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Further Study of the Transmission of Citrus Huanglungbin by a Psyllid, *Diaphorina citri* Kuwayama

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ABSTRACT. The huanglungbin agent was successfully transmitted from diseased citrus plants to healthy Ponkan mandarin seedlings by fourth and fifth instar nymphs of *Diaphorina citri* Kuwayama and not by first to third instar nymphs. The effect of number of adults on transmission showed that a single adult could transmit the pathogen very efficiently. In serial transmissions to healthy Ponkan mandarin seedlings by single adults, the minimum latent period of the pathogen was 1-2 days, and the maximum latent period was 25 days after acquisition feeding. The infected adult retained infectivity throughout its life. When the adult was newly emerged from an infected nymph, it could transmit the pathogen to healthy seedlings without an acquisition feeding. In ultrathin sections of salivary glands and other organs of infected adults under electron microscope, the pathogen was found in the cells of salivary gland, the filtration chamber of the foregut, and the cells of midgut and hindgut.

Citrus huanglungbin (Citrus yellow shoot) is widespread in south and southwest China, and destroyed much of the developed citrus industry of the country (11). Bacteria-like organisms (BLO) (rickettsia-like organism (RLO) as used in earlier reports) are always associated with citrus huanglungbin, and its envelope structure (3 layers, 20-25 nm thick) is very similar to that of the greening organism, but it is quite different from mycoplasma-like organisms (unit membrane, 10-12 nm thick) (3, 8, 10, 15).

Citrus leaf-mottle in the Philippines, citrus vein phloem degeneration in Indonesia, citrus dieback in India and citrus greening in Thailand are similar to citrus huanglungbin disease, and are transmitted by the oriental psylla, *Diaphorina citri* (4, 13, 17, 18, 19). The citrus greening disease is transmitted by the African psylla, *Trioza erytrea* Del Guercio (5, 16), but under experimental conditions, the vector of African citrus greening was able to transmit Asian citrus dieback (14).

In China, the relationship between the oriental psylla and citrus huanglungbin was shown early in Guangdong and Guangxi provinces (1, 2). Since then, many authors (6, 7, 9, 12, 20) have indicated that the oriental psylla is the vector transmitting citrus huanglungbin in nature. This is based on the fact that citrus plants infected by psylla not only show typical symptoms, but the citrus huanglungbin agent is found in ultrathin sections of salivary glands of the psylla vector. In transmission tests, the transmission efficiency is low even though numerous psyllids are used on one citrus seedling. In contrast, citrus huanglungbin often spreads quickly in citrus plantations, especially in the young plantations. Fifty to seventy % of the citrus trees were infected before fruit production began during the epidemic years of 1981-1983 in Guangdong and Fujian provinces. So, it was necessary to further study the transmission of citrus huanglungbin by psylla.

MATERIALS AND METHODS

Pathogen source plants. Diseased seedlings of Tankan and Fuji mandarins, which were inoculated by psylla and which showed typical symptoms and contained characteristic BLOs upon electron microscope examination, were used as pathogen source plants.

Psylla. Adults of the oriental psylla collected from citrus plantation were fed on orange jessamine. After oviposition, the adults were killed.

The freshly hatched nymphs of the new generation were transferred to other seedlings of orange jessamine for propagation.

Test plants. Ponkan mandarin seedlings with 3-4 leaves were used as test plants. They were grown for half a year in the greenhouse from seed treated in 56 C water for 50 min.

Test of transmission by nymphs or their freshly emerged adults. Nymphs were divided into two groups: first to third instar nymphs and fourth to fifth instar nymphs. The former group was fed on diseased Tankan mandarine seedlings until the first or second exuviation, the latter was fed until third or fouth exuviation. Then each group was tested separately on Ponkan mandarin seedlings (5 nymphs per seedling). When nymphs emerged into adults, they were killed. Some of the fourth to fifth instar nymphs after aquisition feeding were transferred to healthy seedlings of orange jessamine until they became adults. These freshly emerged adults were transferred to ten Ponkan mandarin seedlings (3 nymphs per seedling).

Effect of numbers of adults on transmission. Adults were fed on diseased Tankan mandarin seedlings 20 days, then transferred singly or in groups of 2, 4, 6, 8, or 10, to ten Ponkan mandarin seedlings.

Minimum infection feeding period. The freshly emerged adults were fed on diseased Tankan mandarin seedlings for 20 days. Then one adult was transferred to each one of 10 Ponkan seedlings for an infection period of 1, 3, 5, and 7 hr (a total of 40 seedlings).

Transmission in serial transfers by a single adult. Adults used for transmission were divided into 3 groups: A) The freshly emerged adults from healthy nymphs were fed on diseased Tankan mandarin seedlings for 2 days. B) The freshly emerged adults from nymphs given an aquisition feeding were not fed on diseased seedlings. C) The freshly emerged adults from nymphs given an acquisition feeding were fed on diseased Tankan mandarin seedlings for 2 days more. A single adult was collected from each group and transferred serially to Ponkan mandarin seedlings for an infection feeding period of 24 hr. The first transfer was made at 1 day, the second and third transfers were made at intervals of 4 days, and the others were made at intervals of 2 days until the adult died.

All the above mentioned tests were conducted in insect-proof greenhouses and the temperatures were maintained between 24 and 28 C. Two groups of 20 healthy Ponkan mandarin seedlings fed with pathogen-free, fourth to fifth instar nymphs and adults were treated as controls.

Electron microscopy. Specimens of the salivary gland, foregut, midgut, hindgut and Malpighian tubules used for the electron microscopic study were collected from adults which were fed on diseased Tankan mandarin seedlings for 1 day, 5 days and 30 days. Specimens were fixed in 4% glutaraldehyde for 24 hr and postfixed in 2% osmium tetraoxide for 2 hr. After dehydration in a graded ethanol series, the specimens were embeded in Epon 812, and sectioned with an LKB-V ultramicrotome. Thin sections were doubly stained with uranyl acetate and lead citrate, and were examined with a JEM-100CXIl electron microscope (10).

RESULTS

Transmission by nymphs and their freshly emerged adults. The results show that none of ten Ponkan mandarin seedlings infected by first to third instar nymphs after acquisition feeding exhibited symptoms. However, in transmission by fourth to fifth instar nymphs after acquisition feeding and freshly emerged adults from nymphs given an acquisition feed, three and five of ten Ponkan mandarin seedlings exhibited symptoms, respectively. Symptoms appeared in 4-10 months. It is clear that the first to third instar nymphs even grown on a diseased plant were unable to transmit the disease, but the fourth to fifth instar nymphs were able to transmit the disease and were able to pass the pathogen to the freshly emerged adults which then transmitted the pathogen.

Transmission effect of numbers of adults. The results show that one adult after an acquisition feeding on diseased Tankan mandarin seedlings for 20 days was able to acquire the pathogen and to infect Ponkan mandarine seedlings with an efficiency of 80%. On transmission to ten Ponkan mandarine seedlings by group of 2, 4, 6, 8, and 10 adults per plant, Ponkan mandarin seedlings were infected at 20, 40, 50, 50, and 20%, respectively.

Minimum infection feeding period. When a single adult was transferred to each of ten Ponkan mandarin seedlings after an acquisition feeding for a infection feeding period of 1 and 3 hr, none of the seedlings tested was infected. When the infection feeding period was prolonged to 5 and 7 hr, 3 and 2 of 10 Ponkan mandarin seedlings were infected, respectively. This indicated that an adult could transmit huanglungbin with a minimum infection feeding of 5 hr.

Serial transmission of huanglungbin to Ponkan mandarine seedlings by a single adult. In serial transmission tests by a single adult of group A, the single

TABLE 1
SERIAL TRANSMISSION OF HUANGLUNGBIN TO PONKAN MANDARIN SEEDLINGS BY SINGLE ADULTS OF *DIAPHORINA CITRI*

Psyllid		Ponkan seedlings serially exposed to a single adult in continuous transfer at different intervals (days)[z]													
Group no.[y]	Insect no.	1	5	9	11	13	15	17	19	21	23	25	27	29	31
A	1	−	−	−	+	−	−	+	−	−	+	+	−	−	−
	2	−	+	+	−	−	−	−	−	+	−	−	−	−	−
	3	−	+	−	−	−	+	−	−	−	+	−	−	−	−
	4	−	−	+	+	−	−	−	−	−	+	−	−	+	−
	5	−	−	+	−	−	−	−	−	−	−	+	−	+	−
	6	−	+	+	+	+	−	−	+	−	−	−	−	−	−
	7	+	+	−	−	−	−	−	+	−	−	−	+	+	−
	8	−	−	−	−	−	−	−	−	−	−	+	+	−	−
	9	−	+	−	−	−	−	−	−	−	−	−	+	+	+
	10	−	+	−	−	+	−	+	−	+	+	−	+	−	−
B	1	−	+	−	−	−	−	−	+	−	−	+	+	−	−
	2	+	−	−	+	+	−	−	−	+	−	+	+	−	−
	3	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	4	−	+	−	−	−	−	−	−	+	−	−	−	−	−
	5	+	−	+	+	−	−	−	−	−	−	+	−	−	−
	6	−	−	−	−	−	−	−	−	−	+	−	−	−	−
	7	+	+	+	−	−	+	−	+	−	−	−	+	−	−
	8	−	−	−	−	+	−	−	−	−	−	−	+	−	−
	9	−	+	−	−	−	−	−	−	+	−	−	−	−	−
	10	−	+	+	−	−	−	−	−	−	−	−	−	−	−
C	1	−	+	−	−	−	−	−	−						
	2	+	+	−	−	−	+	−	+						
	3	−	+	−	−	−	−	−	−						
	4	+	+	−	−	−	−	−	−						
	5	+	−	−	+	−	−	−	−						

[z]+ indicates a diseased plant, − indicates a healthy plant.

[y]Adults of group A were freshly emerged from nymphs with no acquisition feed and were fed on diseased Tankan mandarin seedlings for 2 days. Adults of group B were from nymphs after acquisition feeding were not fed on diseased Tankan mandarin seedlings. Adults of group C were from nymphs after acquisition feeding and were fed on diseased Tankan mandarin seedlings again.

adult no. 7 infected Ponkan seedlings on the first day of transmission with 10% efficiency. On the fifth day of transmission, 6 single adults, i.e., no. 2, 3, 6, 7, 9 and 10 infected each of 6 Ponkan seedlings with 60% efficiency. But, single adult no. 8 infected the first one of a series of Ponkan mandarin seedlings on the twenty-fifth day of continuous transfer. Among ten single adults, 4 single adults, no. 4, 5, 7, and 9 were still able to infect Ponkan mandarin seedlings on the twenty-ninth and thirty-first day. A single adult from groups B and C gave results similar to those in group A. Of ten single adults of group B, three infected Ponkan mandarin seedlings on the first day of transmission with 30% efficiency, and eight single adults infected Ponkan mandarin seedlings on the fifth day of serial transfer with 80% efficiency. Among five single adults of group C, three infected Ponkan mandarin seedlings on the first day of serial transfers. All single adults infected Ponkan mandarin seedlings on the fifth day of transmission with 100% efficiency. It is clear that latent period of pathogen in the adult may be as short as 1-2 days or as long as 25 days. The adults retained infectivity after acquisition throughout their life. For example, no. 10 of group A and no. 2 and 7 of group B were able to infect 6 Ponkan mandarin seedlings in a serial transmission. The nymphs after acquisition feeding were able to transfer the pathogen as freshly emerged adults, in which the latent period of pathogen was absent or shortened. No symptoms appeared on the 40 Ponkan mandarin seedlings used as controls.

Electron microscopy. Ultrathin sections of specimens of salivary gland, filtration chamber of the foregut, midgut, hindgut and Malpighian tubules were examined under the electron microscope. The huanglungbin agent was found in the cells of the salivary gland, cells of filtration chamber of the foregut, and cells of midgut and hindgut of those adults after aquisition feeding for 1, 5 and 30 days. Especially large numbers of the agent were present in the cells of midgut and the outer layer cells of salivary gland (fig. 1, 2) Pleomorphic BLO had an envelope system composed of three layers 20-25 nm thick which is the same as those of BLO found in diseased Tankan mandarin seedlings. But no BLO were found in the cells of Malpighian tubules. Thus, the huanglungbin agent in the adult after acquisition feeding for 2 days was able to circulate from its mouth parts to the salivary gland within 1-2 days.

DISCUSSION

Previous studies indicated that the transmission efficiency of huanglungbin by psylla was low. The Guangxi Research Group (2) reported that 271 citrus seedlings were tested for transmission of hanglungbin by psylla, but only 18 seedlings showed symptoms (6.6%). Huang *et al.* (9) reported that the efficiency of transmis-

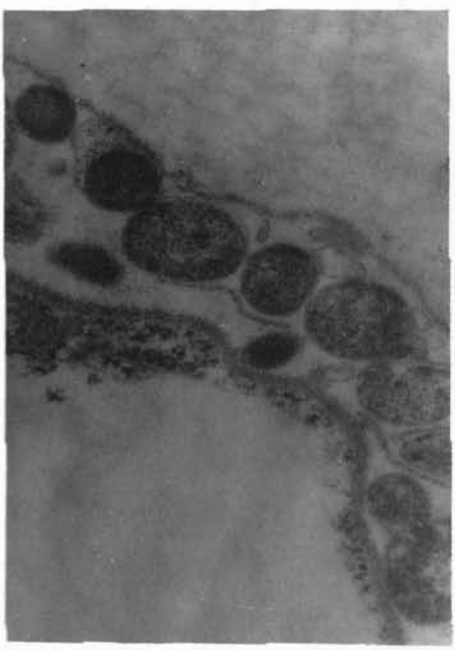

Fig. 1. Ultrathin section from salivary gland of insect vector showing the outer layer cell of salivary gland with bacteria-like organism. (17,000 X).

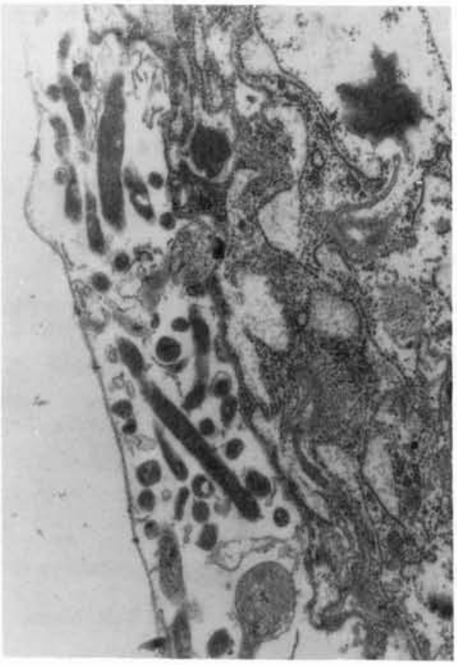

Fig. 2. Ultrathin section of the digestive organ of insect vector showing the cell of mid-gut with bacteria-like organism. (28,000 X).

sion was extremely low. Of 380 plants tested, only 5 plants showed likubin symptoms (1.3%). In the preliminary experiments by the authors (20), 40 of 329 citrus seedlings tested exhibited symptoms (12.2%). However, the results of present experiments showed that psyllids were able to transmit huanglungbin highly efficiently. One adult per plant gave 80% transmission. This may be due to the following modified conditions: 1) the young diseased Tankan mandarin seedlings used as source plants were inoculated by psylla and were examined to be sure they contained BLO. 2) young Ponkan mandarin seedlings with 3-4 leaves used for inoculation which may be in the most susceptible stage. 3) psylla were fed on a diseased source plant not collected directly from field. 4) Test plants were put in the greenhouse with an appropriate controlled shade level which may be good conditions for symptom development.

Present results show that the fourth to fifth instar nymphs were able to transmit huanglungbin agent, but first to third instar nymphs were unable to transmit it, and one adult per plant can give a high transmission efficiency. In addition, there was no evidence for transovarial transmission, because when nymphs freshly hatched from eggs, which had been laid by adults on diseased plants, were transferred to 15 Ponkan mandarin seedlings till the nymphs became adults and were killed, none of these seedlings showed symptoms after one year. These results correspond with those for transmission of citrus dieback by the oriental psylla (4).

Moll (16) showed that the greening agent was acquired by the adult vector within 5 days of feeding, and entered the salivary gland of adult on twenty-first to thirtieth day after acquisition feeding. Indicating that the latent period is more than 21 days. Capoor (4) showed that the latent period of citrus dieback agent in adult psylla was 8-12 days. Present results indicate that the latent period for huanglungbin is variable and may be as short as 1-2 days or as long as 23-25 days depending on the individual psylla carrier.

Since the huanglungbin agent was found in the cells of gut and salivary gland of psylla after acquisition feeding on diseased citrus for 1 day and 5 days, and a great number of BLO were found in the cells of the midgut and outer layer cells of salivary gland, it appears that the agent multiplied there. So, the relationship between the huanglungbin agent and the psyllid is quite similar to that between a persistent virus and its insect vector.

Because the fourth and fifth instar nymphs after acquisition feeding were able to transmit the huanglungbin agent, and deliver it to their freshly emerged adults, the latent period in the freshly emerged adults from infected nymphs is shortened. It is possible that most psylla in orchards have acquired pathogen as fourth to fifth instar nymphs or as adults and thus make the huanglungbin epidemic rapid in nature.

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