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Partial Purification of Thai Isolate of Citrus Huanglongbing (Greening) Bacterium and Antiserum Production for Serological Diagnosis

Yoshihiro OHTSU[1]*, Maitree PROMMINTARA[2], Seiichi OKUDA[3], Tomoaki GOTO[3,4], Takeshi KANO[5], Kazuo NAKASHIMA[6], Meisaku KOIZUMI[7], Jun IMADA[1] and Koji KAWASHIMA[8]

ABSTRACT

We aimed to improve the purification of citrus Huanglongbing (greening) bacterium (HB), *Candidatus* Liberobacter asiaticum and to produce an antiserum against HB. Periwinkle plants *Catharantus roseum* L. graft-inoculated with HB were used to produce an antiserum. All young leaves of new shoots incubated at 20-25°C and 25-30°C, a few mature leaves incubated at 20-25°C, and all mature leaves incubated first at 25-30°C and later transferred to 20-25°C developed yellowing symptoms and were then used to prepare immunogen. The HB was partially purified from these leaves by an improved method that included a macerating enzyme treatment of the midribs of infected leaves and homogenization of infected phloem sieve tissues. An antiserum raised against partially purified HB reacted clearly at a dilution of 1/16 with HB-infected citrus extract prepared at a concentration of 40 times, but did not react with healthy or tristeza virus-infected citrus extract in microprecipitin tests.

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Key words : citrus huanglongbing, greening, diagnosis, Liberobacter asiaticum, partial purification, microprecipitin test.

INTRODUCTION

Since the citrus greening was first reported in China by the name Huanglongbing[21,28], the disease has become one of the most destructive diseases of citrus, occurring in Africa[1] and Asia[4,9] and on the Arabian Peninsula[3]. In 1995, citrus greening was designated as "Huanglongbing" at the 13th Conference of International Organization of Citrus Virologists. Therefore, the disease is referred to as Huanglongbing hereafter in this article. The pathogen of the Huanglongbing is a bacterium (HB), which is restricted to the phloem of infected plants. HB has two strains, one Asian and the other African, that are transmitted by psyllid vecters *Diaphorina citri* in Asia and *Trioza erytreae* in Africa. Because of the diversity of the 16 S rDNAs sequences of HB, the scientific names of *Candidatus* Liberobacter africanum for the African isolates and *Candidatus* Liberobacter asiaticum for the Asian isolates were proposed[11]. The disease can be diagnosed by its typical symptoms[19,20], by graft transmission to indicator citrus plants[22], by electron microscopy[6], by hybridization with a DNA probe[10,25,26], by enzyme-linked immunosorbent assay with monoclonal antibodies[5,7,8], and by polymerase chain reaction diagnosis[11,12,17]. However, a serological diagnosis using polyclonal antibodies has been long-awaited because it is the simplest, cheapest, most reliable method.

Villechanoux *et al.*[24] purified the Poona strain of HB from an infected periwinkle plant *Catharantus roseum*

[1] Plant Protection Division, National Institute of Fruit Tree Science (NIFTS), 2-1, Fujimoto, Tsukuba 305-8605, Japan
[2] Plant Pathology and Microbiology Division, Department of Agriculture (DOA), Chatuchak, Bangkok 10900, Thailand
[3] Faculty of Agriculture, Utsunomiya University, 350, Minemachi, Utsunomiya 321-8505, Japan
[4] Present address: Tochigi Agricultural Experiment Station, 1080, Kawaraya-cho, Utsunomiya 320-0002, Japan
[5] Headquarters of National Agricultural Research Organization (NARO), 3-1-1, Kannondai, Tsukuba 305-8517, Japan
[6] Biological Resources Division, Japan International Research Center for Agricultural Sciences (JIRCAS), 1-2, Ohwashi, Tsukuba 305-0851, Japan
[7] Formerly Citrus Division, National Institute of Fruit Tree Science (NIFTS), 2-1, Okitsu, Shimizu 424-0204, Japan
[8] Formerly Production and Post Harvest Division, Japan International Research Center for Agricultural Sciences (JIRCAS), 1-2, Ohwashi, Tsukuba 305-0851, Japan
* Corresponding author (E-mail : yotsu@affrc.go.jp)

(L.) G. Don, using a monoclonal antibody MA (10A6) in affinity chromatography. The purified HB bodies have a filamentous structure, 1-4 μm long and 0.15-0.3 μm in diameter. A round form of HB with a diameter 1.0 μm also exists[24]. Whitlock and Chippindall[27] produced an antiserum against an extract of HB-infected citrus plants; however, the antiserum was not specific to HB. Ke *et al.*[14] produced a polyclonal ascitic antiserum against a partially purified HB from periwinkle infected with the HB strain, but the titer of the antiserum was low, and nonspecific interference concealed an expected specific reaction.

In this study, symptom development and HB concentration in periwinkle plants after graft-inoculation with the Thai-isolate[18] of HB were first studied at different temperatures. Then, we produced an antiserum against partially purified HB from periwinkle. We tried to remove the disease shock protein[27], using a 20% sucrose cushion during purification, and to prepare a citrus sample as highly concentrated as possible for the serological test. We believe that this is the first report of a serological diagnosis by a microprecipitin test[23] using a polyclonal antiserum developed for the Huanglongbing.

MATERIALS AND METHODS

Thai isolate of Huanglongbing bacterium The Thai isolate of HB (HB) was originally collected in Nakhon Pathom, Thailand, in 1985. HB was initially transmitted from a naturally infected sweet orange plant to acidless sweet orange plants by the psyllid vector, *Diaphorina citri*. Then HB was transmitted to mandarin plants by graft-inoculation. HB was also transmitted to periwinkles by dodder, then from periwinkle to periwinkle by grafting. The infected plants were then maintained in a greenhouse at the Department of Agriculture (DOA), Thailand[18].

Periwinkle plants and growth conditions Periwinkle (*Catharantus roseum* L.), rough lemon (*Citrus jambhiri* Lush) and sweet orange 'Madam Vinous' [*C. sinensis* (L.) Osb.] were used in the study. All periwinkle plants were grown in pots of 15-cm diameter, two plants in each pot. Four-month-old plants were graft-inoculated with HB-infected buds and were maintained in a greenhouse at 24-27°C until they were placed in NK LH-200-RDMP incubators (Nippon Ika Co. Ltd., Japan). Ten plants were kept at 25°C for 12 hr a day and 20°C for 12 hr at night. The other 10 plants were kept at 30°C for 12 hr a day and 25°C for 12 hr at night. The day illumination intensity was 10,000 lux. Healthy rough lemon plants and rough lemon and sweet orange plants infected with HB were maintained in a screen house at the DOA. Citrus rough lemon plants infected with mild strains of citrus tristeza virus were maintained in an air-conditioned greenhouse at the National Institute of the Fruit Tree Science (NIFTS)[13].

Symptom appearance after graft-inoculation To determine the time needed for HB-infected periwinkle plants to show yellowing symptoms, nine plants were graft-inoculated with HB and were incubated at 26-28°C (28°C for 12 hr day with illumination intensity of 10,000 lux, 26°C for 12 hr night).

To examine the effect of temperature (20-25°C and 25-30°C) on the appearance of yellowing symptoms in young leaves of new shoots and in mature leaves present before graft-inoculation of periwinkle plants infected with HB, 10 plants were graft-inoculated with HB and were incubated at each temperature range. The effect of the temperature was evaluated by the proportion of mature leaves with yellowing.

Electron microscopy One-mm length midribs with typical symptoms were cut from leaves at different leaf positions and grown at different temperature ranges (20-25°C and 25-30°C), then fixed with 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for several days, mainly at 4°C, and were post-fixed with 1% osmium tetroxide in Dalton chrome buffer for 1 hr. Then the specimens were serially dehydrated in ethanol and acetone, stained with uranyl acetate dihydrate between 60% and 70% ethanol, and embedded in Epon 812. The embedded midribs were sectioned with a Sorvall MT-1 ultramicrotome, stained with lead citrate, and examined with a JEM-100SX electron microscope.

Partial purification of HB from phloem tissue of diseased periwinkle Phloem sieve tissue was prepared with a modification of the method of Lee and Davis[15]. Midribs (5-9 g) were separated 35-60 g of leaves with sharp forceps. The midribs were surface-sterilized with 1% sodium hypochlorite for 5 min, and then rinsed three times in sterile distilled water in a small polyethylene bag. The washed midribs were placed in three to four petri dishes containing an enzyme solution of 100 ml sterile distilled water, 0.8 g cellulase "ONOZUKA" RS (Yakuruto Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.4 g MACEROZYME R-10 (Yakuruto Pharmaceutical Industry Co., Ltd.), 1.0 mM $CaCl_2 \cdot H_2O$, 0.5 g polyvinylpyrrolidone-40 and 12.5 g mannitol. The midribs were incubated at room temperature (24-31°C) overnight. The digested vascular bundles were transferred to a petri dish containing extraction solution (0.6 M mannitol, 25 mM Tris-HCl, 5 mM magnesium acetate)[16]. With sharp forceps, the phloem tissues were carefully stripped from the xylem tissues in the petri dish and were stored at 4°C overnight. The phloem sieve tissues were washed to remove other bacterial contaminants in a fresh extraction solution, then washed five times with

fresh extraction solution in a new petri dish. The washed tissues (0.3-0.7 g) were homogenized in 5 ml of extraction solution. The homogenate was centrifuged for 5 min at $29\times g$, and the supernatant was carefully collected and centrifuged for 30 min at $11,200\times g$. The pellet was resuspended in 1 ml of extraction solution. The suspension was then dialyzed overnight with 4% glutaraldehyde in extraction solution, then dialyzed in the extraction solution for 24 hr with four solution changes. The solution was then centrifuged at $29\times g$ using 20% sucrose cushion. The supernatant was carefully collected and centrifuged on a 20% sucrose cushion for 30 min at $17,000\times g$. The pellet was resuspended in 500-1000 μl of 0.85% NaCl solution.

Production of antiserum against HB Partially purified HB in 0.85% NaCl solution was emulsified with Freund's incomplete adjuvant for the injections. The emulsion was injected into the muscles of a rabbit (about 2 kg) five times at alternating 17- and 30-day intervals. The emulsion was then injected under the skin of the rabbit 3 days after the last muscular injection. Antiserum against HB was collected from the rabbit 3 weeks after the subcutaneous immunization.

Microprecipitin test The microprecipitin test was done as described[23]. HB antiserum was diluted to 50 % in 0.85%NaCl solution containing 0.1% NaN_3 and reserved until use. Further dilutions were made with a solution of 0.85% NaCl in 0.05 M Tris-HCl buffer at pH 8.0.

Diseased midribs of citrus leaves were digested in enzyme solution for 2 days, and then phloem tissues were separated from the xylem. The phloem tissue was macerated in extraction solution (20 ml) in a mortar with a pestle. After filtration through cheesecloth, the homogenate was centrifuged for 5 min at $29\times g$. The supernatants were centrifuged for 30 min at $20,000\times g$, and the pellets were resuspended in 1 ml of 0.85% NaCl solution containing 0.1% NaN_3. Partially purified HB concentrated up to 40 times of the midrib weight was obtained from the final pellets after one cycle of low and high speed centrifugation of the homogenate of the phloem tissues. The final suspension was used as the antigen. For the control, healthy citrus and citrus infected with the mild strain of citrus tristeza virus[13] were prepared as infected tissue. To determine a rough titer of the antiserum, extract of bark tissue of infected periwinkle was prepared as described previously and used as antigen.

One droplet of antigen (5 μl) was added carefully to a droplet of antiserum (5 μl) without mixing in a petri dish. A set of droplets in the petri dish was overlaid with liquid paraffin to prevent drying. After incubation of the reaction drops for 2 hr at 37°C and one night at 4°C, precipitations were examined under a stereomicroscope.

RESULTS

Symptom development in periwinkle

Symptoms appeared first on at least one leaf of the new shoots immediately below the graft insertion at 26-35 days after graft-inoculation, and on all leaves of the lower new shoots at 29-41 days after graft-inoculation at 26-

Table 1. Timing of appearance of yellowing symptoms on leaves of new shoots of periwinkle plants graft-inoculated with Huanglongbing bacterium and incubated at 26-28°C

Symptoms	Days after inoculation								
	26	27	29	34	35	37	38	39	41
First appearance on any leaf	3[a]	1(4)		2(6)	3(9)				
Appearance on all leaves			1[b]	3	4	5	7	8	9

a) Number of plants with first symptom appearance on any leaf (total no. of plants).

b) Number of plants with symptoms/9 plants inoculated and treated. Graft-inoculation on 27 Sep., 1994; incubated at 28°C for 12 hr with the illumination intensity 10,000 lux day and 26°C for 12 hr night.

Table 2. Effect of temperature (20-25°C/25-30°C) on the appearance of yellowing symptoms in mature leaves of periwinkle plants inoculated with Huanglongbing bacterium

	Replication		
	I	II	III
Graft-inoculated on	5 Jan. '95	25 Jan. '95	20 Feb. '95
Treatment started on	9 Feb. '95	13 Feb. '95	15 Mar. '95
Symptoms observed on	13 Feb. '95	28 Feb. '95	29 Mar. '95
20-25°C[a]	17.2% (11/64)[b]	30.4 (32/105)	63.5 (30.5/48)
25-30°C[a]	3.1% (2/63)	7.2 (6.5/90)	10 (4.9/50)

a) Temperature at 20-25°C : 25°C for 12 hr day and 20°C for 12 hr night, at 25-30°C : 30°C for 12 hr day and 25°C for 12 hr night; illumination intensity was 10,000 lux day.

b) Proportion (%) of leaves with yellowing (leaves with yellowing/all mature leaves observed).

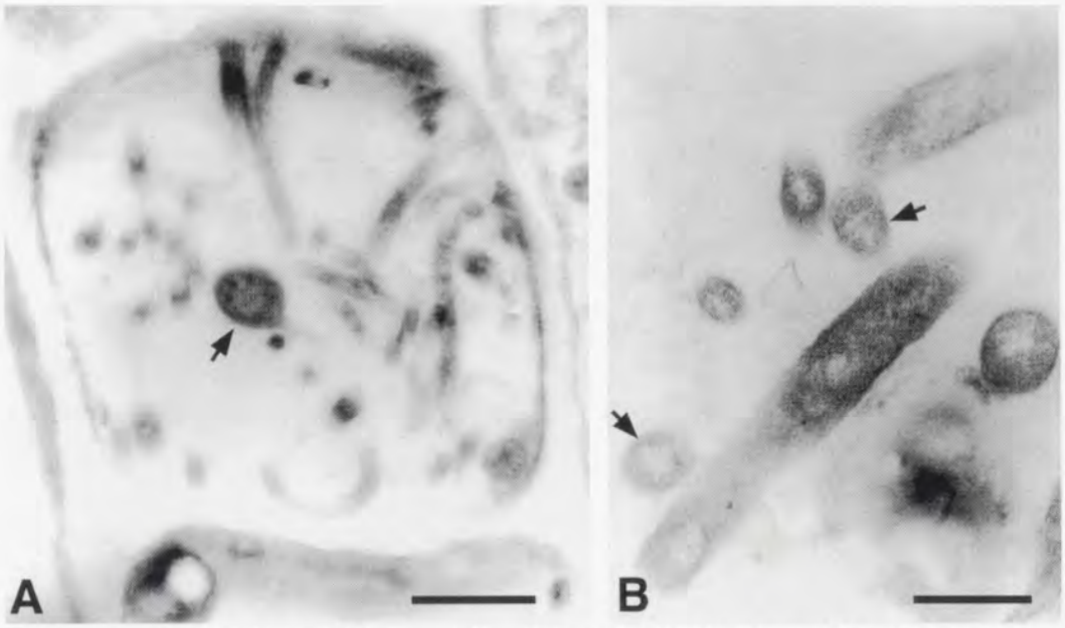

Fig. 1. Citrus Huanglongbing bacteria (HB) in sieve tubes of infected periwinkle plant with yellowing symptoms. A: HB in a mature leaf with yellowing, incubated at 25-30°C. The round form of HB was also visible (arrow); B: HB in a mature leaf with yellowing, incubated at 20-25°C. An *ca.* 25-nm-thick envelope characteristic of HB was clearly visible (arrows). Scale bars are 1 μm (A) and 500 nm (B).

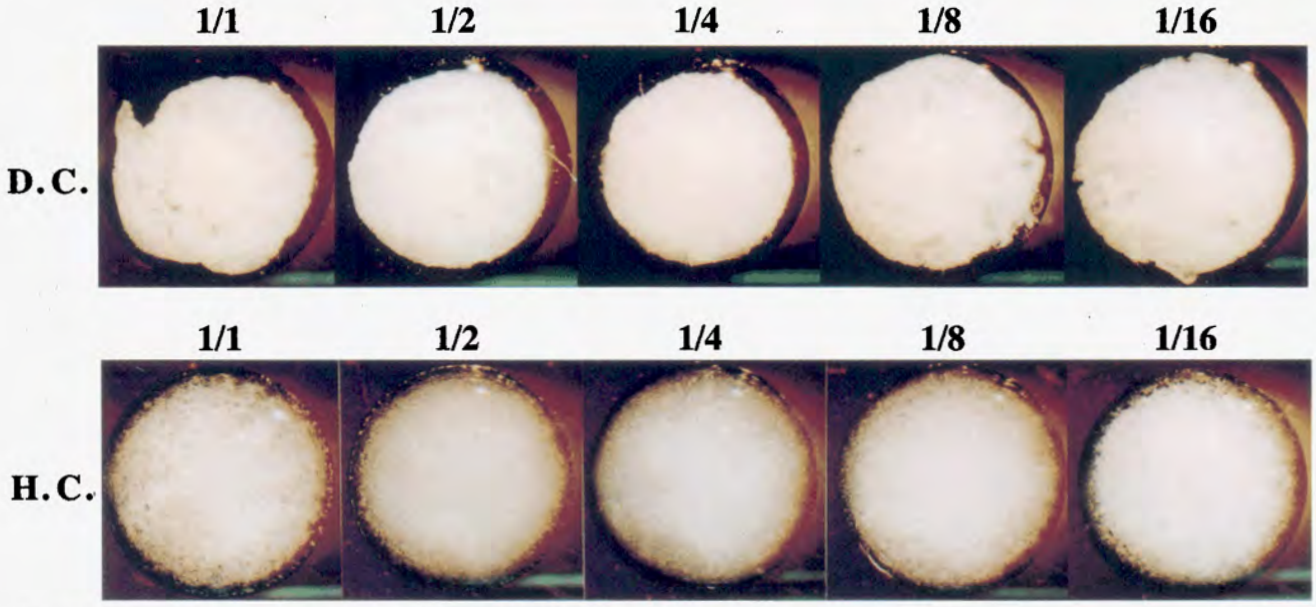

Fig. 2. Microprecipitin test of Thai-HB isolate of Huanglongbing bacterium in citrus. Citrus extracts were concentrated up to ×40 of midrib weight. Each droplet of extract was added carefully to a droplet of antiserum without mixing. The droplets of both diseased and healthy citrus extract without antiserum did not aggregate. D.C. row shows strong positive reactions of the antiserum with partially purified Huanglongbing bacterium (HB). H.C. row shows no reaction with healthy citrus extracts of sieve tissues. H.C.: Healthy extracts of citrus phloem; D.C.: Disease extracts of citrus phloem; 1/1-1/16: Dilutions of the antiserum against HB.

28°C (Table 1). The ratio of mature leaves with yellowing to all mature leaves observed at 20-25°C was four to six times higher than that at 25-30°C (Table 2). Infected periwinkles that had not developed yellowing on mature

leaves at 25-30°C for 15 days were transferred to an incubator at 20-25°C. Leaves of these plants developed yellowing on the 8th day after the change in incubation temperature. The number of yellowing leaves later increased.

Electron microscopy

Electron microscopy revealed the presence of a number of prokaryotes in sieve elements of midribs of periwinkle leaves with yellowing symptoms (Fig. 1A, B). The round form body[29] of HB was visible (Fig 1A, arrow). At a higher magnification, an *ca.* 25 nm-thick envelope characteristic of the HB was clearly visible (Fig. 1B, arrows). Numerous HB-like bodies were observed in most of the young leaves of new shoots with yellowing that were incubated at 20-25°C and 25-30°C, in a few mature leaves with yellowing incubated at 25-30°C, and in all mature leaves with yellowing that were incubated first at 25-30°C and were transferred later to 20-25°C. All these leaves were processed to be used as immunogen.

Microprecipitin test to detect HB in citrus extract

Antiserum diluted to 1/16 reacted with partially purified HB from the diseased periwinkle with strong precipitation but did not react with a preparation of healthy periwinkle in the microprecipitin test and or with a preparation of rough lemon infected with citrus tristeza virus in the test (data not shown).

The antiserum diluted to 1/16 reacted specifically with partially purified HB in citrus extracts with strong precipitations, and a completely transparent edge of the droplets was clearly visible (Fig. 2, D. C.). A similarly prepared component solution from a healthy plant did not react with the antiserum at any concentration (Fig. 2, H. C.).

HB resuspended in 1 ml of 0.85% NaCl after partial purification was detected by the antiserum at dilutions of 1/16.

DISCUSSION

Only a few reports have dealt with the effect of temperature on symptom expression of Huanglongbing. Citrus plants graft-inoculated with the Asian Huanglongbing (greening) strain developed symptoms at both warm (27-32°C) and cool (20-25°C) temperatures[2], and periwinkle plants graft-inoculated with the Indian strain of the Huanglongbing (greening) bacteria and kept at 32°C developed symptoms after 6 weeks, and only two of those kept at 25°C developed symptoms after 3 months[6]. Our study showed that Huanglongbing symptoms appear on all new shoots of periwinkle plants 5-6 weeks at 26-28°C after graft-inoculation with the Thai-HB isolate (Table 1). The leaves infected with HB had brighter yellowing at 20-25°C than leaves at 25-30°C (Table 2, photo not shown). For our results on the effect of temperature on symptom expression in periwinkle, we incubated the graft-inoculated plants first at 25-30°C and then transferred them to an incubator at 20-25°C. This sequence may be adopted as a good measure to promote symptom development of the Huanglongbing on indicator citrus plants.

Our results show that mature leaves having bright yellowing symptoms on the older portion of the main stem of infected periwinkle plants that have been kept at 20-25°C for 1-2 weeks after a 4 week incubation at 25-30°C, should be used for pathogen purification as can young leaves with symptoms on new shoots.

Improvements in HB purification in this study were : 1) mature leaves with bright yellowing symptoms were used for purification as well as young leaves of new shoots, 2) midribs were digested in macerating enzyme solution before homogenization, 3) separated phloem tissues were washed to remove any other bacteria during the digestion, 4) a low centrifugation at $29 \times g$ for 5 min was used to avoid losing the round form[24] of HB, 5) a 20% sucrose cushion at both low and high speed centrifugation was used to remove the disease shock protein[27], 6) HB was fixed in 4% glutaraldehyde to keep it intact until used as an antigen to prepare the HB antiserum.

In this study, a clear serological diagnosis of HB-infected citrus was developed using a microprecipitin test. Clear positive reactions were obtained with droplets containing diseased citrus extracts and antiserum, but not with droplets with healthy citrus extracts or with citrus extracts infected with the mild strain of citrus tristeza virus. This method is simple and reliable for the diagnosis of citrus Huanglongbing.

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