name of pest	
Common Name:	
Growth habit:	
Habitat characters:	
Species Name:	
Family	
Order:	
Life stage of pest	Actively dividing or dormant spore, etc
Scientific Name of host	
Variety	
Common Name:	
Species Name	
Family	
Plant parts affected:	[ ] Leaves; [ ] Buds, [ ] Fruits; [ ] Seed ; and [ ] Whole plant
Stage of plant:	[ ] Seedling stage; [ ] Vegetative Growth stage; [ ] Flowering stage; and [ ] Fruiting stage
Locality	
Village	
VDC	
District	
Province/State:	
Date , time and GPS location of pest Collection	
Method of collection	
Name of the Collector	
Species accumulation curve	
Method of Identification	
Name of the Identifier	
Date of Verification	
Method of verification	
Name of the Verifier	

Pest Record	
Method of pest preservation (detail)	
Suitable for:	Taxonomic analysis or DNA analysis
Life stage of pest affecting alternate host	Actively dividing or dormant spore, etc
Scientific Name of alt. host Common Name: Species Name Family	
Alt. host. Plant parts affected:	[ ] Leaves; [ ] Stem; [ ] Roots; [ ] Buds/Flowers; [ ] Fruits; [ ] Seed ; and [ ] Whole plant
Stage of alt. host plant:	[ ] Seedling stage; [ ] Vegetative Growth stage; [ ] Flowering stage; and [ ] Fruiting stage
Locality, Village, VDC, District, Province/ State:	

Survey Protocol for Asian Citrus Psyllid (ACP)

NPPO Nepa*l endorsement on 6th May, 2013*
First amendment on 17th May, 2023

1. Background information

Asian Citrus Psyllid (ACP), *Diaphorina citri* Kuwayama is one of the notorious pests of citrus fruit in the country which helps in the transmission of phloem-limited bacterium *Candidatus Liberibacter asiaticus* associated with economically devastating citrus greening disease (Bist & Bista, 2020). This Hemipteran insect is primarily linked to plant species belonging to the Rutacea family (Singerman & Rogers, 2019). Both nymphs and adults suck the cell sap from newly emerged leaves, tender shoots and flowers causing curling of leaves and defoliation leading to de-blossoming and dieback. Psyllid is also known to inject toxin in plant due to which die-back of shoot occurs (Nath & Sikha, 2019) but its significance lies in the fact that it is responsible for causing the most economically important disease of citrus i.e. Huanglongbing disease. The ACP inflicts direct harm on citrus trees by not only feeding but also by secreting honeydew which attracts the growth of fungus, adversely affecting the photosynthesis. A well designed survey protocol for ACP is crucial for early detection, effective pest management, regulatory compliance, and scientific research, ultimately aiding in the protection of citrus crops and the prevention of citrus greening disease.

Citrus psyllid

Adult citrus psyllid

Infestation caused
(Source: Food Forward, 2023)

Taxonomic tree of insect:

Domain:Eukaryota
Kingdom: Metazoa
Phylum: Arthropoda
Subphylum: Uniramia

Class: Insecta
Order: Hemiptera
Suborder: Sternorrhyncha
Super family: Psylloidea
Family: Liviidae
Genus: *Diaphorina*
Species: *citri*

2. Surveillance purpose

In citrus growing nations where ACP has not yet been introduced, it is regarded as quarantine pest. Early detection of ACP is vital to prevent economic losses, implement effective management strategies of the pest and ensure food security within the country.

3. Scope

For the purpose of performing detection and management of Asian citrus Psyllid, the survey surveillance program of ACP safeguard citrus orchards, prevent yield loss, thereby protecting the livelihood of the farmers relying on citrus cultivation.

4. Justification for surveillance

Through survey and surveillance of Asian citrus Psyllid, it is easier to monitor its presence and population levels, to find out the potential risk of devastating Huanglongbing disease (HLB) transmission in citrus and take timely actions to prevent its introduction or minimize its impact if detected.

5. Insect biology

The Asian citrus Psyllid is a multi-voltine species, possesses a high reproductive capacity and have a short life cycle. With the availability of young and tender foliage, adult female insects can lay between 500 to 800 eggs throughout their lifespan (Nava *et al.,* 2007). Eggs have an oval shape and are transparent or slightly yellow when newly deposited. However, as they mature, they develop a vibrant yellow-orange color and display two noticeable red eyes marking. Eggs are deposited on the terminal growth of emerging plant tissue, leaf folds, petioles and younger leaves and stems and at 24ºC adult male and female survived for approximately 21-25 days and 31-32 days respectively (Tsai and Liu, 2000). The ACP undergoes five nymphal stages, with the younger instars being relatively stationary, while the older instars become increasingly mobile (Tsai & Liu, 2000). Adults are around 2-4 mm in length and typically have an abdomen that is yellowish brown, although variations in color such as greenish brown or pinkish brown can be observed (Martini *et al.*, 2014). During the feeding process, adults position themselves at an approximate angle of 40 degrees in relation to the surface of the plant tissues

(Ammar *et al.,* 2013). This insect has a preference for young citrus trees, as they offer a more extended and frequent growing period, making them the favored host (Croxton & Stansly, 2014).

7. Symptoms

S. N.	Signs and Symptoms
1	Plants/Fruit/abnormal shape
2	Plants/Growing point/die back
3	Plants/Growing point/distortion
4	Plants/Leaves/abnormal forms
5	Plants/Leaves/abnormal leaf fall
6	Plants/Leaves/honeydew or sooty mould

Adult, Asian citrus psyllid, *Diaphorina citri*

Eggs of Asian citrus psyllid

The five nymphal instars of the Asian citrus Psyllid

(Source: Hall, 2020)

8. Design of survey program

The survey program of Asian citrus Psyllid incorporates multiple sampling techniques and comprehensive methodology to ensure the collection and preservation of the collected samples.

8.1 Time of observation

Citrus tree phenology during February-March, April, May, June and July-August is the appropriate phenomenal seasons to observe Asian citrus Psyllid (ACP) activities including its life cycle. The population fluctuation of Psyllid breeding on citrus are closely correlated with flushing rhythm, because eggs are laid exclusively on young flush points and nymphs develop on immature leaves (Hall, 2008). The heavy and prolong flushing of young trees makes them very attractive to the vector; this partly explains the rapid spread of huanglongbing (HLB) in replanted citrus groves. Moreover, peak movements of ACPs appear to occur following the spring flush of citrus foliage.

In the citrus crop cycle,

- Mid-February to mid-March (Baisakh) remains the critical period for spring flush and flower development.
- Mid-March to mid-April (Chaitra) remains the period of flowering and fruit settings.
- Mid-April to mid-May (Baisakh) remains developing fruits at soybean-grain-size during first week of Baisakh, and initiating summer flush development.
- Mid-May to mid-June (Jestha) remains developing fruits at about marble size (10 mm in diameter), and expanding summer flush during third week of Jestha.
- Mid-June to mid-July (Ashadh) remains mandarin fruits increased in size; greater than a marble but lesser than a lemon size. Sweet orange fruits remain of lemon size in structure. Rainy flush may start in trees.
- Mid-July to mid-August (Shrawan) remain expanding in size, and mandarin fruits attaining at lemon size and sweet orange fruits at oval (egg-like) size.

8.2 Selection of trees for observation

Citrus trees with spring and rainy flushes should be selected for ACP observations typically during April, May, June and July. Relevant causes to select citrus trees in these months are illustrated in the above section of the text.

8.3 Number of sample trees under observation

For the purpose of sampling, the field in each location should be divided into five areas each of 10 x 2 m in dimension. At weekly intervals, one shoots (about 6-10 cm long) should be selected at random from each square meter area by throwing a pointed object. Thus a total of 20 shoots needed to select from each of the 5 areas on each sampling

date. Numbers of citrus Psyllid adults per shoot should be counted and recorded.

- Five places in an orchard
- Two trees in one place.
- Thus, 10 trees in an orchard.

Psyllid tend to be found along edges, and thus it is acceptable to focus sampling efforts on the edges of orchards. Adults can be found along the mid rib on the underside of leaves. Collected Diaphorina citri samples must be diagnosed for bacterial contamination through PCR process. On the basis of bacterial contamination, further delimitation process need to be adopted.

- Status of alternate host of *Diaphorina citri* must be included on the detection process.
- Almost all citrus cultivars are potential hosts of the Asian citrus Psyllid and are able to be infected by HLB. Some species and varieties are better hosts of the Psyllid than others. *Murraya spp.* (native and ornamental forms of mock orange/orange jasmine) and *Bergera koenigii* (curry leaf) are favored hosts. (Biosecurity Qld fact sheet).
- Every detection survey point – meteorological data throughout the citrus phenological stages should be collected.
- GPS coordinates along with the altitude of the survey location need to be recorded.

8.4 Part/s of a tree under observation

- Shoots each length ranging from 6 to 10 cm in twigs.
- Ten shoots in one tree. Thus, 20 shoot in one place of the orchard.
- Altogether 100 shoots needed to observe for ACPs in the orchard (5 places included in the orchard mentioned earlier)

8.5 Frequencies of observations

- Weekly interval

9. Ways to collecting sample insect specimens

- Observe the sample shoot and aspirate adult ACPs by means of a mouth aspirator for 5-man-minute into the glass container in built with aspirator.
- Using a pipette, ACP should be transferred into a vial containing 95 percent non-denatured ethanol.
- ACP nymphs should be collected with a small paint brush or forceps and placed in a vial with 95 percent non-denatured ethanol.
- Adult ACP and nymphs should be placed in separate vials.
- All the vials should be kept in a zippered plastic bag.
- Count the number of ACPs and print it in a data record sheet.

9.1 Sweep net sampling

- Sweep net sampling is effective only for adults.
- Stuff a citrus branch into a large sweep net and shake vigorously.
- Examine the contents of the bag for adult Psyllid.

9.2 Sticky trap

- Sticky trap can be used especially for trapping in the hot spots.
- Yellow sticky trap cards may are used to trap and detect adult Psyllid at a rate of one trap per 400 meters (6 per square km).
- Trap Servicing Interval: Monthly
- Trap Relocation and Replacement: Traps should be relocated and replaced every four to eight weeks to another host with a minimum relocation distance of 500 feet.

9.3 Stem tapping method for sampling

9.3.1 Required materials for Tap Sampling

- A white clipboard or plastic covered sheet of paper with a grid to help count Psyllid quickly
- Squirt of detergent mixed with 500 ml of water
- 300 mm foot section of PVC pipe or other device to beat branches

Instructions

- Mist the detergent solution onto the clipboard to hold the Psyllid in place
- Hold the clipboard 300 mm below a branch and strike the branch 3 times with the PVC pipe
- Count and record the number of winged Psyllid (adults) collected on the clipboard
- Scrape the Psyllid off the clipboard and reapply detergent solution if needed

10. Ways to preserving collected insect specimens

ACPs sample specimens can be preserved in 1) Dry method and 2) Wet method

10.1 Dry preservation method: Knock down the collected insects putting inside a polyethylene bag of 1 kg capacity contained with ethyl acetate imbibed speck of cotton. Some of the dead insects individually pinned in micro-pin at a proper height and thus prepared specimens individually mounted on a thick paper piece and the latter is pinned with collection data described in a piece of paper and placed in the display box for future process of its identification. Mentions made in collection data are:

a. Collector's name
b. Host name
c. Location name

d. Collection date

Some of the specimens individually can be glued with quick-fix material on a piece of paper and the latter can be pinned and placed in the display box with its collection data. Pinning insect specimens should be performed while insects are fresh. Dry insects are relaxed first into a humidity container for some hours before pinning.

Wet preservation method:

The knock down insects are transferred into a glass vial (5-10 ml) with 70-75% alcohol and collection data (written in a piece of paper by soft pencil) should be attached. Each vial should be screw capped.

11. Ways to packing insect specimens

Packing insect specimens to dispatch them to a national expert and or international expert for their authentic identification or verification of their identity needs special insect care while packing. Prior consent of the national and international insect taxonomist is matter of great concern before dispatching the insects for identification. Some customary insect packing should observe the following care-takings.

11.1 Dry preserved insect specimen packing management

Pin mounted insect specimens should be deeply pinned into the thermocole base on the bottom of wooden or stiff cardboard box. Each specimen should bear with collection data (mentioned above) and museum code (Hem., Heter., Psyl., Ent. Bio. #x) developed in the country of origin.

- Maintain ample space between pinned insects to avoid collision while transporting.
- The outer surface of stout closer piece of the box must bear addresses of the sender and receiver parties.
- Packing wrapper with destination address must be durable in nature so that it should remain intact until it reaches the destination.
- Proper documents concerning to custom clearance and legal document for the quarantine clearance sent by the institute of the international insect taxonomist must be accompanied with the insect packing.

11.2 Wet preserved insect specimen packing management

- Wet preserved insect specimens in glass vials (5-10 ml) with collection data should be put inside a wooden or stiff cardboard box.
- Each vial must be thickly wrapped with tissue paper to avoid damage in collision while transporting the package.
- Other requirements are as similar to dry preservation.

12. Collection and Preservation Materials

i. Yellow sticky trap
ii. Aspirators
iii. Insect collection kit:
Collection bottles (plastics), glass vials with tight cap, killing jar, killing agent (ethyl acetate), forceps, scalpel, camel hair brush, lead pencil, insect pins, cotton rolls
iv. Insect sweeping net
v. PVC pipe
vi. Hand-held pressure sprayer
vii. Insect Pinning block
viii. Insect spreading board
ix. Entomological needle and camel hair brush
x. Magnifier (10X-20X lens)
xi. Microscope (dissecting)
xii. Absolute alcohol (ethanol)
xiii. Relaxing jar
xiv. Tissue paper
xv. Scissors
xvi. Secateurs
xvii. Data observation sheet
xviii. White paper sheet, Color photo sheets
xix. Gel pen
xx. Labels (acid free card stock)
xxi. Permanent black ink pen
xxii. Insect rearing cage
xxiii. Insect rearing net-cage
xxiv. Zippered plastic bag
xxv. Wooden or stiff cardboard box.
xxvi. Insect display box, container etc.

13. Place and persons for insect specimen diagnosis

National Institutes

- National Entomology Research Center, NARI, NARC, Khumaltar, Lalitpur.
- Plant Quarantine and Pesticide Management Centre, Hariharbhawan, Lalitpur.
- Central Agriculture Laboratory, Hariharbhawan, Lalitpur.
- Natural History Museum, Tribhuvan University, Swayambhu, Kathmandu.
- Department of Entomology, AFU, Rampur, Chitwan.
- Department of Entomology, TU/IAAS, Kirtipur.

International Institutes

- British Museum (Natural History), Cromwell Rd, London SW7 5BD, United Kingdom
- Commonwealth Agriculture Bureau International, Nosworthy Way, Wallingford, Oxfordshire OX10 8DE, UK
- CSIRO, Canberra, Australia;
- East West University, Hawaii, USA;
- ICAR-NBAIR, Bengaluru
- Department of Entomology, Indian Agriculture Research Institute, New Delhi
- Indian Institute of Horticulture Research, Hassergatta, Bengaluru, India
- Central Institute for Sub-Tropical Horticulture Research, Lucknow
- Molecular diagnosis: ICAR-NBAIR, Bengaluru, India

Literature cited:

Ammar, E.D., Hall, D.G., Shatters, R.G., Jr. (2013). Stylet morphometrics and citrus leaf vein structure in relation to feeding behavior of the Asian citrus Psyllid *Diaphorina citri*, vector of citrus huanglongbing bacterium. PLoS ONE, Volume 8(3) e59914.

Asian citrus Psyllid - *Diaphorina citri* Kuwayama (ufl.edu)

Bist, S. & Bista, S. (2020). A Review On: Citrus Greening, Its History, Symptoms and Management Tactics. *Tropical Agrobiodiversity (TRAB)*, Volume 1, Issue 1. pp. 92-94.

Croxton, S.D., Stansly, P.A. (2014). Metalized polyethylene mulch to repel Asian citrus Psyllid, slow spread of huanglongbing and improve growth of new citrus plantings. Pest Management Science, Volume 70(2), pp. 318-323.

Hall, D.G. (2008). Biology, history and world status of *Diaphorina citri*. In Proceedings of the Taller Internacioinal Sobre Huanglongbing y el Psilido Asiatico de los Citricos, 7-9 May 2008. Hermosillo, Sonora.

Hall, D.G. (2020). Asian Citrus Psyllid Life Cycle and Developmental Biology. CAB International 2020. Asian Citrus Psyllid: Biology, Ecology and Management of the Huanglongbing Vector (eds. J.A. Qureshi and P.A. Stansly). pp.1-11.

https://citrusaustralia.com.au/wp-content/uploads/Asian-Citrus-Psyllid-surveillance-protocol-updated-10-9-18.docx

Food Forward. (2023). What is Asian Citrus Psyllid? https://foodforward.org/fruit-tree-care/asian-citrus-Psyllid

https://www.cdfa.ca.gov/citrus/pests_diseases/acp/docs/2022/ACP-HLB-PEP_OrangeCounty_2022-01-28.pdf

Martini, X., Hoyte, A., Stelinski, L.L. (2014). Abdominal color of the Asian citrus Psyllid (Hemiptera: Liviidae) is associated with flight capabilities. Annals of the Entomological Society of America, Volume 107(4), pp. 842-847.

Nath, R. & Sikha, D. (2019). Insect pests of citrus and their management. *International*

Journal of Plant Production. 12(2). pp. 188-196.

Nava, D.E., Torres, M.L.G., Rodrigues, M.D.L., Bento, J.M.S. & Parra, J.R.P. (2007). Biology of Diaphorina citri (Hem., Psyllidae) on different hosts and at different temperatures. Journal of Applied Entomology, Volume 131, pp. 709–715.

Singerman, A. & Rogers, M.E. (2020). The economic challenges of dealing with citrus greening: The case of Florida. *Journal of Integrated Pest Management,* Volume 11, Issue (1): 3.pp. 1–7.

Tsai, J.H. & Liu, Y.H. (2000). Biology of *Diaphorina citri* on four host plants. *Journal of Economic Entomology*. Volume 93, pp. 1721–1725.

Annex 1. Population density of adult *Diaphorina citri* in shoot* in an orchard

Farmer's name: Locality:

Sampling occurrence: 1st 2nd 3rd to 20th Sampling date:

Sub-field→	**Farmer's citrus orchard number- 1**						**Mean**
Sample↓	**1**	**2**	**3**	**4**	**5**	**Total**	**± S.E.**
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							
16							
17							
18							
19							
20							
Total							

*about 6-10 cm long

Protocol of Phytosanitary Requirements for the Export of Citrus Fruit from Nepal to China- 2012

Protocol of Phytosanitary Requirements for the Export of Citrus Fruit From Nepal to China

between

General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China

and

Department of Agriculture of the Government of Nepal

For the purpose of safe exports of Nepalese citrus fruits to China and on the basis of the pest risk analysis, the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (hereinafter referred to as AQSIQ) and the Government of Nepal, Department of Agriculture (hereinafter referred to as DoA), have exchanged views and reached a consensus as follows:

Article 1

Nepalese citrus fruits exported to China, including ***sweet*** orange (*Citrus sinensis*), mandarin orange (*Citrus reticulata*) and lemon (*Citrus limon*) (hereinafter referred to as citrus) must comply with the relevant phytosanitary laws and regulations of China and satisfy the phytosanitary requirements as stipulated in this Protocol.

The citrus fruits exported to China should be limited in Xizang Autonomous Region for consumption and use.

Article 2

Citrus shall be free of any quarantine pests concerned by China (Appendix).

If any other pests are newly detected in citrus growing areas that have not

been assessed by AQSIQ, DoA shall inform AQSIQ as soon as possible, in order to determine if they are quarantine pests and adopt proper quarantine measures if required.

Article 3

The citrus orchards and packinghouses shall be registered officially by DoA, and jointly approved by AQSIQ and DoA. Before each export season, DoA shall provide AQSIQ with a list of citrus orchards and packinghouses.

Article 4

The citrus orchards shall be monitored and found free of the following seven quarantine pests. If any of these pests are detected, the relevant orchards will be banned from exporting citrus to China for the season.

Bactrocera correcta (Bezzi)

Bactrocera cucurbitae Coquillett

Bactrocera dorsalis (Hendel)

Bactrocera tsuneonis (Miyake)

Bactrocera zonata (Saunders)

citrus huanglongbing (greening) disease

Xathomonas campestris pv. citri (Hasse)

Article 5

Under the supervision of DoA, the citrus orchards and packinghouses shall undertake effective monitoring, precaution and Integrated Pest Management (IPM) to avoid and control the occurrences of quarantine pests of concern to Chinese side; and ensure that orchards and packinghouses maintain the phytosanitary conditions.

Upon request by AQSIQ, DoA shall provide AQSIQ with relevant

procedure and results of the above-mentioned pest monitoring, precaution and IPM programs.

Article 6

The processing, packing, storage, and transportation of citrus ***shall*** be conducted under the quarantine supervision of DoA.

Before the packing, citrus shall be culled and sorted, those with the color or surface are abnormal shall be removed, to ensure that citrus are free of insects, mites, rotten fruits as well as twigs, leaves, roots and soil.

The citrus processed ***(selected for packing)*** shall be stored separately in the chamber to avoid re-infestation.

The packaging material shall not be of raw plant material, and for citrus shall be clean, sanitary, unused.

Every citrus packaging carton shall have markings in English indicating the place of origin, the name or registration numbers of orchards and packinghouses.

The cartons must be marked with Nepalese, Chinese and English characters "For Export to the People's Republic of China".

Article 7

DoA will sample no less than 2% of fruits in a consignment for export quarantine inspection. In cases where live quarantine pests of concern to China are detected, the whole consignment shall not be exported to China.

On completion of quarantine inspection, DoA shall issue a Phytosanitary Certificate, with the following declaration: "The consignment is in compliance with Protocol of Phytosanitary Requirements for the Export of citrus fruit from Nepalese to China and is free of any quarantine pest

o/c

concern to China". The Certificate shall indicate in English the producing area, the orchard and the packinghouse.

DoA shall supply AQSIQ with a template of the Phytosanitary Certificate in advance of trade, for confirmation and keeping record.

Article 8

When citrus arrives at entry port, CIQ (the port branch of AQSIQ) will examine relevant certificates and labels, and conduct a quarantine inspection.

In cases where citrus comes from unapproved orchards or packinghouses, the shipment shall not be allowed entry.

In cases where any quarantine pest or other non-compliances is found, the citrus shipment shall be re-exported, destroyed or quarantine treated (only limited to cases where pests can be exterminated effectively).

Accordingly, AQSIQ may suspend the importation of citrus from relevant orchards and/or packinghouses or even suspend the whole program until both parties conduct investigations to find out the causes and take relevant corrective measures. If such situation arises AQSIQ will facilitate to DOA for technical support from the People's Republic of China to comply with the quarantine requirements of China.

Article 9

After signing of this Protocol, if necessary and agreement is reached by both sides, AQSIQ will send two quarantine inspectors to Nepal to conduct on-site investigation, audit and inspection of the citrus growing areas, orchards, packinghouses, to examine pest monitoring and control.

Article 10

AQSIQ shall conduct further risk assessment according to the pest

0/2

occurrences and interceptions information. In consultation with DoA, the quarantine pest list and relevant quarantine measures ***shall*** be adjusted.

Article 11

In order to ensure the efficient performance, all operations and activities described in this Protocol could be reviewed and evaluated.

This protocol may be revised by mutual agreement between the two countries.

The Protocol will come into effect from the date of signature. It has two-year validity. In the case that neither party requests revision nor termination within two months before its expiration date, the Protocol shall be extended automatically for every year. 2068/3/20 (4 July, 2012)

This protocol is done in Triplicate in…on…in Nepali, Chinese and English languages, each side shall retain a copy of all texts. All texts shall be equally authentic and in case of divergence of interpretation, the English text shall prevail.

For the Nepalese Government
Department of Agriculture

For the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China

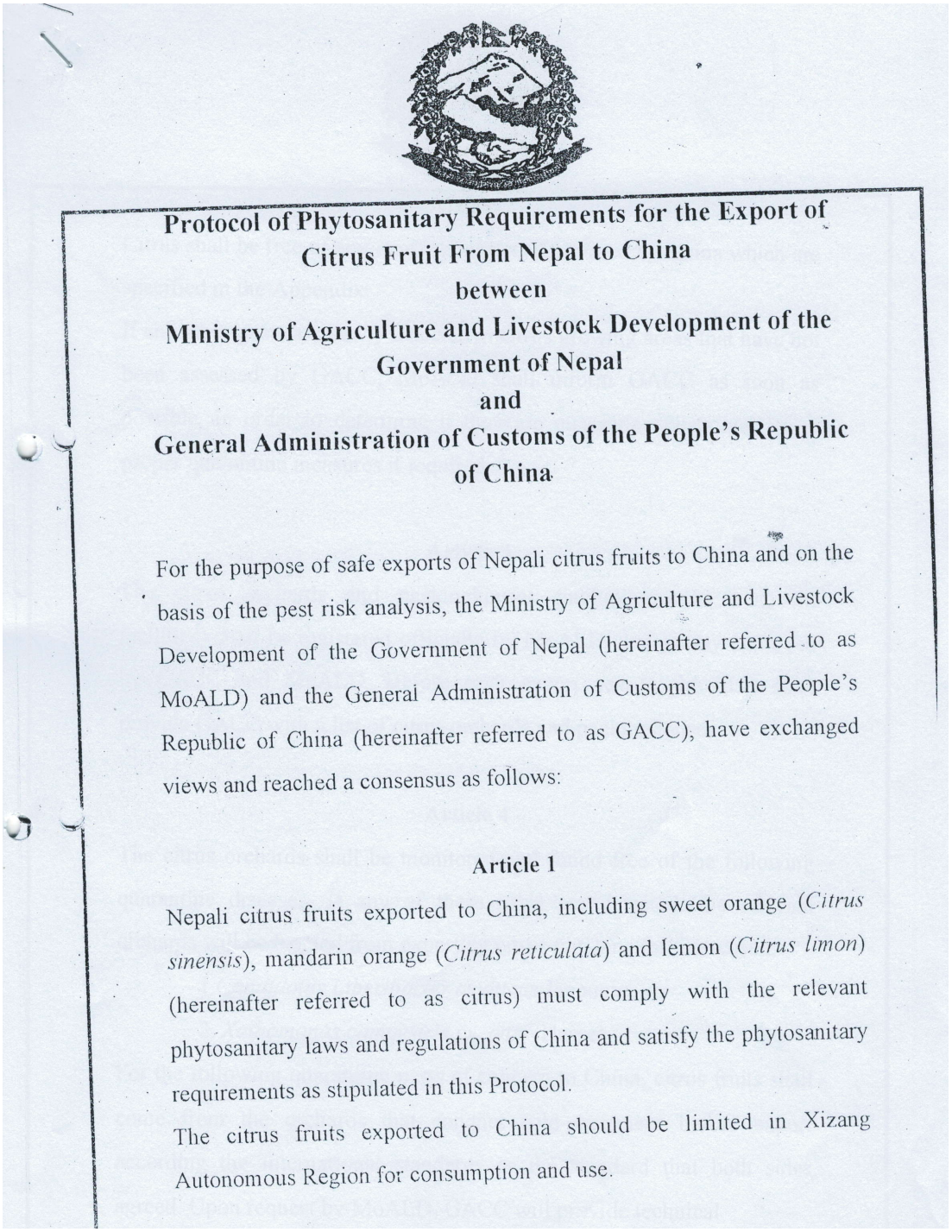
-5-

Appendix

Pests of Quarantine Concern to China

1）*Aleurocanthus woglumi* Ashby）
2）*Bactrocera correcta* (Bezzi)
3）*Bactrocera cucurbitae* Coquillett
4）*Bactrocera dorsalis* (Hendel)
5）*Bactrocera tsuneonis* (Miyake)
6）*Bactrocera zonata* (Saunders)
7）citrus huanglongbing (greening) disease
8）*Colletotrichum acutatum*
9）*Xathomonas campestris pv. citri* (Hasse)

Protocol of Phytosanitary Requirements for the Export of Citrus Fruit from Nepal to China- 2019



Protocol of Phytosanitary Requirements for the Export of Citrus Fruit From Nepal to China

between

Ministry of Agriculture and Livestock Development of the Government of Nepal

and

General Administration of Customs of the People's Republic of China

For the purpose of safe exports of Nepali citrus fruits to China and on the basis of the pest risk analysis, the Ministry of Agriculture and Livestock Development of the Government of Nepal (hereinafter referred to as MoALD) and the General Administration of Customs of the People's Republic of China (hereinafter referred to as GACC), have exchanged views and reached a consensus as follows:

Article 1

Nepali citrus fruits exported to China, including sweet orange (*Citrus sinensis*), mandarin orange (*Citrus reticulata*) and lemon (*Citrus limon*) (hereinafter referred to as citrus) must comply with the relevant phytosanitary laws and regulations of China and satisfy the phytosanitary requirements as stipulated in this Protocol.

The citrus fruits exported to China should be limited in Xizang Autonomous Region for consumption and use.

Article 2

Citrus shall be free of any quarantine pests concerned by China which are specified in the Appendix.

If any other pests are newly detected in citrus growing areas that have not been assessed by GACC, MoALD shall inform GACC as soon as possible, in order to determine if they are quarantine pests and adopt proper quarantine measures if required.

Article 3

The citrus orchards and packinghouses (including cold treatment facilities) shall be registered officially by MoALD, and jointly approved by GACC and MoALD. Before each export season, MoALD shall provide GACC with a list of citrus orchards and packinghouses.

Article 4

The citrus orchards shall be monitored and found free of the following quarantine diseases. If any of these pests are detected, the relevant orchards will be banned from exporting citrus to China for the season.

1. *Candidatus Liberibacter asiaticus* Jagoueix
2. *Xathomonas campestris pv. citri* (Hasse)

For the following quarantine pests of concern to China, citrus fruits shall come from the orchards that conduct cold treatment before export according the international standards or the standard that both sides agreed. Upon request by MoALD, GACC will provide technical

assistance for the establishment of cold treatment facilities and shall support capacity enhancement activities for the concerned MoALD officials and farmers.

1. *Bactrocera correcta* (Bezzi)
2. *Bactrocera cucurbitae* Coquillett
3. *Bactrocera dorsalis* (Hendel)
4. *Bactrocera tsuneonis* (Miyake)
5. *Bactrocera zonata* (Saunders)

Article 5

Under the supervision of MoALD, the citrus orchards and packinghouses shall undertake effective monitoring, precaution and Integrated Pest Management (IPM) to avoid and control the occurrences of quarantine pests of concern to Chinese side; and ensure that orchards and packinghouses maintain the phytosanitary conditions.

Upon request by GACC, MoALD shall provide GACC with relevant procedure and results of the above-mentioned pest monitoring, precaution and IPM programs.

Article 6

The processing, packing, storage, and transportation of citrus shall be conducted under the quarantine supervision of MoALD. Before the packing, citrus shall be culled and sorted, those with the color or surface are abnormal shall be removed, to ensure that citrus are free of insects,

mites, rotten fruits as well as twigs, leaves, roots and soil.

The citrus processed (selected for packing) shall be stored separately in the chamber to avoid re-infestation. The packaging material shall not be of raw plant material, and for citrus shall be clean, sanitary, unused.

Every citrus packaging carton shall have markings in English indicating the place of origin, the name or registration numbers of orchards and packinghouses. The cartons must be marked with Nepali, Chinese and English characters "For Export to the People's Republic of China".

Article 7

MoALD will sample no less than 2% of fruits in a consignment for export quarantine inspection. In cases where live quarantine pests of concern to China are detected, the whole consignment shall not be exported to China.On completion of quarantine inspection, MoALD shall issue a Phytosanitary Certificate, with the following declaration:

"The consignment is in compliance with Protocol of Phytosanitary Requirements for the Export of citrus fruit from Nepal to China and is free of any quarantine pest concern to China". The Certificate shall indicate in English the producing area, the orchard and the packinghouse. The Phytosanitary Certificate of shipments having undergone cold treatment before export must indicate the cold treatment temperature and duration, together with the facility name or code.

MoALD shall supply GACC with a template of the Phytosanitary

Certificate in advance of trade, for confirmation and keeping record.

Article 8

When citrus arrives at entry port, China Customs (the port branch of GACC) will examine relevant certificates and labels, and conduct a quarantine inspection. For items having undergone cold treatment before export, the cold treatment results with attached MoALD's sign-offs, as well as fruit temperature sensor record table, must also be delivered.

In cases where citrus comes from unapproved orchards or packinghouses, the shipment shall not be allowed entry.

Any shipment that is determined as the cold treatment is invalid shall undergo a cold treatment at the destination port (such as in the container itself), or be returned or destroyed.

In cases where any quarantine pest or other non-compliances is found, the citrus shipment shall be re-exported, destroyed or quarantine treated (only limited to cases where pests can be exterminated effectively).Accordingly, GACC may suspend the importation of citrus from relevant orchards and/or packinghouses or even suspend the whole program until both parties conduct investigations to find out the causes and take relevant corrective measures.

If such situation arises GACC will facilitate to MoALD for technical support from the People's Republic of China to comply with the quarantine requirements of China.

Article 9

After signing of this Protocol, if necessary and agreement is reached by both sides, GACC will send two quarantine inspectors to Nepal to conduct on-site investigation, audit and inspection of the citrus growing areas, orchards, packinghouses, to examine pest monitoring and control.

Article 10

GACC shall conduct further risk assessment according to the pest occurrences and interceptions information. In consultation with MoALD, the quarantine pest list and relevant quarantine measures shall be adjusted.

Article 11

In order to ensure the efficient performance, all operations and activities described in this Protocol could be reviewed and evaluated. This protocol may be revised by mutual agreement between the two countries.

The Protocol will come into effect from the date of signature. It has two-year validity.In the case that neither party requests revision nor termination within two months before its expiration date, the Protocol shall be extended automatically for every year.

This protocol is signed in Kathmandu on 13 October 2019 in duplicate in Nepali, Chinese and English languages, each side shall retain a copy of

all texts. All texts shall be equally authentic and in case of divergence of interpretation, the English text shall prevail.

For the Ministry of Agriculture and Livestock Development of the Government of Nepal	For the General Administration of Customs of the People's Republic of China
.....	.....
Name: Yubak Dhoj G.C.	Name: Hou Yanqi
Designation: Secretary	Designation: Ambassador
Date: 2019/10/13	Date: 2019/10/13