Field Test of Citrus Greening: the Scratch Method

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ABSTRACT

Huanglongbing (HLB) commonly known as citrus greening caused by Candidatus Liberibacter is a major destructive disease of citrus. As farmers are encouraged to use grafted saplings due to root-rot and foot-rot problems, infected scion used for grafted sapling production can be initial source of HLB infection. Established citrus orchards show various symptoms similar to HLB infection, and thus identifying HLB is difficult merely based on physiological disorder and nutrient deficiencies. Therefore, it is necessary to develop efficient, quick and simple diagnostic method for HLB. A scratch method, which is efficient, quick and simple diagnostic, was tested with newly developed LAMP method, which is more sensitive to HLB than conventional PCR. The study revealed that about 74% results from scratch method agreed with the results from LAMP. Moreover, the scratch method that required small effort and simple equipments proved to be reliable and its possibility to use for HLB diagnosis in field condition.

Key words: Citrus-greening, huanglongbing, iodine-test, LAMP, scratch-method

INTRODUCTION

Huanglongbing, previously known as citrus-greening, is a major destructive problem of citrus growing areas in the tropics and subtropics. It has been most serious threat to citrus industry in Asian countries like China, Thailand, Indonesia, India, Nepal, Pakistan and Bhutan. Graça (1991) presents an overview of citrus-greening worldwide. Roistacher (1996) and Ohtsu *et al.* (1998) reported that citrus-greening is one of the most destructive diseases of citrus in Nepal and a great threat to the future of citrus industry there. Unless the disease is understood and controlled, citrus will slowly but surely decline. Traditionally seedlings were used as planting materials. Recently, because of root-rot and foot-rot problems, trifoliate is being used as rootstock in mandarin and sweet orange. Free of HLB scion selection from mother plant is very important for quality saplings production. Therefore, establishment of HLB free mother-plant block and, for that, identification of HLB infected plant in established orchard is much crucial.

As of now, there are three types of HLB diseases known: the Asian (*Candidatus Liberibacter asiaticus*), the African (*Candidatus Liberibacter africanus*) and the American (*Candidatus Liberibacter americanus*) types. The disease is vector transmitted; commonly known as psylla. Of which two types namely Asian citrus psyllid (*Diaphorina citri* Kuwayama) and African citrus psyllid (*Trioza erytreae* del Guercio) are identified.

Rapid, sensitive and accurate diagnosis of HLB is an important step to control the disease. Ohtsu *et al.* (1998) classified 7 types of the HLB symptoms as important tools for its rapid diagnosis. HLB can be diagnosed by visual plant symptoms, HLB-specific fluorescent substance, Enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, Polymerase chain reaction (PCR), quantitative PCR (qPCR) and Loop-mediated isothermal amplification (LAMP). PCR, qPCR and LAMP are used to detect low-level *Candidatus Liberibacter asiaticus* infection in citrus trees (Okuda *et al.*, 2009), but these methods are

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time-consuming, expensive and not suitable for large number of samples in field condition. Because distinguishing HLB-infected trees from those of physiological disorder due to zinc and manganese deficiency is very difficult, PCR is commonly used method of HLB diagnosis. However, PCR assay is expensive, not suitable for a large number of samples and time-consuming. Such delays timely removal of infected-trees and allows the trees' longer stay in field to complicate the problem. Therefore, a rapid and accurate diagnostic test of HLB is important to control the disease, and, for the test, it is necessary to develop an efficient, quick and simple method. Previous study reveled that HLB infected citrus leaf accumulates starch granules (Schneider, 1968), and histological method using iodostarch reaction can be used for distinguishing HLB from other factors (Onuki *et al.*, 2002). In this study, a simple scratch method for investigating HLB was considered, and the reliability of this method was analyzed.

MATERIALS AND METHODS

Collection of samples

Samples were collected from trees with and without typical symptoms, and used as mother plant for sapling production at Central Horticulture Centre, Kirtipur, Kathmandu, Nepal. Nine different citrus lines containing 22 varieties used as mother plants were selected. Among the sample trees, 100 were grafted (18 varieties) and 15 were seedling (4 varieties). Fully grown leaf samples were taken from half of the tree height. Samples were immediately stored in ice box and used for both scratch and LAMP method.

Fig. 1: Iodine-starch reaction in the scratch method; yellow color HLB negative (a) and black color HLB positive (b)

Scratch method

This method is based on iodine-starch reaction. HLB-positive leaf accumulates more starch than HLB-negative leaf. Whole leaf sample was taken for the test. The leaf surface was ruptured by rubbing on it a small piece of abrasive paper (that should not produce any color in water) gently for 15-20 times. The piece of abrasive paper thus rubbed on the leaf surface that contained the leaf tissues was put into a small plastic bag with sealer. Roughly a milliliter of pure or distilled water was put into the bag followed by 2-3 drops (25 µl) of 0.05 M iodine solution. The plastic bag was then sealed and the iodine solution and the piece of abrasive paper with small pieces of leaf tissues were mixed gently by pressing and rubbing from outside. After mixing, the color of the water changes to black or dark brown if the sample is HLB infected or to yellow or pale yellow if the leaf sample is HLB-negative (fig.1).

LAMP

LAMP is known as sensitive detection method for confirmation of HLB symptoms (Manjunath *et al.*, 2008) and in this study it is used as reference method. LAMP kit is recently developed method which has alkali solution (NaOH) for extraction, Acetic acid solution for neutralization, Premix HLB, Fluorescent color, Enzyme and miliQ. In addition to these chemicals Iso-propanol and 80% ethanol need to be prepared. In this method, the midrib of a leaf sample (about 5 mm square) was excised with a razor blade. Two to three pieces of leaf samples were put in 1.5ml plastic tube. Alkali solution (250 mM NaOH) was used for DNA extraction. Samples were incubated at 95ºC for five minutes. Acetic acid

solution (concentration 2.5 M) was used for neutralization. Grinding sample is not necessary like conventional PCR. After removing the homogenate, propanol was added, and then centrifuged at 15000 rpm for 15 minutes. Upper phase of the liquid was discarded and DNA pellet remaining at the bottom of the tube was cleaned with 80% ethanol. After cleaning miliQ was added to dissolve the pellet (DNA sample) and stored at -20ºC. Premix HLB, Fluorescent color and Enzyme were mixed to prepare reaction mixture. Two µl of extracted DNA sample was mixed with 23 µl of prepared reaction mixture. Mineral oil was put little bit in each reaction tube and mixed properly. After mixing, it was incubated at 65ºC for an hour. After an hour the reaction tube was immediately removed and the color of liquid was observed. Green fluorescent color indicated HLB-positive sample and colorless negative (fig.2).

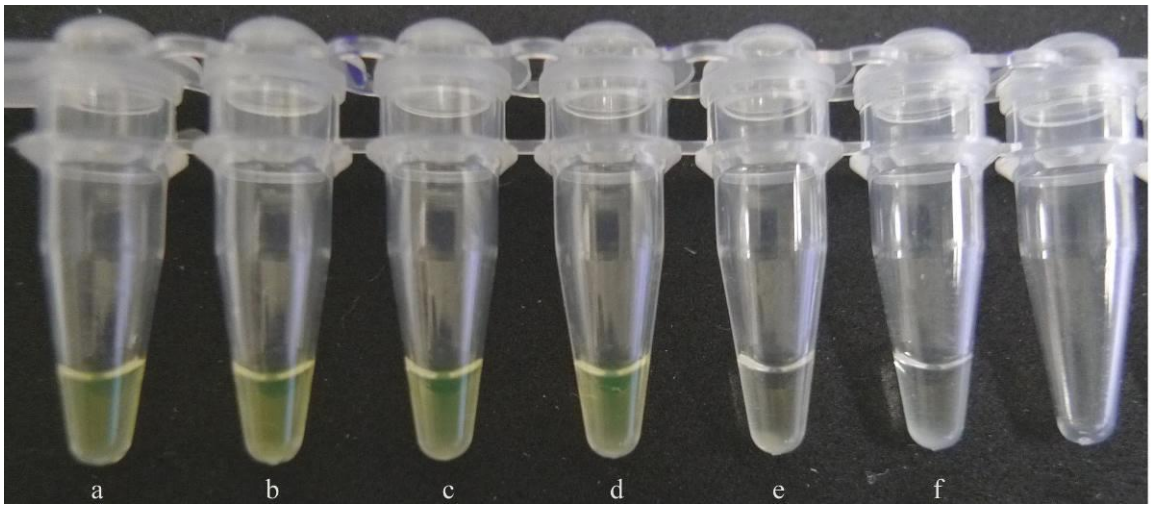

Fig.2: HLB detection by LAMP method; a, b, c and d - HLB positive and e and f - HLB negative

Based on LAMP result of the 115 samples, Yoshida Ponkan from Mandarin line, Miyagawa Wase from Satsuma Mandarin line, Taracco Nucellar, Blood Malta and Local selection from Orange line and Marsh Seedless from Grape Fruit showed HLB positive (Table 1). 13 samples from the six varieties showed positive result. Interestingly, all positive samples were grafted trees. Based on results of scratch method 22 samples from eight varieties showed HLB positive (Table 1). Three samples of Okitsu Wase and a sample of Thai selection gave positive result in this method but they were negative in LAMP. In this method the results of 16 samples were unclear. These unclear samples from scratch method had both positive and negative results in LAMP method.

Table 1: HLB diagnosis in citrus leaves by scratch and LAMP methods

Citrus Line	Name of Varieties	Tree Type	No. of Samples	Positive (+)		Negative (-)		Unclear (±)	
				LAMP	Scratch	LAMP	Scratch	LAMP	Scratch
Mandarin	Yoshida Ponkan	G	23	4	4	19	17	0	2
	Ohta Ponkan	S	4	0	0	4	4	0	0
	Thai Tangerin	G	3	0	0	3	3	0	0
	Hayaka	S	1	0	0	1	1	0	0
	Dekopong	S	1	0	0	1	1	0	0
	Commun	G	1	0	0	1	1	0	0

	Local	G	15	0	0	15	11	0	4
King Mandarin	Kinnow	G	1	0	0	1	1	0	0
Satsuma Mandarin	Miyagawa Wase	G	4	1	1	3	0	0	3
	Okitsu Wase	G	11	0	3	11	7	0	1
Tangors	Murkott	S	9	0	0	9	6	0	3
Orange	Taracco Nucellar	G	5	1	3	4	2	0	0
	Yoshida Navel	G	1	0	0	1	1	0	0
	Blood Malta	G	3	1	1	2	2	0	0
	Oroval	G	1	0	0	1	1	0	0
	Local selection	G	25	5	8	20	15	0	2
Tangelos	Orlando	G	2	0	0	2	2	0	0
Grape Fruit	Marsh Seedless	G	2	1	1	1	1	0	0
Pummelo	Thai Selection	G	1	0	1	1	0	0	0
	Local selection	G	1	0	0	1	0	0	1
Kumquat	Round Fruit	G	1	0	0	1	1	0	0

Table 2: Comparing diagnosis result of scratch method with LAMP

Citrus Line	No. of samples	Agreement	Disagreement	Result not clear
Mandarin	48	39	3	6
King Mandarin	1	1		
Satsuma Mandarin	15	7	4	4
Tangors	9	6	0	3
Orange	35	27	6	2
Tangelos	2	2		
Grape Fruit	2	2		
Pummelo	2		1	1
Kumquat	1	1		
Total	115	85 (73.9%)	14 (12.2%)	16 (13.9%)

RESULTS AN DISCUSSIONS

Table 2 indicated comparison of diagnostic result of scratch method and LAMP. Result indicated that 85 samples out of 115 (73.9%) gave same results in both diagnostic methods. However, scratch method results of 14 samples (12.2%) were opposite from the LAMP results. In addition, scratch method was unable to give clear results for 16 samples (13.9%). Since LAMP is more sensitive than scratch method, LAMP detected HLB from samples without any visual symptom. 73.9% results of scratch method agreed with the results of LAMP. Taba *et al.* (2006) found 75% agreement for citrus leaf samples between PCR results and iodo-starch assay. This finding supports our study and indicates the consistency of the result regardless of detection process modification and plant samples. In this study, some disagreement might be due to low infection to accumulate starch in leaves that did not produce any clear HLB symptom. From this evidence, scratch method can be used as simple, easy, quick and economical for large samples in field condition.

CONCLUSION

Scratch method, which is efficient, quick and simple diagnostic method for HLB, indicated 73.9% results agreed with LAMP. Thus it indicated that scratch method can be used to diagnose HLB infection. Furthermore, this method is useful to diagnose large number of samples in field condition. So, it might be important for quality sapling production in nursery and citrus orchard management.

ACKNOWLEDGEMENT

Our sincere thanks go to Japan International Cooperation Agency (JICA) Nepal Office for providing financial support for this study. We would also like to acknowledge Dr. Shinji Kawano, Plant Disease and Insect Pest Management Section, Okinawa Prefectural Agricultural Research Center, Japan and Mr. Dhan Bahadur Thapa, Central Horticulture Centre, Kirtipur, Kathmandu, Nepal for their technical support.

REFERENCES

Graça, J. V. da, 1991. Citrus greening disease. Annu. Rev. Phytopathol., 29: 109-136.

Manjunath, K. L., S. E. Halbert, C. Ramadugu, S. Webb and R. F. Lee, 2008. Detection of *Candidatus Liberibacter asiaticus* in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. Phytopathol., 98(4): 387-396.

Ohtsu, Y., N. Kazuo, P. Maitree and T. Yuichi, 1998. Typical symptoms of citrus greening on mandarin trees in Nepal, supported by detection and characterization of ribosomal DNA of the causal organisms. Ann. Phytopathol. Soc. Jpn., 64: 539-545.

Okuda, M., S. Kawano, T. Takushi and T. Iwanami, 2009. Differences in the rate of detecting low-level *Candidatus Liberibacter asiaticus* infection in spontaneously infected samples among DNA amplification methods. Kyushu Pl. Prot. Res., 55: 62-67.

Onuki, M., T. N. T. Nguyen, N. Hirohisa, T. T. H. Le and K. Hiromi, 2002. Useful histological method for distinguishing citrus yellowing leaves infected with huanglongbing from those caused by other factors. Proceedings of the 2002 annual workshop of JIRCAS Mekong Delta Project, pp.114-118.

Roistacher, C. N., 1996. A general certification program for citrus in Nepal with emphasis on the greening disease problem. Report of a consultancy visit to Nepal, April 24 to May 16, 1996.

Schneider, H., 1968. Anatomy of greening diseased sweet orange shoots. Phytopathology, 58: 1155-1160.

Taba, S., K. Nasu, K. Takaesu, A. Ooshiro and Z. Moromizato, 2006. Detection of citrus huanglongbing using an iodo-starch reaction. University of the Ryukyus Repository, 53: 19-24.