Useful Histological Method for Distinguishing Citrus Yellowing Leaves Infected with Huanglongbing from Those Caused by Other Factors

Masatoshi Onuki[1], Nguyen Thi Ngoc Truc[2], Hirohisa Nesumi[3], Le Thi Thu Hong[2] and Hiromi Kobayashi[4]

1. *Japan International Research Center for Agricultural Sciences, Okinawa Subtropical Station, Ishigaki, Okinawa, 907-0002 Japan*
2. *Southern Fruit Research Institute, Box 203 My Tho, Tien Giang, Vietnam*
3. *National Institute of Fruit Tree Science, Shimizu, Shizuoka, 424-0292 Japan*
4. *Japan International Research Center for Agricultural Sciences, Tsukuba, Ibaraki, 305-8686 Japan*

Abstract

Nhằm đơn giản hóa phương pháp giám định bệnh VLG ở Việt Nam, phương pháp khảo sát tế bào học qua phép thử iod đối với phản ứng tinh bột trong lá, kết quả phương pháp thử này đã được so sánh với phương pháp PCR. Từ các thí nghiệm chứng minh được rằng phép thử iod là một phương pháp giám định hiệu quả bệnh HLB trên cây có múi.

1. Introduction

HLB is a major constraint in citrus growing areas of the tropics and subtropics. Rapid, sensitive and accurate diagnosis of HLB is an important first step to control the disease. However, it is difficult to distinguish HLB-infected citrus trees from those of physiological disorder, e.g. zinc/manganese deficiency. Currently, accurate diagnosis of HLB is conducted by PCR or Southern hybridization. These molecular methods are time-consuming, expensive and sometimes not suitable for quite a number of samples. To resolve this problem, a simple histological method for investigating IR in citrus leaves was considered because it was revealed that starch granules accumulate in HLB-infected yellowing leaves (Schneider, 1968). In the preliminary experiments in Japan, accumulation of starch granules was recognized only in the HLB-infected leaves. In this brief report, we show the result of observation of IR using citrus samples collected in southern Vietnam.

2. Materials and Methods

1) Citrus plants

Yellowing citrus leaves were collected in several orchards in Tien Giang and Can Tho provinces in summer of 2002.

2) Iodostarch reaction

Small pieces (about 10×2mm) were cut out from the yellowing portion of citrus leaves using razor blades. The piece was put between pith of elderberry, then thin sectioning was carried out using a razor blade. The section was placed on a glass slide then drops of 1-2% of 0.5 M iodine solution (Nacalai tesque) were added. After that, sample was mounted by a cover glass. IR of the sample was observed by light microscope (OLYMPUS). For comparison, citrus leaves infected with citrus tristeza virus (CTV), citrus exocortis viroid (CEVd), *Phytophthora* sp. and physiological disorder were subjected to the observation of IR.

3) PCR

To evaluate practical utilization of IR for diagnosing HLB, polymerase chain reaction (PCR) was conducted and its results were compared with those of IR. Total nucleic acid from citrus plant was extracted using CTAB method according to 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) cetyl trimethyl ammonium 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 microcentrifuge 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 removed 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 with ultraviolet light after staining in an etidium bromide solution.

3. Results and Discussion

The results of IR and PCR of citrus leaves collected in Tien Giang province are shown in Table 1. Of citrus 5 samples No.1-5, the results of IR were consistent with those of PCR. Two samples (No.2 and 4) did not show any positive reaction. Sample No.2 did not show any mottling, but only yellowing: sample No.4 showed a net-like vein yellowing which was similar to the symptom caused by physiological disorder or insect damage in Japan (data not shown). On the other hand, three samples (No.1, 3 and 5) showing positive reactions both in IR and PCR (Fig. 1, Table 1) had typical mottling or zinc/manganese deficiency-like symptoms. These typical symptoms were evaluated as useful markers of the infection of HLB.

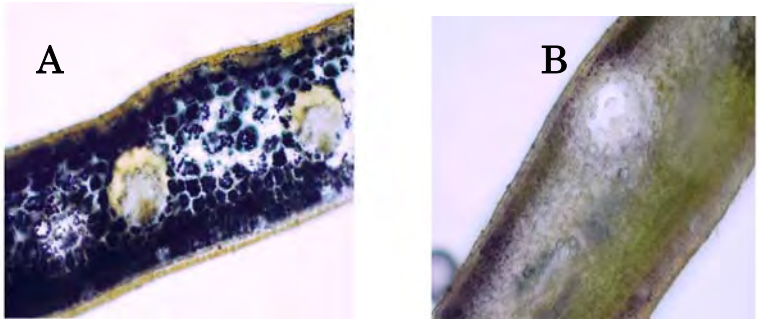

Fig. 1. Positive and negative iodostarch reaction observed under light microscope
(A) HLB-infected citrus leaf sample (B) Healthy leaf sample

As shown in Table 1, HLB-infected sample had been already checked to be positive by PCR, however typical symptom was not recognized and no positive reaction was observed in IR. This is indicating that appearance of IR may be correlated to the symptom development of HLB. Schneider (1968) demonstrated that leaf yellowing of HLB was caused by accumulation of starch granules. Other factors such as CTV, CEVd and *Phytophtora* sp. or physiological disorder were not related to the appearance of IR (Table 1). From these evidences, it was strongly suggested that only HLB is related to the accumulation of starch granules in citrus leaves.

Table 1. Comparison of iodostarch reaction and PCR on yellowing citrus leaves collected in Tien Giang province

Citrus sample	Symptom	Iodostarch reaction	PCR
No.1 (orchard A)	M, VY	+	+
No.2 (orchard A)	Y	-	-
No.3 (orchard B)	D, VY	+	+
No.4 (orchard B)	VY	-	-
No.5 (orchard C)	D	+	+
*HLB-infected	None	-	NT
*CTV-infected	YF	-	NT
*CEVd-infected	YF	-	NT
*Phytophthora sp.-infected	MY	-	NT
*Physiological disorder	MY	-	NT

+: positive reaction -: negative reaction
NT: not tested D: Zinc/Manganese deficiency-like
M: Mottling MY: Marginal yellowing
VY:Vein yellowing Y: Yellowing YF: Yellow fleck
**:Samples maintained in Southern Fruit Research Institute, Vietnam*

Table 2. Relationship among the symptom, iodostarch reaction (IR) and PCR on citrus leaf samples collected in Can Tho province

Sample	Symptom	IR	PCR	Sample	Symptom	IR	PCR
No.1	D	+	+*	No.23	M, VY	+	+
No.2	D, M, VY	+	+	No.24	D	+	+
No.3	D	+	+	No.25	D	+	+
No.4	M, VY	+	+	No.26	None	-	+
No.5	Not clear	-	+	No.27	D	+	+
No.6	D, VY	+	+	No.28	Not clear	-	+
No.7	None	-	+	No.29	D	+	+
No.8	D	+	+	No.30	None	-	+
No.9	M	+	+	No.31	D	+	+
No.10	None	-	+	No.32	M	+	+
No.11	D	+	+	No.33	D, M	+	+
No.12	Not clear	-	+	No.34	M	+	+
No.13	D, M, VC	+	+	No.35	M	-	-
No.14	Not clear	-	+	No.36	D	-	+*
No.15	Not clear	+	+	No.37	D	+	+
No.16	D	+	+	No.38	D, M	+	+
No.17	M, VC	+	+	No.39	D	+	+
No.18	Not clear	+	+	No.40	M, VY	+	+
No.19	M, VC	+	+	No.41	M	+	+
No.20	M, VY	+	+	No.42	M	+	+
No.21	D, M	+	+	No.43	M	+	+
No.22	M	+	+	No.44	None	-	+

+: positive reaction -: negative reaction
+: positive but weak reaction*
D: Zinc/ Manganese deficiency-like
M: Mottling VY: Vein yellowing VC: Vein corking

From Can Tho province, 44 leaf samples were collected in the summer of 2002. Symptoms of the leaves and the results of IR and PCR are shown in Table 2. Of 44 samples collected in Can Tho, the results of IR were not consistent with those of PCR. Samples No.5, 7, 10, 12, 14, 26, 28, 30, 36 and 44 showed negative reaction in IR, though positive in PCR (Table 2, Fig. 2). Of these 10 samples, except sample No.36, nine samples did not show any clear symptom, suggesting obvious positive IR may appear along with or after the symptom development. In case of sample No.36, zinc/ manganese deficiency-like symptom was apparently recognized though the IR was negative, suggesting that the clear symptom of sample No. 36 was due to other factors, not HLB.

Samples No.15 and 18 had indistinct symptom, whereas IRs were recognized. These samples were not yet yellowing, however dark and light coloring portions were weakly recognized, indicating accumulation of starch granules actually starts before yellowing. Concerning this, the IR of sample No.18 was partially recognized in one leaf. Although it is unknown how the accumulation of starch granules starts in HLB-infected leaf, this phenomenon is important because it is closely involved in symptom development.

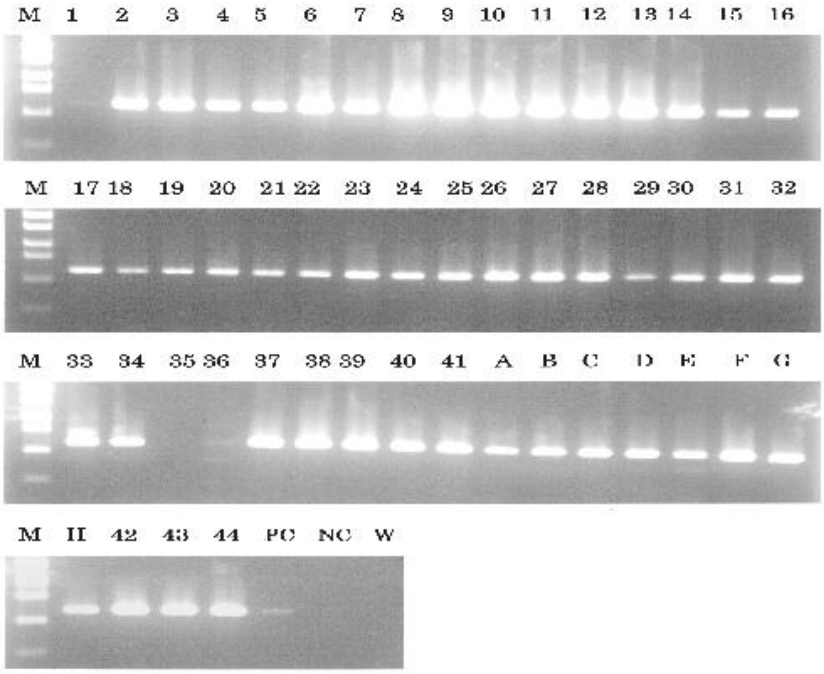

Fig 1.
Amplification of HLB-specific DNA fragments using O11 and O12C primers
M; DNA marker, λ /EcoT14I 1-44; Citrus leaf sample
A-H; Citrus bark sample PC; Positive control
NC; Negative control W; Water

IR-positive samples were all PCR-positive (Table 2, Fig. 2). This fact suggests that IR can be useful as an index of infection of HLB. Thus, in actual diagnosis of HLB, only IR-negative samples should be checked by PCR. This means diagnosis of HLB will be improved together with checking IR.

Ohtsu *et al*. (1998) classified 7 types of the symptoms of HLB and showed the symptoms were important indices for rapid diagnosis. In this report we demonstrated the utility of IR, therefore combination of

observing symptoms and checking IR may provide a more reliable tool for diagnosing HLB.

Literature Cited

Jagoueix, S., Bove, J.-M. and Garnier, M. (1994). The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the *Proteobacteria*. Int. J. Syst. Bacteriol. 44: 379-386.

Nakashima, K., Prommintara, M., Ohtsu, Y., Kano,T., Imada, J. and Koizumi M. (1996). Detection of 16S rDNA of Thai isolates of bacterium-like organisms associated with greening disease of citrus. JIRCAS Journal 3:1-8.

Ohtsu, Y., Nakashima, K., Prommintara, M. and Tomiyasu, Y. (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.

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