ヨウ素デンプン反応を利用したカンキツグリーニング病の検出

Detection of Citrus Huanglongbing using an Iodo-starch Reaction

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Abstract: A rapid, low cost method for the detection of physiological changes associated with the causal agent of huanglongbing (greening) in citrus was developed. A leaf of citrus was cut into 5×5 mm pieces, crushed and softened with hot water for 10 min. A 100 μl sample of sap containing the infectious agent placed in a micro plate with 40 μl 5 mM iodine solution turned purple as a result of the iodo-starch reaction. Leaf samples from trees with deficiency symptoms gave a reaction ranging from light blue to blue, and healthy tree samples became yellow or brown, a result that distinguished diseased trees from healthy ones with the naked eye. Moreover, diseased samples had 20 times the optical absorbance as healthy ones at 540 nm. A comparison of PCR results with the iodo-starch assay resulted in 75 % agreement for leaf samples, and 95 % for trees.

Key words: assay, greening, iodine, naked eye, starch

Introduction

Citrus huanglongbing disease (HLB) was first reported and named in China.[1)] The disease inflicts severe damage to citrus in Asia and Africa.[2-6)] The disease had been named by symptomology depending on its geographic location, including 'likubin' in Taiwan, Citrus dieback in India, leaf mottle in the Philippines, vein phloem degeneration in Indonesia and yellow branch, blotch-mottle and greening in South Africa[7)], until adoption of the universal designation 'huanglongbing' in 1995 (The 13th Conference of International Organization of Citrus Virologists). In Japan, the first occurrence of the disease was recognized in Taketomi-cho, Okinawa prefecture.[8)] Afterwards, the disease was found on Okinawa[9-11)] and Yoron Island, Kagoshima Prefecture.[12)]

It has been reported that the pathogen of HLB is a Gram negative, phloem-restricted obligate parasitic bacterium. Jagoueix *et al.* [13)] classified the bacterium into two species, *Candidatum liberobacter asiaticum* (Asian type) and C. *liberobacter africanum* (South Africa type) according to a genetic analysis using the sequence of 16 S ribosomal DNA. The causal bacterium in Okinawa is considered to be of the Asian type.[14)] The disease is persistently transferred by two psyllae, *Diaphorina citri* (Asian type) and *Trioza erytreae* (African type) and by grafting.[15)]

Like other phloem-limited bacterial pathogens, it has been assumed that direct control of the bacterium in citrus is difficult, so that control methods are limited to detection at early stage, removal of infected trees, and control of the insect vector by spraying insecticides. Detection has depended on PCR using two universal primers. Hybridization with a DNA probe[16)], electron microscopy, immuno-detection methods[17)] and immuno-gold staining[18)], grafting on sensitive varieties[19)] and symptomology[11,20)] are also commonly used. However, other than observation symptoms, these methods are expensive, require extensive technical training and can be time consuming.

The observation that large amounts of starch are accumulated in the palisade mesophyll tissue of infected leaves[21,22)] suggested that a detection method using the iodo-starch reaction would be possible and would have the advantages of reliability, simplicity, rapidity and cost efficiency. In this work we examine the use of the iodo-starch reaction as an assay for huanglongbing in citrus. In addition, this research was conducted in 2000 to 2001.

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Materials and Methods

1. Primary test for the optimum concentration and supplemental iodine

To determine optimum concentrations, 20 μl aliquots of a dilution series of iodine ranging from 0.5 M to 0.5 mM (Nakarai tesque Co. Ltd) were added to 100 μl extracts of 0.1 g infected leaves from a diseased ponkan mandarin (*Citrus reticulata Blanco*) that had been ground and heat-treated for 10 min. For the quantitative test, 5 to 50 μl of 5 mM iodine were added to a specimen prepared as above. Source trees for all experiments were maintained and managed in a glasshouse.

2. Iodo-starch reaction

Ten healthy leaves from seedlings of ponkan mandarin, 30 leaves showing deficiency symptoms such as yellowing, midrib yellowing or green veining (5 samples for each type) and 35 diseased leaves that had developed severe chlorosis with green vein, vein corking or mottling, including 5 symptomless infections were tested (Fig.1) as summarized in Fig. 2.

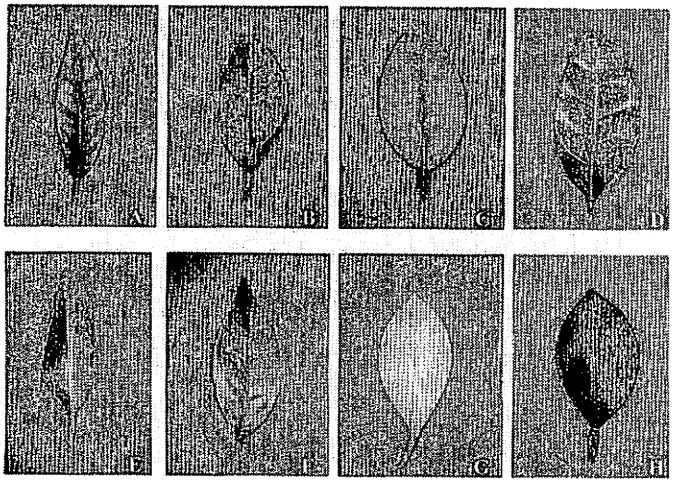

Fig.1. Symptom of leaves used in iodine reaction assays.
The upper row is diseased leaves. A : chlorosis with green veins, B : motteling, C : yellowing, D : vein corking. The lower row is representative of leaves with deficiency symptom. E : midrib, F : chlo-rosis with green veins, G : yellowing, H: without symptoms (healthy).

1) Manual tearing of the leaf sample about 5×5 mm.
↓
2) A 0.1 g sample is put in a 1.5 ml micro tube, 0.3 ml tap water is added and crushed with chopstick. Tap water to a total volume of 1.0 ml is added.
↓
3) Hot water for 10 minutes.
↓
4) 0.1 ml of sap and 40 μl of the 5 mM iodine solution are added to the micro plate wells.
↓
5) Scoring results as positive (purple) or negative (blue, light blue, brown).

Fig. 2. Detection method by iodo-starch reaction for HLB.

3. The effect of grinding method on detection efficiency

The variable of tissue maceration was addressed using the iodo-starch reaction on leaves with yellowing symptoms. Detection efficiency for the tearing-only treatment was compared with fine razor cutting and grinding with a chopstick.

4. Detection by micro plate reader

Samples were measured for absorbency at 540 nm with a micro plate reader (BIO-RAD) after appearance of the iodo-starch reaction with various symptoms. A healthy ponkan mandarin leaf was used as a check and city water as a blank.

5. Comparison of PCR and the iodo-starch reaction in the field

The yellow leaves of four tankan (*C. tankan* Hayata) trees and seven satsuma mandarin trees (*C. unshu* Marc) from orchards in Yaka, Kin-cho and nine shiikuwasya (*C. depressa*) trees lining a street in Oonishi, Nago City, Okinawa, Japan were tested with the iodo-starch reaction. Five leaves were collected from each tree. In the visual test, a single positive leaf sample was judged to be representative of a positive result for the tree. Results of the iodo-starch assay were compared with the results of PCR.[23]

Results

1. Optimum concentration and supplemental iodine

Iodine concentrations from 0.5 M to 50 mM were too high to distinguish healthy leaves from diseased ones. An iodine concentration of 0.5 mM gave a recognizable but transient positive reaction. A 5 mM solution gave stable and reliable purple color and provided a clear distinction between healthy and infected leaves. Addition of 40 μl was found to be the optimum volume that gave consistent results lasting up to 10 minutes.

2. Detection by the iodo-starch reaction

Extracts from healthy leaves turned light blue, yellow or brown with the iodo-starch reaction, and those from leaves with deficiency symptoms turned light blue or blue. Extracts from all infected leaves turned purple

and extracts from symptomless leaves turned blue. It was apparent that color reactions can be used to distinguish healthy or mineral deficiency symptom leaves from infected ones (Table 1).

Table.1. Detection of HLB using iodo-starch reaction (Only purple)[a]

Sample	Symptom [b]					
	C	Mi	Mo	Y	V	W
Healthy leaf	—	—	—	—	—	0/10[c]
Deficiency symptom leaf	0/10[d]	0/10[e]	—	0/10	—	—
Diseased leaf	10/10	—	10/10	10/10	10/10	0/5[f]

[a] Healthy leaves are represented by 10 samples, leaves of deficiency symptoms are represented by 30 samples, diseased leaves (PCR positivity) were represented by 45 samples collected from individual ponkan trees.
[b] C:chlorosis with green veins, Mi: midrib yellowing, Mo: mottling, Y: yellowing, V: vein corking, W: without symptom.
[c] Three samples were light blue.
[d] Five samples were blue.
[e] All sample were light blues.
[f] All sample were light blue to blue.

3. The effect of grinding on detection efficiency

The use of fine maceration was found to be marginally more effective than course maceration and was adopted for subsequent assays (Table 2).

4. Detection using a micro plate reader

Although it was possible to detect positive samples, distinguishing blue from purple was not possible based on the single absorbance value used by the microplate reader. If the sample turned blue, absorbency values ranged from 1.5 to 2.0. Infected samples could be detected when the positive value was set at 2.0 or more, a level which exceeds the water check by at least 20 fold (Table 3).

5. Validation by PCR

The iodo-starch method and detection by PCR had the same result for 95% of leaf samples and 75% of tree samples (Table 4). The iodo-starch assay gave 11 false negatives: one tree sample and ten leaf samples which were negative in the iodo-starch reaction were positive with PCR.

Table.2. Effect of grinding method on the detection efficiency

sample	Treatment [a]	
	A	B
Diseased sample [b]	4/5[c]	5/5

[a] A: manual tearing only treatment, B: fine razor cutting and grinding with chopstick
[b] Yellowing (symptom).
[c] Number of positives.

Table.3. Detection result for each symptom on a micro plate reader[a]

Sample [b]	W	H	D1	D2	D3	C	M	V	Y
Optical absorbance	0.04	0.13	0.71	0.95	0.25	3.39	3.76	3.34	3.79

[a] Optical absorbance (wavelength 540nm) was measured by the micro plate leader.
[b] W: tap water, H: healthy leaf, D1: deficiency symptom leaf (midrib yellowing), D2 : deficiency symptom leaf (chlorosis with green veins) , D3: deficiency symptom leaf (yellowing), C: diseased leaf (chlorosis with green veins), M: diseased leaf (mottling), V: diseased leaf (vein corking), Y: diseased leaf (yellowing).

Table.4. Validation of the iodo-starch assay with PCR[a]

	Tree			Leaf		
Detection method	Positivity	Negativity	Agreement rate (%)	Positivity	Negativity	Agreement rate (%)
Iodo-starch reaction	15	5	95%(19/20)	58	42	75%(75/100)
PCR	16	4		68	32	

[a] Five leaves per a tree were collected from 4 tankan and 7 satsuma mandarin with yellowing in the citrus orchards of Okinawa prefecture, Kin-cho Yaka. Fifty-five leaves in all were collected. Moreover, 45 leaves were similarly collected from 9 shiikuwasya trees of Nago City, Oonishi (100 total samples). Assays were performed on individual leaves. In the visual test one positive sample out of 5 changed was judged as a positive.

Discussion

In a preliminary examination, when a 0.5 M iodine solution was dripped onto the midribs of leaves sliced with a razor, diseased leaves turned purple, whereas a healthy leaf did not. However, repeating the same test several times gave blue false positives. Since blue can easily be confused with purple, an improvement of the method was required that would allow the two colors to be clearly distinguished. In hot water, starch turns purple in the presence of iodine due to depolymerization and subsequent complexing of the iodine with starch fragments.[24] Depolymerization and reaction of iodine with leaf starch contents has made it possible to detect infectious leaves with a high degree of accuracy by direct examination of the color of the sap/iodo-starch solution.

As a result of examining an optimum concentration and amount of iodine, we found that addition of 40 μl of a 5 mM solution of iodine gave stable and reliable purple color and addition of 30 μl or less of the same concentration of iodine is sufficient for the assay despite the disappearance of color in about a minute, unless there are many samples. Disappearance of color by the time the iodine solution is added to all samples may give inaccurate results in some of the samples. However, there is generally enough residual color to give a reliable result in each sample. The addition of 4-5 drops of boiled sap and a drop of the iodine solution gave a clear result for each sample.

The results of the iodo-starch reaction assays using samples from plants that present various symptoms indicate that it is possible to detect the diseased samples, and to differentiate disease from mineral deficiencies and healthy tissues based on the color of the reaction (Table 1). Starch is a compound of amylose and amylopectin. It is thought that the amylose combines with iodine to give a blue color, that the amylopectin in diseased leaves becomes purple and that yellow and brown are due to the added iodine. As necrosis and shrinkage of sieve tissues progresses during the course of pathogen proliferation in the sieve tubes[21, 22, 25], the movement of glucose synthesized by the leaf may be inhibited, resulting in starch accumulation in mesophyll cells. However, the cause of the increase in amylopectin content remains to be demonstrated.

As for the effect of grinding on detection efficiency, the detection rate increases with the maceration of the leaf. This is simply thought to be a result of the increase in starch grains released by more extensive crushing of the cells. It will be necessary to examine other typical symptoms to determine the overall reliability of this testing method, because only one of the greening symptoms was examined in this research.

Use of the micro plate reader to allow for more quantitative or reliable results was partially successful in that a critical point value could be established for positive samples (Table 3). It will be necessary to re-examine the assay after enzymatic digestion of the amylase, or chemical treatment, to remove the amylose, which causes the confounding blue coloration.

As a result of this indirect huanglongbing pathogen detection in several citrus species samples collected from the field, it was found to be preferable to collect several leaf samples with symptoms from the same tree, and to use individual leaves in separate assays. Moreover, it was clear that this method could be used with several citrus species. From these results, the detection of HLB can be accomplished using the rapid, simple, portable and inexpensive iodo-starch reaction. It is thought that an accurate visual diagnosis can be effectively complemented with the iodo-starch reaction. In the future, the influence of the season, leaf age, white-spotted longicornis beetle (*Anoplophora malasiaca* Thomson) damage and the preservation period of the sample will be examined, as will the mechanism of starch accumulation in the leaf.

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ヨウ素デンプン反応を利用したカンキツグリーニング病の検出

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要　約

本研究では低コストで，迅速に多量のサンプルを検定できるカンキツグリーニング病の検定法の開発を行った．まず，カンキツの葉を約５×５mm大にちぎって破砕し，これを10分間熱湯で糊化した後、その上清を100μl取ってマイクロプレートに入れ，これに５mMのヨウ素溶液を40μl添加すると，カンキツグリーニング病に罹病したサンプルはヨウ素デンプン反応により紫に，要素欠乏症状は淡い青～青色に，健全サンプルは，淡い青，黄または茶色に発色するため，肉眼での判別が可能であった．また波長540 nmで吸光値を測定した結果，罹病サンプルは健全サンプルの20倍以上の吸光値を示した．PCRとヨウ素・デンプン反応による検定結果を比較した結果では，葉当たりでは75%，樹当たりでは95%の一致率を示した．