Iodine Reaction Quick Detection of Huanglongbin Disease

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Abstract

PCR has been considered as the most effective, quick and correct methodology of Huanglongbin detection in citrus of Mekong Delta Vietnam (Hong et al 2001). However, in order to help farmers for quick detection on field, Iodine Reaction (IR) showed a potential practice (Truc and Hong 2002, Onuki et al 2002). Therefore, this investigation aimed to improve and evaluate IR detection on NCM for Huanglongbin quick detection. The method gave 8.9% false negative, 3.0% false positive, 88.8% sensitive Index and 65.9% specific index.

Introduction

Starch is formed in leaf during the day and translocated during the night. HLB infected yellowing leaves showed this starch granules accumulation (Schneider, 1968). When infected with HLB disease, the accumulation of starch on citrus leaves was 16-20 times higher than that of other virus/stressed citrus plants (Hong et al, 2002).

This phenomenon was closely involved in symptom development and were confirmed by histological method with positive iodostarch reaction (Masatoshi Onuki et al, 2002). The IR on NCM (Nitro cellulose membrane) was investigated resulted a potential quick detection of HLB (Truc et al, 2003). Therefore, this investigation was conducted to study the effect of other factors on the accuracy of IR quick detection for citrus field condition.

Materials and Methods

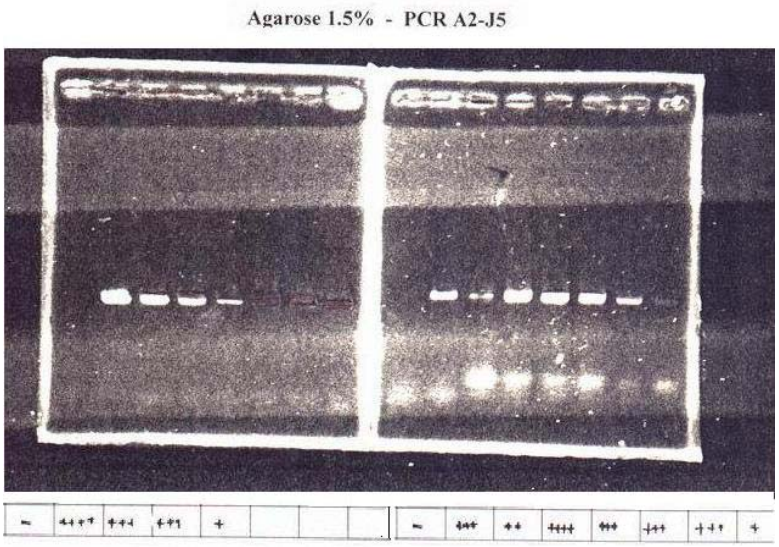
Citrus samples: Citrus leaves were collected from Tien giang, Dong thap, Ben tre, Da lat, Dong nai and Can tho provinces.

IR test on NCM was simply done by steps of samples in plastic bag with distilled water (1:1). Drop 2 µl of this liquid into the Nitrocellulose membrane (NCM). Wait 5 minutes until the liquid dry. Drop 2 µl of 2% of 0.5M iodine solution. Observe the changing color in the NCM.

PCR: To evaluate practical utilization of IR for HLB diagnosis, polymerase chain reaction (PCR) was conducted and its results were compared with those of IR. Total nucleic acid of citrus samples was extracted using CTAB method of Nakashima *et al*. (1996) with slight modification. The midrib of a citrus leaf was excised with a razor blade and homogenized in a mortar and pestle with CTAB solution (2% (w/v) cetyltrimethylammonium bromide, 100 mM Tris-HCl (pH8.0), 1.4 M NaCl, 20 mM EDTA, 1% (w/v) polyvinylpyrrolidone, 1% β-mercaptoethanol). After removing the homogenate to 1.5 ml micro centrifuge tube, the homogenate was incubated at 65^0 C for 10 min, then centrifuged at 15,000 rpm for 10min. The supernatant was transferred to a new tube and mixed gently with an equal volume of chloroforme-isoamyl alcohol (24:1). After centrifugation of 15,000 rpm for 10min, The supernatant was transferred to a new tube and mixed with an equal volume of ice-cold isopropanol, then centrifuged at 15,000 rpm for 5 min. The precipitate was washed with 70% ethanol, dried and resuspended in a small amount of sterile distilled water (ca. 30 μ l).

PCR was carried out using Ready-To-Go PCR Beads (Amersham pharmacia biotech) or *Ex Taq* kit (TaKaRa) according to the instruction manuals. The 25 μ l of PCR reaction mixture contained 1 μ l of nucleic acid solution as a template and primers at a concentration of 0.4 μ M. The primers, OI1 and OI2C, originally designed by Jagoueix *et al*. (1994) for amplification of 16S rDNA region of HLB pathogen were used. The thermal conditions for PCR were as follows: 95^0 C for 2 min; 35 cycles, each consisting of 95^0 C for 40 s, 60^0 C for 1 min and 72^0 C for 1 min; and 72^0 C for 10 min. Eight μ l of PCR-terminated solution was electrophoresed in a 1% agarose gel in Tris-acetate-EDTA buffer (40 mM Tris-acetate, pH8.0, 1 mM EDTA). DNA bands were visualized using ultraviolet light after staining in an ethidium bromide solution.

505 samples of total from 130 trees, with all kinds of symptom, and different parts of trees. The results were evaluated from: + to ++++ by the size of DNA band (PCR) and by the color reaction (IR)



Evaluated grade with IR

++++ (4 +) +++ (3+) ++ (2+) + (1+) - (negative)

Figure 1: Evaluated grade by PCR and IR methods

We used the followings to define the index of method evaluation:

$$\text{* Percentage of false negative A \%} = \frac{\text{False negative samples}}{\text{Total of tested samples}} \times 100$$

$$\text{* Percentage of false positive B \%} = \frac{\text{False positive samples}}{\text{Total of tested samples}} \times 100$$

Total of tested samples = Positive samples + negative samples + False negative samples+ false positive samples

Sensitive index (C) (%)

$$\text{* C} = \frac{\text{Positive samples}}{\text{Positive samples + False negative samples}} \times 100$$

Specific index (D)(%)

$$\text{D} = \frac{\text{Negative samples}}{\text{Negative samples + False negative samples}} \times 100$$

Results and Discussion

1) IR result on NCM of different HLB infected citrus varieties

Different varieties expressed different symptoms of HLB and their reaction to IR and PCR were showed. Almost of varieties gave similar reaction on both IR and PCR from +++ to ++++ (Table 1). Four citrus species, i.e., tau lemon, giay lemon, num lemon, and hanh gave weak IR positive reaction (from + to ++). However, the stained color of positive was clearly differentiated (Figure 2).

Table 1: Effect of different Citrus varieties on the HLB disease IR test (SOFRI, 2003)

Seri. No	Local name	Scientific name	Detection result PCR	IR
1	Cam mat	*Citrus sinensis*	++++	++++
2	Cam sanh	*Citrus sinensis*	++++	++++
3	Cam soan	*Citrus sinensis*	+++	+++
4	Quyt tieu	*Citrus reticulata*	++++	++++
5	Quyt duong	*Citrus reticulata*	+++	++++
6	Quyt ta	*Citrus reticulata*	++++	++++
7	Chanh tau	*Citrus lemon*	+++	+++
8	Chanh giay	*Citrus lemon*	++	+
9	Chanh num	*Citrus lemon*	++	++
10	Buoi5 roi	*Citrus maxima*	++++	++++
11	Buoi long	*Citrus maxima*	+++	+++
12	Buoi duong	*Citrus maxima*	++++	++++
13	Sanh ngot	*Hybrid*	+++	+++
14	Sanh chua	*Hybrid*	+++	++++
15	Hanh	*Citrus microcarpa*	++	+

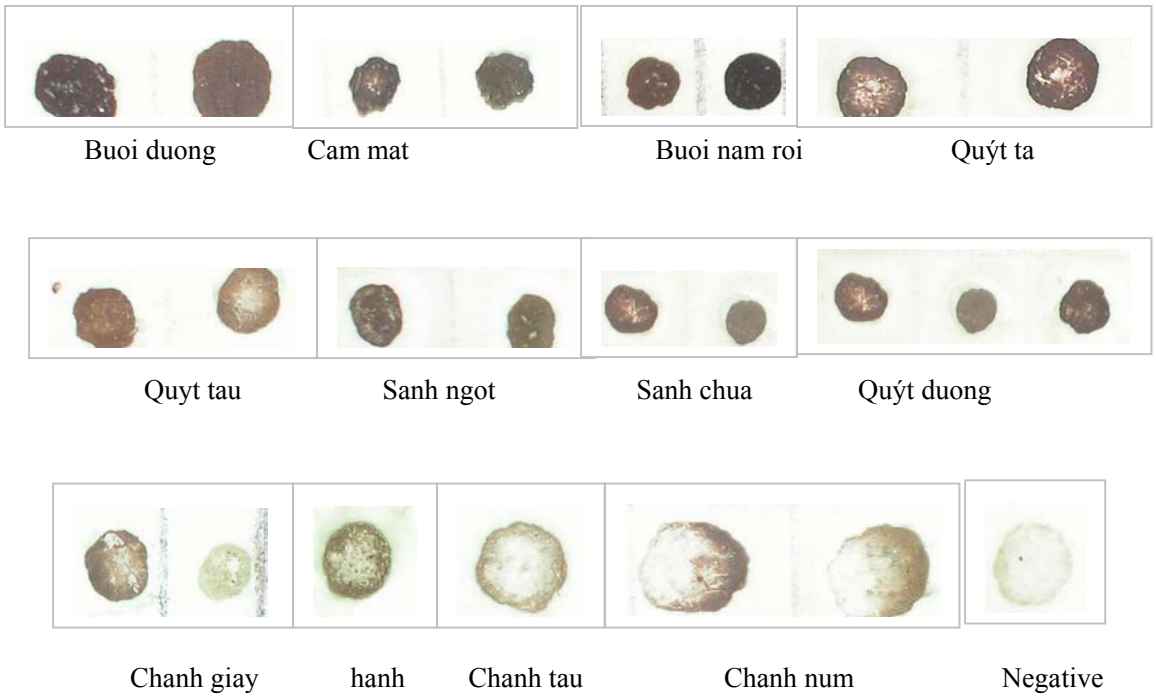


Figure 2: Different citrus varieties to the HLB disease's quick test

2) IR result on NCM of different plant ages of HLB infected citrus

Table 2 showed that all 15 citrus species gave the same result in term of age influence on IR stained: the more infected aged, the better IR result show on NCM color (Table 2).

Table 2: Effect of different plant ages on the HLB IR test (SOFRI, 2003)

Seri.No	Plant age / cultivars	1-3 years old	4-6	> 7
1	Cam mat	+++	++++	++++
2	Cam sanh	+	+++	++++
3	Cam soan	+	+	+++
4	Quyt tieu	++++	++++	++++
5	Quyt duong	+	++++	++++
6	Quyt ta	++	+++	++++
7	Chanh tau	++	+++	+++
8	Chanh giay	+	+	+
9	Chanh num	+	++	++
10	Buoi5 roi	++++	++++	++++
11	Buoi long	++	+++	+++
12	Buoi duong	+	++	++++
13	Sanh ngot	++	+++	+++
14	Sanh chua	+	++++	++++
15	Hanh	+/-	+	+

3) IR result on NCM of different HLB symptoms

All five performance of symptoms of HLB infected trees showed IR positive. Mottling is considered as the identical symptoms of the disease, however, many other symptoms secondary of micro-nutrient deficiency were also observed. Mottling and Zn deficiency gave the IR positive 4+. Mn deficiency, vein corking got the IR positive from 2+ to 3+ (Table 3). IR result were varied in different varieties. Result of positive IR were from mat orange, king mandarin, soan orange, tieu, duong orange, ta mandarin, 5 roi pomelo, long pomelo is much higher than in lemon, hanh, and sanh varieties though collected with similar symptoms (Table 3, Figure 2).

Table 3: Effect of different symptoms to the HLB IR test (SOFRI, 2003)

(Average of 30 samples/ each variety)

Seri .No	Varieties	Symptoms							
		Zn deficiency	Mottling	Mn deficiency	Yellow leaf, yellow vein	Vein corking	Young leaf	Oldest leaf	Note
1	Cam mat	++++	++++	+++	+++	++++	-	+++	
2	Cam sanh	+++	+++	++++	+++	+++	-	+++	
3	Cam soan	+++	+++	++	+++	+++	-	+++	10 years old tree
4	Quyt tieu	++++	++++	++++	++++				
5	Quyt duong	++++	+++	++	++		-		
6	Quyt ta	+++	+++	+++	+++	++++	-	++++	
7	Chanh	+++	+++	+++	+++		-		

	tau								
8	Chanh giay			+	+	+	+		
9	Chanh num		+	++	++	++			
10	Buoi roi	5	++++	++++	++++	++++	++++	-	++++
11	Buoi long		+++	+++	++	+			
12	Buoi duong		++++	++++	-	++++	++++		
13	Sanh ngot			++		+++		-	
14	Sanh chua		++++	+	+	+			
15	Hanh			+			+	-	

In comparison between young leaf and older leaf, the reaction of sample from the older leaf is better than that of young leaf. It maybe due to the accumulation of starch in older leaf is higher in the young leaf.

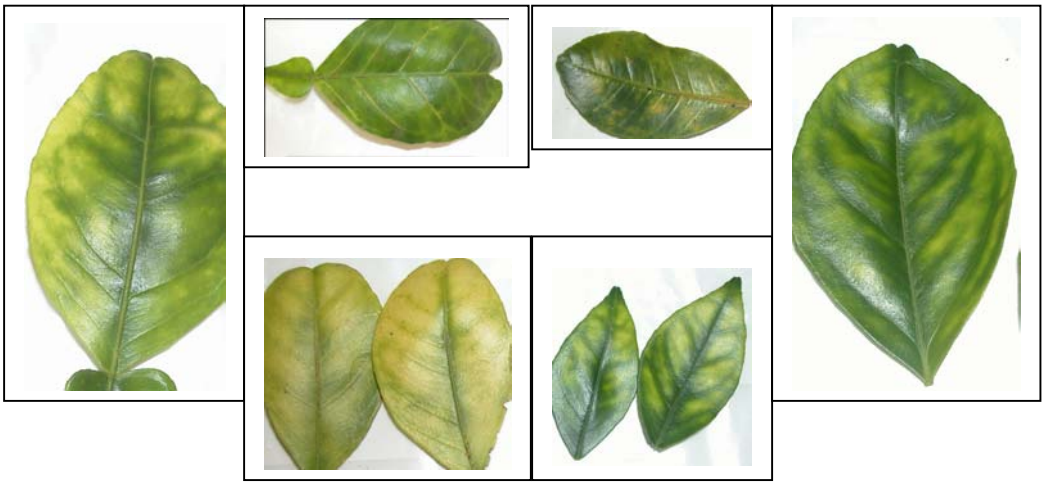


Figure 3: Different symptoms caused by HLB on different Citrus cultivars

4) IR result on NCM of different collecting times of samplings of HLB

Twenty four samples were collected in the morning, noon and evening of the day on the same 6 HLB infected trees, 6 healthy trees. IR positive were The results showed that, the result of samples collect in the morning is better than that of evening time.

Table 4: Effected of colleting time on the result of HLB disease's quick test

Sr.No	Treatment	Timings of sample collecting		
		5 AM	12 PM (noon)	18 PM
1	HLB infected Sweet orange	++++	++++	+++
2	Healthy sweet orange	-	-	-/+

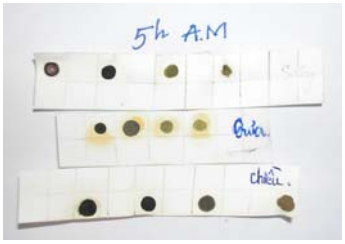


Figure 4: IR result on different collecting timings of samplings

5) IR result on NCM of citrus HLB collect from different locations

There were no different in IR result of almost of citrus cultivars from different locations (Table 5).

Table 5: Reaction of different varieties in different locations on the HLB disease's quick test

Seri.No	Areas / Variety	Mekong delta (low land)			South east (high land)	High mountaineous land
		Tien giang	Can tho	Ben tre		
1	Cam mat	++++	++++	++++	++++	+++
2	Cam sanh	++++	++++	++++	++++	++++
3	Cam soan	+++	+++	+++		
4	Quyt tieu	++++	++++		++++	
5	Quyt duong	++++	++++	++++	++++	
6	Quyt ta		++++			
7	Chanh tau	+++	+++	+++		
8	Chanh giay	++	++	++		
9	Chanh num	++	++	++		
10	Buoi 5 roi	++++	++++	++++		
11	Buoi long	+++	+++	+++		
12	Buoi duong	++++	++++	++++		
13	Sanh ngot	+++	+++	+++		
14	Sanh chua	+++	++++	+++		
15	Hanh	++	+	++		

6) IR result on NCM of different parts of the plants

The starch accumulate in the HLB infected leaf is 20 times this one more than that of the other leaf, but we try to test the Iodine reaction in the other parts of the infected tree such as: root, trunk, fruit etc. The conclusion was that we should not use other parts of the tree for detection, the PCR positive was very light, from 1+ to 2+ (Table 6).

Table 6: Reaction of different parts of the plants to the IR test (SOFRI, 2003)

Seri.No	Parts / Variety	Leaf	Fruit	Root	Trunk
1	Cam mat	++++	++	++	+
2	Cam sanh	++++	+	++	+
3	Cam soan	+++	++	++	+
4	Quyt tieu	++++	++	+++	+
5	Quyt duong	+++	+	+	+
6	Quyt ta	++++	++	++	+
7	Chanh tau	+++	+	+	+
8	Chanh giay	++	+	+	+
9	Chanh num	++	+	+	+
10	Buoi 5 roi	++++	++	++	+
11	Buoi long	+++	++	++	+
12	Buoi duong	++++	++	++	+
13	Sanh ngot	+++	+	+	+
14	Sanh chua	+++	+	+	+
15	Hanh	++	+	+	+
16	Heath plant	-	-	++	-

Figure 5: The different parts of the HLB infected Citrus tree

Minor IR result could be observed from fruit, root, trunk, however, these were significantly different to the best result obtained from leaf sample (Table 6). It was, of course, starch accumulation was mainly in leaf due to its translocation was decreased by the pathogen disturbance.

7) IR evaluation index: False negative percentage, false positive percentage, sensitive index and specific index

Among 505 samples detected, there were 45 false negative; IR false negative means IR negative but PCR positive and the plant had the HLB infected symptoms, 15 false positive; IR false positive means IR positive but PCR negative and the tree looks healthy. IR positive was 358 samples, IR negative was 87 samples (Table 7)

Table 7: The detected samples number by IR and PCR method

PCR	+	-	+	-	Total	Note
	98			32	130	
			15			False Positive
		45				False negative
	373			87	505	
IR	+	+	-	-		

7.1. False negative percentage of this IR method was 8.9 % meaning that 100 samples tested may give 8.9 false negative and similar explaination for false positive percentage with 3.0 %. The IR test were sensitive up to 88.8% for HLB detection.

$$\text{* False negative percentage A \%} = \frac{\text{False negative samples}}{\text{Total of tested samples}} \times 100 = \frac{45}{505} \times 100 = 8.9\ \%$$

$$\text{* False positive percentage B \%} = \frac{\text{False positive samples}}{\text{Total of tested samples}} \times 100 = \frac{15}{505} = 3.0\%$$

Total of tested samples = Positive samples + negative samples + False negative samples+ false positive samples

7.2. Sensitive index (C)(%)

$$\text{* C \%} = \frac{\text{Positive samples}}{\text{positive samples + False negative samples}} \times 100 = \frac{358}{358+45} = 88{,}8\%$$

Specific index (D)(%)

$$\text{D} = \frac{\text{Negative samples}}{\text{Negative samples + False negative samples}} \times 100 = \frac{87}{87+45} = 65{,}9\ \%$$

Specific index revealed that although sensitive index of IR test was relatively high, false negative and false positive percentages could be acceptable, the IR method limited with specific plant part of samplings. Hence only 65.9 % of Specific index was obtained and hence, young leaf, trunk, fruit, roots could not be used for IR test.

Conclusion

IR test for HLB detection were evaluated by PCR and declined syndrome of orchard. The Test had False negative percentage 8.9 %False positive percentage 3% Specific index 65.9%, Sensitive index 88,8% and the additional observation on all aspects affected. This investigation would come up to a recommendation that IR test could be effectively utilized for field citrus HLB detection

Literature Cited

Devlin and Witham, 1983. Plant physiology-fourth edition.1993

Kazuo NAKASHIMA, yoshihiro, 1998. Detection of Citrus Greening Organism in citrus Plants and Psylla Diaphorina citri in Thailand. 1998

Le thi thu Hong et al, 2000. Nghien cuu mot so bien phap bao ve thuc vat trong san xuat cay giong cay co mui o DBSCL. 2000

Le thi thu Hong et al, 2001. Nghien cuu qui trinh cai tien benh VLG tren cay co mui bang phuong phap PCR. Bao cao khoa hoc Bo. 2001

Linder RC, Kirkpatrick HC, weeks TE, 1959. Some factors affecting the suceptibility of cucumber cotyledons to infection by tobacco masaic virus. Phytopathology,1959.

M. Onuki, NTN Truc, LTT hong, 2002. Useful Histological Method for distinguishing Citrus yellowing leaves infected with Huanglongbin from those caused by other factors. Proceedings of the 2002 annual workshop of JIRCAS Mekong Delta Projet

NTN Truc, LTT Hong, 2002. Nghien cuu giam dinh nhanh benh vang la greening bang thuoc thu iod. Bao cao khoa hoc hang nam, Vien nghien cuu cay an qua mien nam.2002

R. P. Srivastava and Sanjeev Kumar, 1998. Fruit and Vegetable presevation

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

Tóm Lược

PCR là phương pháp giám định hiệu quả, nhanh và chính xác nhất đối với bệnh VLG. Tuy nhiên, để có biện pháp xử lý kịp thời nhằm giúp nông dân giám định nhanh ngoài vườn, sử dụng phương pháp IR (Iodine reaction) đơn giản đã được khẳng định hiệu ích của phương pháp (Hồng và ctv,2002; Onuki và ctv, 2002).

Do đó, thí nghiệm này đã đánh giá và hoàn thiện phương pháp IR đã được thực hiện trong năm 2003 cho thấy có tỷ lệ âm tính giả là 8,9 %, tỷ lệ dương tính giả là 3 %, độ nhạy là 88,8 %, độ đặc hiệu là 65,9 **5.**

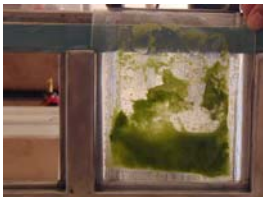
APPENDIX

KIT FOR HUANGLONGBIN DISEASE QUICK DETECTION

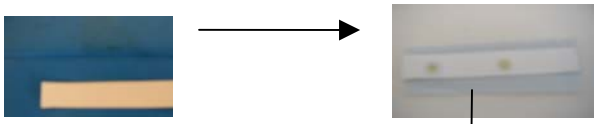
1 plastic bag;1 Iodsolution tube ;1 NCM piece;1 guide paper;

1. Collect the typical symptom leaves of HLB disease. Should not take the leave with the hall, miner leaf, curl, young, …for detect, and should take the leaf in the morning. Don't collect trunk, fruit, root for detect

2. Take 1 gam leaf, well grind with 2 ml distilled water

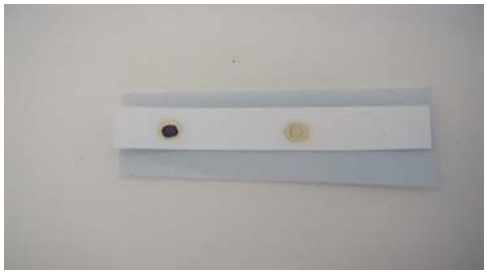


3. Drop 2 µl of leaf & water solution into the NCM (NitroCellulose Membrane), wait 5 minutes, drop 2 µl of Iodine solution in the same place of old drop.



4. Observe the changing color

5. IR result



Positive reaction----------Negative reaction