A presumptive field test for Huanglongbing (Citrus Greening Disease)

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ABSTRACT

Huanglongbing (HLB) or Citrus Greening Disease (CGD) is a serious bacterial disease of citrus and other members of the Rutaceae. The bacterium, "*Candidatus* Liberibacter asiaticus", resides and multiplies in the phloem tissue of the plant, causing the tissues to become necrotic. It is spread through planting material marcotted or grafted from infected source plants and by the insect vector, the Asiatic or Oriental Citrus psyllid, *Diaphorina citri* Kuwayama.

Due to its obligate nature, the bacterium cannot be cultured on any growth medium, hence making its detection difficult. The current international method of detection of this pathogen is by the molecular technique of polymerase chain reaction, PCR. This method is time consuming, but if carried out correctly, can detect very low concentrations of the bacteria in the plant tissue. The precision of this method depends on the sampling technique, tissue processing, DNA extraction and PCR procedures, coupled with the experience of the laboratory staff. Other methods of HLB detection are the use of monoclonal antibodies, DNA-DNA hybridization probes, electron microscopy and thin-layer chromatography to detect the presence of gentisic acid. All these methods are laborious and hence it can take months to screen thousands of trees in a citrus orchard using these methods.

With the introduction of the presumptive field test using the principle of iodine-starch reaction, precious time taken to screen all the trees in a citrus orchard is cut down tremendously. This also reduces the cost of detection, and the manpower requirement. A high level of expertise in carrying out the test is not required, thus enabling growers to carry out the tests themselves. The accuracy of this scratch test ranges from 74.5% to 89.5% in mandarins. For pummelo, the accuracy ranges from 12.5% to 51.7% using this test. However, the accuracy of this test is highly dependent on the correct selection of the infected leaves and the concentration and quality of the iodine solution used in the test.

This paper describes the field testing carried out to verify the accuracy of the presumptive iodine-starch scratch test on honey mandarin (*Citrus reticulata*) and pummelo (*Citrus maxima*) by gauging it against the PCR technique. It also describes the testing of the different iodine formulations, and the type of abrasive paper to be used.

1. INTRODUCTION

Huanglongbing or Citrus Greening Disease is one of the most important diseases of citrus. It is caused by an obligate bacterium, "*Candidatus* Liberibacter asiaticus", a phloem-restricted organism. This bacterium has an insect vector, the Asiatic or Oriental Citrus psyllid, *Diaphorina citri* Kuwayama. Its other modes of spread are through grafting and marcotting using infected material. There is no record of seed transmission.

The disease symptoms are a reduction in leaf size and presence of leaf interveinal chlorosis that are often mistaken as those caused by mineral deficiencies due to zinc,

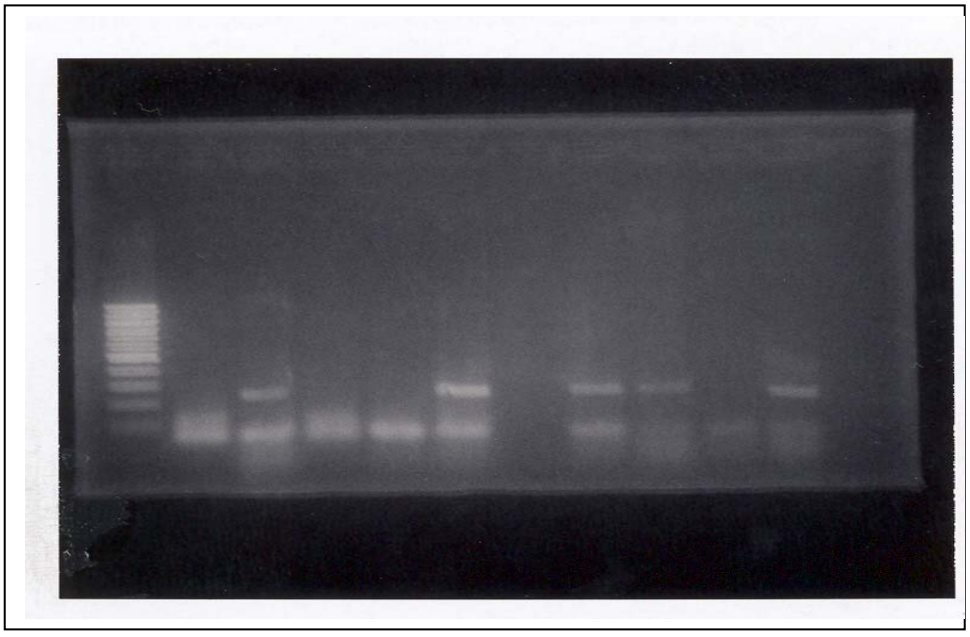
iron or magnesium. The fruit remain small and lopsided, with small, dark aborted seeds. Fruit drop and dieback of branches occur, eventually leading to the death of the tree.

Apart from Malaysia, this disease is prevalent in Thailand, Indonesia, Vietnam, the Philippines, Taiwan, China, Japan, India, Bangladesh and South Africa. In South Africa, the vector is a different psyllid, *Trioza erytreae*, and the bacterium is "*Candidatus* Liberibacter africanus". In 2005, this disease was found in backyard citrus in southern Florida.

Disease detection methods, including the current international standard of PCR, are the use of monoclonal antibodies, DNA-DNA hybridization probes, electron microscopy and thin-layer chromatography to detect the presence of gentisic acid. All these methods require expensive laboratory equipment and are laborious; hence it can take months to screen thousands of trees in a citrus orchard using these methods.

The Plant Pathology Laboratory at the Agriculture Research Centre has been using the PCR technique to test for HLB. The process of PCR can take about 2 days, not including the preparation time for the leaf material after field sampling. After sampling, the leaf laminae have to be stripped off, and the mid-ribs cut and dried. The DNA of the mid-ribs including that of the bacteria (if present) is then extracted. A set of primers specific to the bacteria is then used to isolate the bacterial DNA from the plant DNA, which is then amplified using the PCR technique. The amplified DNA is then separated based on its molecular size by the process of gel electrophoresis. The bacterial DNA (if present on the agarose gel) can be seen when the gel is viewed under UV light (Fig. 1).

Figure 1: An agarose gel showing the results of field samples infected with HLB.
(Lane 1=Molecular Marker; Lanes 3,6,8 & 9 = Infected Samples; Lane 11 = Diseased Control)



Schneider (1968) observed that the HLB-infected leaves have a high accumulation of starch in all the parenchyma cells of the leaves. The disease causes necrosis of the phloem, resulting in the blockage of the translocation in the vascular system. Replacement phloem proliferates to bypass the blockages. This late stage of phloem proliferation is associated with the severe vein corking.

This observation of starch accumulation in HLB-infected leaves by Schneider (1968) was not fully exploited until lately. Starch has two components, amylose and amylopectin. Amylose turns dark blue while amylopectin turns purple when both react with iodine (Shannon & Garwood, 1984). Le and Nguyen of Vietnam used ground infected leaf tissue spotted onto nitrocellulose membrane and then adding 2 µL of 2% of 0.5 M iodine solution before observing the colour change on the membrane. They reported that this method gave 8.9% false negative, 3% false positive, 88.8% sensitive index and 65.9% specific index,

where the Sensitive index, $C\ (\%) = \frac{\text{Positive samples}}{\text{Positive samples + False negative samples}} \times 100$

and the Specific index, $D\ (\%) = \frac{\text{Negative samples}}{\text{Negative samples + False negative samples}} \times 100$.

Taba, Nasu, Takaesu, Ooshiro and Moromizato (2006) tried a different method. They cut a citrus leaf into 5x5 mm pieces, crushed and softened them with hot water for 10 min. A 100 µL sample of the sap was then placed in 40 µL 5 mM iodine solution in a micro-plate well, and then checked for colour change. They reported that a 5 mM solution gave stable and reliable purple colour and provided a clear distinction between healthy and infected leaves. Takushi, Toyozato, Kawano, Taba, Taba, Ooshiro, Numazawa and Tokeshi (2007) demonstrated the scratch method (that is being used in this study) using 50 mM of iodine solution, and observed that the iodine test and the PCR were more than 90% in agreement. They also reported that this method did not give HLB-positive reactions for healthy, nutrient-deficient or other diseased leaves infected with Citrus tatter leaf virus and Hop stunt viroid. Prof. Su of Taiwan also reported that this test does not give a positive reaction for nutrient deficient leaves or leaves infected with other diseases (pers. com.).

2. MATERIALS AND METHODS

2.1 Testing the different iodine formulations

Four formulations of iodine solution were tested using the scratch test. They were 1.2% (47mM), 1% (39mM), Gram (0.33% or 13mM) and 5mM (0.13%) iodine formulations. The first formulation was chosen as it is similar to some commercially produced laboratory iodine solution. The 1 % solution was what was first used by this laboratory in the starch test. The Gram formulation is normally used in the laboratory for staining bacterial cell in the Gram stain procedure. The 5 mM was for comparison with Taba *et al.* 's (2006) formulation. Five leaves per tree from 10 honey mandarin (limau madu) trees

that tested positive for HLB using PCR, were tested with the four formulations. For the control, five healthy leaves each from honey mandarin and langkat were tested using the four formulations of iodine solution.

2.2 Testing the different types of abrasive paper

Different types of abrasive paper were tested: Three types of glass paper (Sail brand abrasive papers No. 0, No.1, and No. 80), and three brands of 120 mesh size silicon carbide paper (Eye, Riken and Swallow), were cut into small rectangles of 1x½ inch each. The small piece of abrasive paper was then used to scratch the upper surface of an infected leaf for at least 20 times before being put into a small polythene bag (about 3" x 2") containing 1 ml of distilled water. The tissue scrapings were gently washed off from the abrasive paper into the water by gently rocking the bag by hand. The paper was removed from the liquid suspension and a drop (about 30-50 µl) of iodine solution (1%) was added to it. Healthy controls using healthy leaves were also tested similarly for each type of abrasive paper. Each type of paper was tested five times. A background colour control for each type of paper was also carried out. Here each type of the abrasive paper was used to scrape a piece of aluminium foil, before being put into 1 ml of distilled water in a polythene bag. A drop of iodine was added to see if there was any colour change. This background colour control test was to check if the abrasive paper itself contained any starch.

2.3 Comparison between the PCR and scratch methods on honey mandarin (*Citrus reticulata*)

In the first study here, fifty-five honey mandarin trees in a citrus orchard in Asajaya, which were tested positive for PCR were used in this study. Five leaves per tree were used in the iodine scratch test described above. The concentration of the iodine was 1%.

In the second study, 30 honey mandarin trees in a citrus orchard in Batu Kawa with mineral deficiency symptoms were used. A PCR test was carried out on the 30 trees to determine its HLB-infection status. At the same time, five leaves per tree were used in the 1% iodine scratch test.

2.4 Comparison between the PCR and scratch methods on pummelo (*Citrus maxima*)

In this study, eight pummelo trees from a citrus orchard in Asajaya were used. Since the pommelo trees have three categories of symptoms: no symptoms, mineral deficiency/HLB symptoms and vein corking symptoms, five leaves of each type of symptom from each tree were collected for the 1% iodine test. Similar leaf samples had been collected for PCR analysis to determine their HLB status.

3. RESULTS AND DISCUSSION

3.1 Testing the different iodine formulations

Out of the four formulations, the 1.2% and 1% of iodine formulations had nine trees with all five leaves showing positive reactions, giving a 90% positive result (Table 1). For both the Gram iodine and the 5 mM iodine solutions, none of the trees have all 5 leaves showing positive reactions. All the leaves for all the 10 HLB-infected trees in the 5 mM formulation of iodine did not give any positive reaction. However, if the criterion of having a minimum of three leaves with positive reaction is taken into consideration, the percentages would be 100%, 100%, 40% and 0% for the 1.2%, 1%, Gram and 5 mM iodine formulations, respectively. There were no positive reactions for all the control healthy leaves from honey mandarin and langkat tested. The possible explanation for the non-reaction of the 5 mM solution here, when compared with the method used by Taba *et al.* (2006), was that they used only 100 µL of the diluted leaf sap in the 5 mM iodine reaction, whereas one mL was used here. This dilutes the reaction and hence the reaction results cannot be seen in this scratch method. It can be determined here that the 1.2% and 1% formulation are more suitable for the scratch test.

Table 1: Comparisons between the different iodine formulations using leaf samples from HLB-infected trees

Tree No.	1.2 % (47mM) iodine			1% (39mM) iodine			Gram (0.33% / 13mM)			5mM (0.13%) iodine		
	No. of positive	No. of negative	Overall iodine result	No. of positive	No. of negative	Overall iodine result	No. of positive	No. of negative	Overall iodine result	No. of positive	No. of negative	Overall iodine result
1	5	0	+*	5	0	+	2+, 3±	0	+/-	0	5	-**
2	5	0	+	4	1	+/-	3+, 1±	1	+/-	0	5	-
3	5	0	+	5	0	+	1+, 3±	1	+/-	0	5	-
4	4+, 1±	0	+/-***	5	0	+	3+	2	+/-	0	5	-
5	5	0	+	5	0	+	3±	2	+/-	0	5	-
6	5	0	+	5	0	+	4+	1	+/-	0	5	-
7	5	0	+	5	0	+	0	5	-	0	5	-
8	5	0	+	5	0	+	1+, 2±	2	+/-	0	5	-
9	5	0	+	5	0	+	2±	3	+/-	0	5	-
10	5	0	+	5	0	+	3+, 2±	0	+/-	0	5	-
Percentage of trees with 5 leaves that tested positive with iodine =			90%			90%			0%			0%
Percentage of trees with at least 3 leaves that tested positive with iodine =			100%			100%			40%			0%

*+ = positive reaction -** = no reaction ±*** = mild reaction

3.2 Testing the different types of abrasive paper

Except for the glass paper (Sail brand No.1) there were no reactions for all the background colour control tests. When iodine was added, the suspension for the former turned purplish black immediately, indicating the presence of starch in the suspension, even though no leaves were used. This starch could be used in the manufacturing of this glass abrasive paper.

The silicon carbide brands, especially the Eye and Swallow brands, produced a more determinative colour than the glass abrasive papers. This could be due to the silicon carbide papers being more efficient in scraping off the leaf tissues. It is advised here that background colour control tests be carried out before using any abrasive paper of unknown quality for the presumptive field test to avoid any false positive results.

3.3 Comparison between the PCR and scratch methods on honey mandarin (*Citrus reticulata*)

Table 2 shows that the accuracy of this test ranged from 74.5% to 89.5% in honey mandarin when compared with the PCR technique. The percentage of trees with all five leaves testing positive using iodine was 74.5%, and the percentage of leaves that tested positive for all the 55 trees using iodine was 89.5%. If the criterion of trees having at least 3 leaves testing positive using iodine is taken into consideration, the percentage was increased to 92.7%.

Table 2: Iodine test results of 55 honey mandarin trees that tested positive by PCR in a citrus orchard in Asajaya

Tree No.	No. of positive reactions	No. of negative reactions	Overall iodine result	Tree No.	No. of positive reactions	No. of negative reactions	Overall iodine result
1	5	0	+	**29**	5	0	+
2	5	0	+	**30**	5	0	+
3	4	1	+/-	**31**	5	0	+
4	5	0	+	**32**	5	0	+
5	5	0	+	**33**	3	2	+/-
6	5	0	+	**34**	5	0	+
7	5	0	+	**35**	5	0	+
8	5	0	+	**36**	5	0	+
9	5	0	+	**37**	5	0	+
10	5	0	+	**38**	3	2	+/-
11	5	0	+	**39**	5	0	+
12	5	0	+	**40**	5	0	+
13	3	2	+/-	**41**	5	0	+
14	2	3	+/-	**42**	5	0	+
15	5	0	+	**43**	5	0	+
16	5	0	+	**44**	5	0	+
17	3	2	+/-	**45**	5	0	+
18	5	0	+	**46**	3	2	+/-
19	5	0	+	**47**	2	3	+/-
20	5	0	+	**48**	5	0	+
21	2	3	+/-	**49**	5	0	+
22	5	0	+	**50**	5	0	+
23	5	0	+	**51**	5	0	+
24	3	2	+/-	**52**	4	1	+/-
25	1	4	+/-	**53**	5	0	+
26	5	0	+	**54**	5	0	+
27	5	0	+	**55**	4	1	+/-
28	4	1	+/-	**-**	**-**	**-**	
	122	**18**	**20 totally positive**		**124**	**11**	**21 totally positive**
Percentage of trees with all 5 leaves testing positive using iodine = 41/55 x 100 = 74.5%							
Percentage of trees with at least 3 leaves that tested positive using iodine = 51/55 x 100 = 92.7%							
Percentage of leaves that tested positive for all the 55 trees using iodine = 246/275 x 100 = 89.5%							

Table 3 shows that the possibility of having a false negative can be 10%. Both the percentages of trees with false negative iodine results and of leaves that tested negative with iodine were 10% for all the 30 trees tested. This 10% of false negatives concurs with the 89.5% of positives detected by the iodine test in the previous test (Table 2). All the 30 trees showed mineral deficiencies, but only three of them tested positive for HLB. This could indicate that the trees could possibly have mineral deficiencies due to lack of fertiliser or other causes. It was noted that some of these trees have trunk symptoms similar to those caused by the woody gall virus (Prof. Su H.J., pers. comm.).

Table 3: Comparison between the PCR and 1% iodine tests in a honey mandarin orchard in Batu Kawa

Tree No.	PCR result	No. of leaves with negative iodine result out of a total of 5 leaves tested	Tree No.	PCR result	No. of leaves with negative iodine result out of a total of 5 leaves tested
1	-	5	**16**	-	5
2	-	5	**17**	-	5
3	-	5	**18**	-	5
4	+	5	**19**	-	5
5	+	5	**20**	-	5
6	-	5	**21**	-	5
7	-	5	**22**	-	5
8	-	5	**23**	-	5
9	-	5	**24**	-	5
10	-	5	**25**	-	5
11	-	5	**26**	-	5
12	-	5	**27**	-	5
13	+	5	**28**	-	5
14	-	5	**29**	-	5
15	-	5	**30**	-	5
TOTAL	3+, 12-	75	TOTAL	15-	75

Percentage of trees with false negative iodine results = 3/30 x 100 = 10%
Percentage of trees that tested negative with iodine = 30/30 x 100 = 100%
Percentage of leaves with false negative iodine results = 15/150 x 100 = 10%
Percentage of leaves that tested negative with iodine = 100%
Percentage of trees that tested negative using PCR = 27/30 X 100 = 90%

3.4 Comparison between the PCR and scratch methods on pummelo (*Citrus maxima*)

Table 4 shows that in the case of pummelo, the accuracy of the results between the starch test and PCR is less than that for honey mandarin. In this case, the percentages where all five leaves tested positive for leaves with no symptoms, with deficiency/ HLB symptoms and with vein corking symptoms were 0%, 25% and 12.5%, respectively. If the criterion of having at least 3 leaves testing positive was taken into consideration, the values were increased to 12.5%, 75% and 75% for no symptoms, with deficiency/HLB symptoms and with vein corking symptoms. The percentage of leaves that tested positive using iodine for all eight trees was 51.7%.

In four cases, the starch test had some positive reactions whereas the PCR results were negative. This could be due to the fact that the protocol for PCR for testing mandarins was not optimised for pummelo. There could be inhibitors in the DNA extracts for pummelos interfering with some of the samples during the PCR process, thereby hindering its reaction. Spectrophotometric readings of the DNA extracts for all the eight pummelo trees showed a wide variation in the concentrations of the DNA.

Table 4: Comparison between the PCR and 1% iodine tests on pummelo leaves, with different symptoms

Tree No.	Leaves without symptoms			Leaves with mineral deficiency/ HLB symptoms			Leaves with vein corking		
	PCR result	Individual leaf result	Overall iodine result	PCR result	Individual leaf result	Overall iodine result	PCR result	Individual leaf result	Overall iodine result
1	+	3+, 2±	+/-	+	5+	+	+	3+, 2±	+/-
2	+	2+,1±, 2-	+/-	+	4+, 1-	+/-	-	5±	+/-
3	-	2+, 3-	+/-	+	5+	+	+	2+, 1±, 2-	+/-
4	+	2+, 3-	+/-	+	4+, 1-	+/-	+	4±, 1-	+/-
5	+	1+, 1±, 3-	+/-	+	3+, 1±, 1-	+/-	+	5+	+
6	+	3±, 2-	+/-	+	2+, 3-	+/-	+	1+, 1±, 3-	+/-
7	+	2+, 3-	+/-	-	2+, 1±, 2-	+/-	+	4+, 1±	+/-
8	+	2+, 1±, 2-	+/-	-	4+, 1±	+/-	+	4+, 1±	+/-
Percentage of trees with all 5 leaves testing positive using iodine =			0%			25%			12.5%
Percentage of trees with at least 3 leaves testing positive =			12.5%			75%			75%
Percentage of leaves that tested positive using iodine for all the 8 trees = 62/120 x 100 = 51.7%									

4. CONCLUSIONS

This technique of starch testing with 1-1.2% iodine solution is reliable enough to detect HLB in mandarin leaves. For pummelo, more than five leaves have to be tested, and careful selection of leaves with HLB symptoms, including vein corking, must be made. This technique can be recommended to growers so that they can carry out their own field disease monitoring. However, they should be taught the technique properly to ensure the accuracy of the tests. In the areas of uncertainty, more leaves can be tested, bearing in mind the sectorial pattern of disease expression by HLB on trees infected through the psyllid vectors.

The technique is cheap and rapid, without a need for sophisticated equipment. It can be used widely and can hasten the detection of HLB throughout the State of Sarawak.

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