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Clean Citrus Planting Materials Production through Shoot tip Grafting and Biological Indexing

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ABSTRACT

Huanglongbing (Citrus Greening) is the most devastating citrus disease caused by phloem-inhabiting bacteria carried by the insect vector, Asian citrus psyllid (Diaphorina citri). In Bhutan, the production and movement of citrus seedlings and new orchard establishment have been restricted after the Citrus Greening disease was detected in most citrus orchards. To address this problem, Agriculture Research and Development Centre-Wengkhar has initiated research on clean citrus propagation through shoot tip grafting. This study assessed the efficiency of shoot tip grafting for the production of clean planting materials. Sweet orange was used as an indicator plant to study disease transmission and symptom expression in some of the potential and released citrus varieties. A total of 15 plants, 3 plants of each cultivar (AREP-1, AREP-2, Aoshima, Wengkhar Tshelu-2 and Yoshida Ponkan) produced through shoot tip grafting were used for bio-indexing. The cultivars were grafted on indicator plants using different grafting techniques (T-budding, side grafting, wedge, and split grafting). A total of twenty-six samples with two samples per cultivar was sent to the National Plant Protection Centre for PCR analysis. The result of PCR showed negative result for Huanglongbing. Thus, shoot tip grafting could be one potential method for clean citrus planting material production.

Keywords: *Bio-indexing; Greening; Cultivar; Grafting, Symptoms; Shoot tip grafting*

1. Introduction

The citrus fruit is one of the most important cash crops - both for local as well as exports - among which the mandarin (*Citrus reticulata* Blanco) is pre-dominate and the most widely distributed in Bhutan, both in quantity and value. It occupies more than 5,086 hectares of land (RSD, 2020) in Bhutan. Though it is grown in 17dzongkhags, certain dzongkhags like Samtse, Sarpang, Dagana, Tsirang, Chhukha and Pemagatshel have emerged as the leading producers. The average annual production of citrus is about 38,100 Mt, but both annual production and yield have decreased over the years due to various factors such as incidence of pests (citrus

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fruit fly, leaf miner) and diseases (Huanglongbing, citrus canker, tristeza, exocortis, phytophthora) and improper management of orchards by the farmers. Most of the citrus orchards in Bhutan are grown in hilly areas where farmers cannot manage their orchards timely and properly. In some dzongkhags like Sarpang and Tsirang, the citrus orchards were established illegally on state land near forest areas which encourage wild animals, particularly monkeys to feed on the crop, further jeopardizing citrus production in the country. The low productivity of the citrus is also attributed to the use of seedlings produced from seeds of unknown sources instead of using seedlings produced through grafting and budding where rootstocks such as Wengkhar Rato-1, Wengkhar Rato-2 and C-35 play a significant role in combating abiotic and biotic stresses. The seedlings produced through seeds not only have long unproductive juvenile phases but also the young plants are more prone to pests and diseases, thereby resulting in further decline of the citrus industry in Bhutan. The most common factor associated with citrus decline is Huanglongbing (HLB) disease, which was first reported in Bhutan in 2003 (Doe, Om, Dorji, Dorji et al., 2003). The disease is caused by *Candidatus Liberibacter asiaticus* (a fastidious phloem limited bacterium), an obligate gram-negative bacterium (Sheng Li, Wu, Duan, Singerman et al., 2020) and is transmitted through infected budwood and the citrus psyllid, *Diaphorina citri (Batool, Iftikhar, Mughal, Khan et al., 2007).* The disease is also transmitted by the parasitic weed dodder (Sheng Li et al., 2020). Following the detection (PCR test report from NPPC, 2015) of HLB from the Regional Seed Centre at Samtenling, Sarpang, the Department of Agriculture re-emphasized that only the National Seed Centre (NSC) is authorized to produce citrus seedlings. Currently, the Regional Seed Centre in Jachephu, Trashiyangtse (located about 1,800 m above mean sea level) carries out citrus seedling production as Asian citrus psyllid remains active at an elevation of 1000 m and below (Manandhar, Malla, & Sah, 2004).

Huanglongbing results in significant fruit yield and quality losses due to severe deterioration of tree health (Vidalakis, Garnsey, Bash, Greer et al., 2004). The HLB has become one of the major challenges for both farmers and the Royal Government of Bhutan, especially for the Ministry of Agriculture and Forests, as the infected trees become less productive within 2 to 6 years of infection (Kinley Dorji, Lakey, Chophel, Dorji et al., 2016). Asian citrus psyllid, the main vector of the disease in Bhutan (Halbert & Núñez, 2004) is active only below 1000 m, so symptoms of citrus greening do not occur or are not very well expressed or simply disappear at higher elevations (Ajene, Khamis, Ballo, Pietersen et al., 2020), especially in places such as Wengkhar (Mongar) which is located at around 1,700 m above sea level.

Thus, against this backdrop, ARDC Wengkhar initiated the production of clean citrus seedlings through shoot tip grafting followed by bio-indexing and PCR to rule out any possibility of HLB. This is also believed to be the best techniques to obtain seedlings free of virus and other disease such as tristeza, psorosis, exocortis and also HLB (Navarro, Roistacher, & Murashige, 1975). The plants produced through shoot tip grafting are found to be disease free, particularly the HLB virus, when the shoot tip grafted citrus samples are bio-indexed and PCR analysed. The production of citrus through shoot tip grafting is the best techniques in producing disease-free citrus which will ultimately help revive the citrus industry in the country. However, due to the lack of laboratory facilities and limited knowledge and technical expertise in biotechnology, production of clean citrus seedlings both in terms of quality as well as the numbers is a huge challenge.

2. Materials and Method

Disease-free rootstocks (seeds imported from Australia), mainly C35, were raised under laboratory conditions with a room temperature of 25 -27^{o}C. After 2 to 3 weeks, once they have attained graft size, shoot tip grafting was carried out. The vigorous, healthy, and successful STG plants (that attained 2 to 3 leaves stage) were potted in bigger pots and shifted to protected greenhouse for further approach grafting. Out of 55 successful shoot tip grafted plants, to encourage faster growth, 26 plants were further approach-grafted onto the bigger and vigorous C35 rootstocks maintained in an insect-proof greenhouse.

One of the methods to confirm disease (HLB) free status of plants is biological indexing (Razi, Khana, Jaskania, & Basrab, 2012). Biological indexing is a method to detect pathogens before laboratory analysis is carried out. Though there are other indicator plants (plants that can easily express signs and symptoms of the disease) for HLB, such as sour lime, mandarin and grapefruit, sweet orange is considered a more effective and good indicator for greening disease as it expresses greening symptoms very easily as compared to other citrus species (Das, 2008). Hence, in this study, scion woods from the above successfully grafted plants were collected and again grafted (Bhandari, Basnet, & Khanal, 2021) onto the indicator plants (sweet orange) to observe if any signs and symptoms of HLB are expressed. For further reconfirmation of disease-free status, leaves and stem samples were collected from those grafted onto indicators plants and sent to the National Plant Protection Centre for PCR analysis.

2.1 Shoot Tip Grafting

Shoot tip grafting was carried out from January to May 2018 and 2019, where two to three-week-old, etiolated rootstocks were topped and shoot tips were grafted. Grafted plants were grown in a controlled growth room at a temperature of 25-27°C and humidity of 70-75%. The one- to two-month-old STG plants with a height of about 15 to 20 cm and a stem thickness of 0.5 to 1.5 mm were re-potted and kept in a controlled insect-proof citrus greenhouse (K. Dorji & Lakey, 2015). The following are the standard procedures for grafting shoot tips.

(A) (B)

Figure 2. Rootstock seeds planted in potting media (A) and two weeks old rootstocks (B)

2.1.1 Shoot tip grafting rootstock preparation

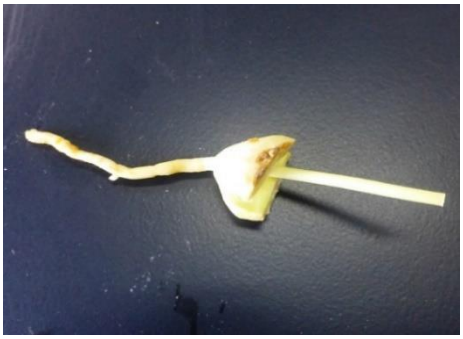
The rootstock seeds imported from Australia were first de-coated and sterilized for 10 min with a 0.5% sodium hypochlorite solution containing 2-5 drops of 0.1% Tween-20. Then, seeds were thoroughly rinsed 3-4 times with sterile distilled water and planted in potting soil (perlite, coco peat) and allowed to germinate for 2-3 weeks under dark conditions at 25-27°C in the germination chamber. The 2-3 weeks old, etiolated rootstocks were used for grafting the shoot tips.

2.1.2 STG scion preparation and grafting

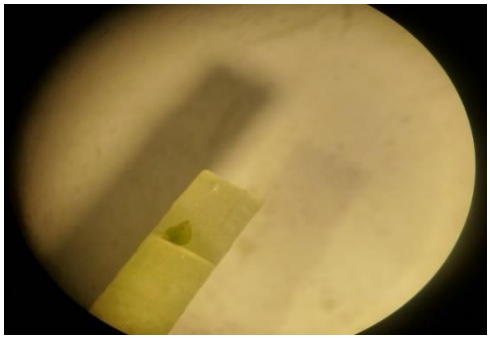
Citrus buds of 15 released cultivars (Table 2) were collected from the germplasm collection greenhouse. The collected bud sticks were 5-10 cm long and contained at least 2-3 opened buds. The leaves of the bud canes were removed, and surface sterilized with 0.5% sodium hypochlorite solution containing 2-5 drops of 0.1% Tween-20 for 5 minutes. They were then thoroughly rinsed (3-4 times) with sterile distilled water. The 3-week old rootstock was decapitated at about 1.5 cm from the hypocotyl (Starrantino & Caruso, 1988). The cotyledons and root tips were cut off. Using a sharp surgical knife, an inverted T-cut is made on the rootstock under the dissecting microscope. Again, under the microscope, the shoot tip (apical

meristem) with a length of about 0.2 mm is removed from the bud stock and aseptically placed on the inverted T-cut of the rootstock. Then the inverted T-cut is carefully wrapped with para-film to prevent immediate drying of the shoot tip (Figure 4 shows the details of shoot tip grafting). The grafted plants were transplanted into the planting medium and kept in a germination chamber with 16 hours light/day at a temperature of 25-27^{o}C.

(A)



(B)



(C)

(D)

Figure 3. STG Scion collection (A), Decapitated rootstock (B), Apical meristem aseptically placed on the inverted T-cut of the rootstock (C) and T-cut is carefully wrapped with parafilm (D)

2.2 Bio-indexing of citrus varieties

T-budding, side grafting, wedge grafting, and split grafting were used to graft onto indicator plants.

T-budding: In this method, an annual rootstock with a height of 25-30 cm and a diameter of 1-2 cm is used for budding. The T-shaped cut is made on the rootstock 12-15 cm from the base with a vertical cut of 1-2.3 cm and a horizontal cut of 0.8-1 cm. Similarly, a 3.4-cm bud is inserted into the T-shaped cut with two flaps of bark and wrapped with a 300-gauge polyethene strip.

Side grafting: In this method, a similar size and height are used. A shallow inward cut of about 2-5 cm is made on the mother plant at the desired height. On the scion, a similar inward cut and a small slanting cut is made as on the rootstock so that the cambium layer of both sides match as much as possible and is wrapped with polyethene and parafilm.

Wedge and split grafting: In this method, the upper part of the rootstock is topped at a height of 15-20 cm from the base and a 3-cm split cut is made. A wedge-shaped cut of 2-3 cm is made on the scion wood, which is put on the base and fixed with a strip of polyethene.

Table 1. Grafting methods employed

S.N.	T-Budding	Side grafting	Wedge and cleft grafting
1	Aoshima	Aoshima	Aoshima
2	AREP-1	AREP-1	AREP-1
3	AREP-2	AREP-2	AREP-2
4	Wengkhar Tshelu-2	Wengkhar Tshelu-2	Wengkhar Tshelu-2
5	Yoshida ponkan	Yoshida ponkan	Yoshida ponkan

The success rate of grafting was promising for all the methods used. However, the new shoots emerging from the side grafting were more vigorous and grew faster compared to those from T-budding, wedge and cleft grafting.

3. Results and Discussion

The results are discussed under three topics: shoot tip grafting, biological indexing, and PCR analysis.

3.1 Shoot tip grafting

Fifteen different cultivars were shoot tip grafted. Out of 167 plants that were grafted, 55 plants were successful, of which 26 were successfully approach-grafted in the protected greenhouse for biological indexing and PCR analysis.

(A)

(B)

(C)

(D)

Figure 4. Plantlet 1-2 week after STG (A), 2-3 weeks old STG plant (B), Successful STG plants (C) and then potted plants (D)

Table 2. Details of shoot tip grafting (STG) and success

S.N.	Citrus variety	Rootstock used	No. of plants grafted (STG)	No. of Successful grafts (STG)	No. of successful approach grafts
1	Tarku	Grapefruit	07	00	None
2	Wengkhar Tshelu II	Grapefruit	08	06	06
3	AREP I	Grapefruit	13	03	None
4	AREP I	C-35	11	03	None
5	Wengkhar Tshelu Ngarm	C-35	10	03	02
6	Otshu-4	C-35	19	04	None
7	Wengkhar Tshelu Drukchu	C-35	06	03	None
8	Local Selection	Swingle Citrumelo	13	04	None
9	Wengkhar Tshelu Ngarm	Swingle Citrumelo	28	10	04
10	Wengkhar Tshelu Ngarm	*Poncirus trifoliata*	08	02	01
11	Aoshima	C-35	10	04	04
12	Yoshida Ponkan	C-35	09	01	01
13	AREP II	C-35	11	07	05
14	Kiyomi	C-35	05	02	02
15	Junar	C-35	09	03	01
		TOTAL	167	55 (32.9%)	26 (47.27%)

3.2 Biological indexing

Visual symptoms were observed 30 days after grafting and persisted for up to 3 months (Table 3) at 30-day intervals. At the first observation, 30 days after grafting, some plants started showing symptoms on the leaves and the symptoms tend to decrease after 40-45 days. The symptoms were blotchy leaves and resembled zinc deficiency. However, at 90 days after grafting, all plants were very healthy. This indicated that no greening pathogens were present, and the symptoms were likely caused by nutrient deficiency or micronutrient deficiency (Silva-Stenico, Pacheco, Pereira-Filho, Rodrigues et al., 2009).

Table 3. Visual observations on indicator plants

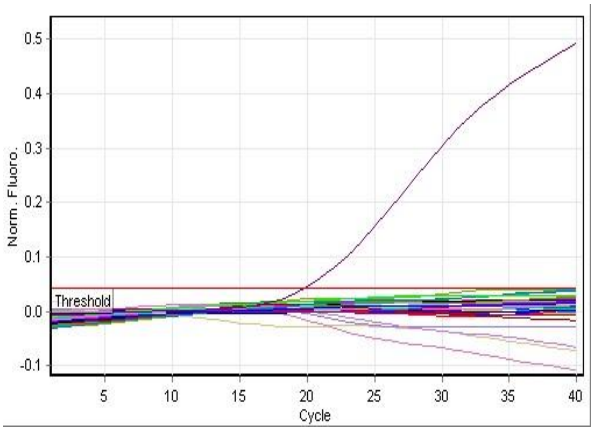
S.N.	Time interval	Citrus varieties	Symptoms expressed	Healthy plants
1	30 days after grafting	Aoshima	Normal leaf size, symmetrical chlorosis in some leaves (1)	2 and 3
		AREP-1	Blotchy appearance of some of the leaves with thickened veins (2&3)	1
		AREP-2	Yellowing and varied chlorotic pattern with downward curling of leaves (1,2 &3)	None
		Wengkhar Tshelu-2	None	All
		Yoshida Ponkan	Symmetrical bright yellowing of outer leaf portion and thickening of the vein (1,2 &3)	None
2	60 days after grafting	Aoshima	Normal leaf size, symmetrical chlorosis in some leaves (1,2&3)	None
		AREP-1	None	All
		AREP-2	Yellowing and varied chlorotic pattern with downward curling of leaves (1&2)	3
		Wengkhar Tshelu-2	Yellowing and blotchy on some leaves (2)	1&3
		Yoshida Ponkan	Symmetrical bright yellowing of outer leaf portion and thickening of the vein (3)	1&2
3	90 days after grafting	Aoshima	None	All
		AREP-1	None	All
		AREP-2	None	All
		Wengkhar Tshelu-2	None	All
		Yoshida Ponkan	None	All

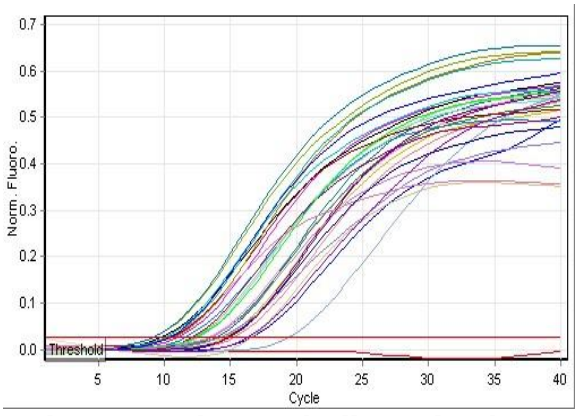
3.3 PCR analysis

In the final analysis, to fully confirm the absence or presence of HLB pathogens, leaves and stem samples from 15 bio-indexed plants (leaves and stems) were sent to the National Plant Protection Centre (NPPC) for polymerase chain reaction (PCR) analysis. Real-time polymerase chain reaction analysis was performed using Rotor-Gene Q (Qiagen) with the primer-probe combinations, for Clas and the internal control, cytochrome oxidase gene (COX), (Shalan Li, Zhang, Liu, Liu et al., 2020). A reaction volume of 25 µL with cycling conditions of pre-incubation at 50 °C for 2 min followed by 95 °C for 10 min and 40 amplification cycles of 95 °C for 30 sec and 58 °C for 40 sec were used. Positive and non-template controls were included. Samples were considered positive if both the Clas and COX probes showed threshold cycle (Ct) values in the range of zero to less than or equal to 36. All the samples were negative for HLB pathogen (Table 4), which shows that citrus plants grafted through the shoot tip are free from HLB pathogens.

Table 4. PCR analysis report from NPPC

Sample Number	Ct values	
	Clas	COX
437-21	0	14.58
438-21	0	16.64
439-21	0	14.81
440-21	0	13.41
441-21	0	11.59
442-21	0	13.96
443-21	0	15.3
444-21	0	13.6
445-21	0	11.02
446-21	0	9.79
447-21	0	10.14
448-21	0	16.55
449-21	0	12.26
450-21	0	12.53
451-21	0	14.32
452-21	0	13.41
453-21	0	10.4
454-21	0	10.45
455-21	0	10.63
456-21	0	9.18
457-21	0	10.23
458-21	0	9.17
459-21	0	14.93
460-21	0	12.18
461-21	0	10.36
462-21	0	12.38





Figures 5 and 6. The standard curve derived during the PCR analysis with all samples showing Clas Ct values equal to zero and the COX probe Ct values for all samples ranged from 9.17 to 19.40

4. Conclusion

The spread of citrus greening has significantly affected the citrus industry in Bhutan since its initial detection, especially in the major citrus growing areas of Bhutan. Therefore, in this research, we studied some of the techniques to produce HLB free seedlings. Citrus plants produced using the shoot tip grafting method proved to be one of the most effective techniques for producing disease-free plants in all crops, especially citrus. The study reconfirms the precept that eliminating pathogens and keeping the same plants in a standard insect-proof greenhouse is very effective in protecting the plants from further spread of the HLB vector. The successful grafts produced through shoot tip grafting and then biological indexing showed no symptoms of greening except some nutritional deficiency symptoms. Besides production and use of cleaning planting materials, proper orchard management and protection from HLB vectors are critical. Therefore, the integrated application of all the above methods will not only help to manage the vectors and HLB disease but also help to revamp the citrus industry in the long run.

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