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Department of Agriculture
Ministry of Agriculture and Forests
Roval Government of Bhutan

PROTOCOL

NATIONAL CITRUS REPOSITORY

EXECUTIVE ORDER

In Accordance with Section 32 and 33 of the "Seed Rules and Regulations of Bhutan, 2018", it is mandatory for any seed producers to meet the minimum standards of seeds or planting materials to have them certified for sale. The minimum standards required for the production and sale of any seeds or planting materials are specifically prescribed in the "Minimum Seed Standards of Bhutan 2019". For citrus, the seeds and planting materials produced must be a released and notified variety; genetically pure; and free from viruses, canker and citrus greening diseases to fulfil the prescribed specific minimum standards.

The National Citrus Repository (NCR) being the basic starting point for development of any citrus industry in Bhutan, it is highly imperative to maintain high-health status planting materials of known horticultural characteristics. Without these minimum standards ensured at the NCR, it will be near impossible for the nurseries that avail basic planting materials from this centre to multiply and produce planting materials with minimum standards required for certification and sale. The NCR also has the mandate to collect and maintain all kinds of citrus germplasm for conservation, research programmes, and hold citrus information repository in the country.

Therefore, this "Protocol" has been issued to help implement the provisions of the Seed Rules and Regulation of Bhutan 2018, safeguard the conservation of citrus germplasm, and improve the availability of high-health status citrus planting materials for nursery production. It is mandatory for NCR, researchers, collaborators, and all other relevant agency that work with NCR to strictly follow this protocol.

Issued for strict compliance on 8 July 2021.

Kinlay Tshering (Ms)
DIRECTOR

ROYAL GOVERNMENT OF BHUTAN
MINISTRY OF AGRICULTURE AND FORESTS
THIMPHU

1 Introduction

A functional and certified citrus repository is the basic starting point for development of any citrus industry in the world. Repository develops and maintain high-health status planting materials with known horticultural characteristics for the citrus nurseries to produce high quality nursery stocks to be supplied for the orchard establishment. Further, it also maintains the collection of all kind of citrus germplasm for future research and development programmes.

With more than 22,158 households growing citrus in Bhutan for their income and livelihoods, citrus is one of the main cash crops that needs to be promoted through a well-established and strategic production system. Bhutan has the huge potentials to upscale citrus production and has assured market both within and outside country.

However, limited availability of quality planting materials is one of the impeding factors towards overcoming rapid citrus decline challenges and overall growth of the industry. Infestation from diseases-Citrus greening/Huanglongbing(HLB), Citrus Tristeza Closterovirus (CTV) and Citrus Exocortis Viroid (CEVd) that are mainly spread through planting materials are causing early orchard decline in the field.

Therefore, it is highly crucial to maintain high-health status planting materials to produce high quality nursery stocks. The varieties maintained and made available also need to be highly diverse and climate resilient; completely free from diseases; and should be with known horticultural characteristics for the commercial production.

In order address these issues and challenges, the Department of Agriculture has established the National Citrus Repository (NCR) in 2012 in Menchuna, Tsirang. The NCR has the critical role and mandate to maintain the citrus repository in the country. The centre collects and maintains both indigenous and exotic germplasms; conduct characterization studies; maintain and source high-health status planting materials for citrus nurseries. It also maintains citrus information repository in Bhutan.

2 Obligation

This standard "Protocol" is developed to guide the implementation of "Minimum Standards for Seed and Planting Materials" prescribed under "Seed Rules and Regulations of Bhutan, 2018". The requirement is exclusively prescribed under the "Minimum Seed Standards of Bhutan, 2019" under section 6.1, with particular reference to citrus planting materials.

The NCR will not make any changes to approved structures and production process without obtaining prior approval of the Department of Agriculture.

3 Mandate and function

The key mandates of National Citrus Repository are:

I. Function as the central repository for citrus genetic resources and genetic related scientific information in Bhutan;
II. Collect and conserve all types of citrus germplasm (both indigenous and exotic);
III. Maintain the primary sources of high health-status and true-to-type budwoods or scion woods of all released citrus varieties for commercial nurseries and research programs; and
IV. Characterize different collections and maintain detailed information.

3.1 Key activities

The National Citrus Repository shall:

i. Collect and acquire germplasm or accessions of citrus including the wild citrus species;
ii. Maintain all accessions under a protected and pest-free environment;
iii. Test for the presence of pathogenic organisms in the collections and discard the infected ones;
iv. Characterize and evaluate the genetic and physiological characteristics of germplasm collections;
v. Identify and conserve desirable clones of local mandarin collections, released varieties and introduced citrus types;
vi. Identify sports or mutants of citrus for breeding and improvement programmes;
vii. Establish a database for accessions detailing acquisition, inventory, evaluation, and gene descriptor data;
viii. Produce and supply high health-status citrus plants to commercial nurseries for the establishment of budwood multiplication, and budwood (depending on availability) for mass production (grafts);
ix. Produce and support research centres with planting materials for research programmes; and
x. The repository stocks must be examined for any pests or diseases on a regular basis with the support of National Plant Protection Centre (NPPC), and get certified by Bhutan Agriculture and Food Regulatory Authority, Bhutan.

4 Repository Facilities

The National Citrus Repository is located in Menchuna, Tsirang, at an elevation of 1450 metres above sea level. It is isolated from commercial citrus orchards and research fields. The centre is established with a required number of structures with double door system to house the collections and pool of germplasm along with screening and quarantine facilities. The centre is equipped with modest laboratory and office facilities. The following structures are developed for the specific purposes as described below:

4.1 Quarantine Block

All kinds of collection, except for imported materials confirmed free of disease(s) will be grown and maintained under quarantine block for a minimum of 2 years. The plants will be observed for expression of symptoms of any pests or diseases by the plant itself, and will also undergo bio-indexing to further study the presence of diseases during this period in the separate indexing house. Further confirmatory test will be conducted by using PCR technology. Any collections made will be thoroughly investigated for the presence or absence of HLB, CTV and CEVd using the above technology. Infected plants, not limited to, will be rescued through shoot tip grafting (STG) technique. The STG process will be repeated until the accession is confirmed completely free of diseases of concern that will be diagnosed through PCR.

Specific requirements:

i. Maintain separate tools and equipment to use for quarantine block; shall not be used in other areas. Tools should be disinfected with 75% ethanol or 20% sodium hypochlorite.
ii. Change footbath solution regularly.
iii. Examine the irrigation water quality from time to time.
iv. Check for any damages on walls, doors, tops, vents, etc.
v. Maintain proper records of different collections.
vi. Observe visually for symptom expression of diseases or emergence of pests.
vii. Conduct bio-indexing and PCR analysis.
viii. Spray collections fortnightly with Plant Protection Products (PPP) recommended by NPPC.
ix. Tag and label the plants properly.
x. Grow all the plants in containers (Polypots, clay or cement pots, plastic containers).

4.2 Foundation Block

All the germplasms (indigenous type, backyard collection, wild collection, released, imported) confirmed free of diseases after the quarantine period will be grown and maintained in the foundation block. A minimum of 3 replications of each germplasm or

varieties will be maintained for conservation and to source germplasm for mother plant development.

The block should be completely under the insect proof structure at all times to safeguard the germplasm from pests and diseases. This block will be strictly quarantined; only the designated personnel assigned to work in this block will be allowed entry.

Specific requirements:

i. Maintain separate tools and equipment to use for foundation block; shall not be used in other blocks. Tools should be disinfected with 75% ethanol or 20% sodium hypochlorite.
ii. Change footbath solution regularly.
iii. Examine the irrigation water quality from time to time.
iv. Check for any damages to walls, doors, tops, vents, etc.
v. Maintain proper records of different collections.
vi. Test the plants at least once in a year for diseases of concern using PCR.
vii. Any plants tested positive for diseases of concern shall be discarded and destroyed.
viii. Spray the grafts fortnightly with PPP recommended by NPPC.
ix. Tag and label the plants properly. Maintain the sketch map for the location of the plants inside the block.
x. Grow all the plants in containers (Polypots, clay or cement pots, plastic containers).

4.3 Mother Block

This block will maintain mother germplasm of only released and promising cultivars for the high health-status budwood production to propagate planting materials to support the establishment of Budwood Multiplication Block (BMB) for nurseries and research programmes. A minimum of five trees of each variety will be maintained based on the recommendation of the Department of Agriculture (DoA) or on availability of space.

The block should be completely under the insect proof structure at all times to safeguard the mother trees from pests and diseases. This block will be strictly quarantined; only the designated personnel assigned to work in this block will be allowed for entry.

Specific requirements:

i. Maintain separate tools and equipment to use for mother block; shall not be used in other blocks. Tools should be disinfected with 75% ethanol or 20% sodium hypochlorite.
ii. Change footbath solution regularly.
iii. Examine the irrigation water quality from time to time.
iv. Check for any damages to walls, doors, tops, vents, and so on.
v. Maintain only released and recommended varieties.
vi. Test the plants at least once in a year for diseases of concern using PCR.

vii. Any plants tested positive for diseases of concern shall be discarded and destroyed.
viii. Spray the grafts fortnightly with PPP recommended by NPPC.
ix. Tag and label the plants properly. Maintain the sketch map for the location of the plants inside the block.
x. Grow all the plants in containers (Polypots, clay or cement pots, plastic container).
xi. Regularly prune the trees to induce and increase the growth of budwoods.
xii. Budwoods shall be supplied to National Seed Centre (NSC) or private nurseries for the production of budwood trees (BMB) or for mass multiplication (if budwoods are sufficient) and research centres for reach programmes.
xiii. Budwoods shall not be supplied to farmers or private citrus growers unless authorized by the department.
xiv. Any trees without the consent of the department shall not be removed from this block

4.4 Propagation Block

All kinds of propagation activities required within the NCR and for supply to Nurseries or Research Centres must be exclusively carried out in the propagation block (PB) only. Germplasm or planting materials must be confirmed free from diseases of concern before propagation. The grafts until attending required size will be raised within the PB.

The block should be completely under the insect proof structure at all times to safeguard the germplasm from pests or vectors and diseases. This block will be strictly quarantined; only the designated personnel assigned to work in this block will be allowed entry.

Specific requirements:

i. Maintain separate tools and equipment to use for propagation block; shall not be used in other areas. Tools should be disinfected with 75% ethanol or 20% sodium hypochlorite.
ii. Change footbath solution regularly.
iii. Examine the irrigation water quality from time to time.
iv. Check for any damages to walls, doors, tops, vents, etc.
v. Use rootstocks raised within the NCR only for propagation.
vi. Collect budwoods for propagation only from the MB and FB depending on the end use.
vii. Test grafts for HLB, CTV and CEVd before shifting to hardening facilities.
viii. Spray the grafts fortnightly with PPP recommended by NPPC.
ix. Tag and label the plants properly.
x. Grow all the plants in containers (Polypots, clay or cement pots, plastic containers and so on).
xi. Planting materials shall be supplied only to NSC for raising budwood trees and research centres for research activities.
xii. NCR neither has an obligation nor right to produce planting materials on a commercial scale for sale, supply to farmers or commercial growers.

5 Other facilities

i. *Hardening house*
   a. The hardening shall be done completely under designated insect proof net houses.
   b. All planting materials intended to be supplied to nurseries and research centres should be hardened for a minimum of 15-30 days prior to supply.
   c. The plants must be sprayed with recommended PPP, at least 15 days prior to supply.
   d. All sanitary measures must be strictly applied.
   e. The plants must be tagged and labeled properly.

ii. *Media*
   a. Only the recommended media must be used for the propagation.
   b. The media must be completely sterilized.
   c. Do not reuse and recycle the used media.
   d. Maintain proper sanitation in and around the media preparation room.
   e. All workers must strictly follow the sanitation measures.
   f. Sterilize the containers, equipment or tools before use.

iii. *Office, laboratory and premises*
   a. Prohibit unauthorized vehicles and trespassers in NCR campus.
   b. Maintain the laboratory and function in accordance with Laboratory protocol.
   c. The daily workers shall use the specified working gear and equipment while working in the NCR.
   d. All approved visitors shall follow dress code specified or being made available by the NCR.
   e. Maintain the office premises neat and clean.
   f. Prevent encroachment of alternate or collateral hosts of HLB and by Rutaceae sub-family.
   g. Prevent run-off entry from surrounding areas.
   h. Prohibit use of the plants and equipment allotted for NCR for other activities.

6 Sanitation Requirements

A precautionary notice board must be placed in front of the gate and in front of every individual block to alert the worker(s) and visitor(s) for adherence to sanitation requirements and for strict compliance to bio-security measures or rules. Sanitation procedure must be clearly written on the precautionary board.

All equipment, plant material, supplies and personnel that enter or remain in a repository area shall subject to sanitation requirements.

6.1 Personnel

i. Repository employees who work with citrus produced outside of the approved structure shall not return to work within the approved structure until the following day.
ii. Prior to entering the repository houses everyone must decontaminate using an approved decontamination product and wear a clean garment provided by the repository. If gloves are used, they must be a disposable glove or decontaminated each day and kept on the site itself.
iii. All persons entering an approved structure, soil or media storage area shall walk through a sanitizing footbath containing a decontaminant that is approved by the National Plant Protection Centre (NPPC), such as copper sulfate products or chlorine bleach.
iv. Designate and retain nurseryman with skills and experiences to operate and function the specific block.
v. The personnel working in the repository must adhere to Occupational Health, Safety and Welfare rules and regulations.

6.2 Propagation Tools or Equipment

i. All equipment entering or leaving the repository must be clean of all plant material and soil, and must be decontaminated using approved decontamination products.
ii. Propagation tools and implements should be sterilized between different groups or lots of propagations[1] during repository plant propagation and between different plants while collecting budwood(s) from MB or FB. Additionally, the following procedures must be strictly followed;
    a. A solution of 20% household bleach (sodium hypochlorite) or 75% Ethanol shall be used to sterilize tools.
    b. Sterilizing solution shall be prepared fresh each day.

[1]Research has shown that viroids can frequently be spread from infected plants to healthy plants on clippers, budding knives, and other mechanical equipment used in pruning and budding. *Exocortis* is a viroid disease detrimental to certain rootstock/scion combinations. Rootstock especially susceptible are *Poncirus trifoliata*, most citranges (Carrizo &Troyer), some citrumelos (Swingle not severely affected), Rangpur lime, and sweet lime.

c. If the bleach solution is used, dip clippers, knives or pruning tools in the solution for a few seconds.
d. Clean and dry the tools and equipment after completing the propagation works.
e. All equipment, if possible, should be kept on site.

6.3 Potting media

i. All soil, peat, sawdust, mulch, manure or other plant-growing or potting media entering the approved site for the production of citrus nursery stock must be free from pests or pathogens of citrus.
ii. All types of media must be completely sterilized before use.
iii. Media must be stored in an appropriate structure to avoid cross contamination.
iv. Media if recycled should be completely sterilized before use.

7 General Site Quarantine Measures

i. Only the Department of Agriculture authorized visitors shall be allowed to visit the repository.

ii. Visitor must adhere to quarantine rules and must strictly follow site sanitation procedures.

iii. Prevent encroachment at the nursery location from Rutaceous subfamilies- *Aurantioideae, Rutoideae*, and *Toddalioideae*, plants and noncertified citrus plant materials, to avoid the nursery site becoming infested with pests or diseases of citrus.

iv. Completely avoid bringing in any Rutaceous subfamilies, unless authorized and certified by Bhutan Agriculture and Food Regulatory Authority or other competent authority.

v. Follow established sanitation procedures to prevent nematode, psyllid, aphid or other common plant pest infestation of the nursery site.

vi. Repository areas and perimeter shall remain weed free.

vii. Any citrus repository stock or tree, except for stocks under quarantine area, found infected or exposed to plant pest infestation shall be subjected to immediate removal from the site and must be completely destroyed.

viii. Nursery compound must be kept well fenced and protected always.

Figure: Schematic Representation of NCR Protocol