Transmission of the Huanglungbin Agent from Citrus to Periwinkle by Dodder

S. Ke, K. B. Li, C. Ke and J. H. Tsai

ABSTRACT. During 1981-1985, several experiments were conducted to transmit the citrus huanglungbin agent from citrus to citrus, periwinkle, cabbage, cucumber, tobacco and and *Solanum nigrum* by means of *Diaphorina citri*, *Cuscuta campestris*, *C. japonica*, and graft inoculations. *Diaphorina citri* transmitted huanglungbin agent to citrus seedlings, but not to the other eight plant species tested. The huanglungbin agent was transmitted to periwinkle by *C. campestris* but not by *C. japonica*. Of the 25 periwinkle plants tested, 96% of the periwinkle plants developed yellowing symptoms in 3-6 months after inoculation. Observations by electron microscopy of thin sections of leaf veins of the inoculated citrus and periwinkle plants revealed the presence of bacteria-like organisms (BLO's) in the sieve tubes. These BLO's were ultrastructurally and morphologically similar to those found in tissues of greening affected citrus and periwinkle plants. The titre of BLO in the sieve tubes of the affected periwinkles was higher than that of citrus. Subsequent transmission from periwinkle to periwinkle was successful only by graft inoculation.

Citrus huanglungbin (citrus yellow shoot) is a devastating disease of citrus in China and was first recognized in 1930 (4). Since then it has become endemic in Fujian, Guangdong and Guangxi provinces. In recent years, this disease has spread to all other citrus growing areas including Sichuan, Yunan, Guizhou, Hunan, Jiangxi and Zhejian provinces affecting most species and cultivars of *Citrus sinensis*, *C. grandis*, *C. reticulata*, and *C. limon*. Among these, *C. reticulata* is the most susceptible to huanglungbin. Trees at 3-8 yr old are the most susceptible to this disease. The loss of yield ranges from 74 to 97% within 2-4 yr after the appearance of symptoms. Following the first demonstration of bacteria-like organisms (BLO's) associated with citrus greening disease (14, 15), BLO's (referred to as rickettsia-like organisms (RLO) in earlier reports) were also found in ultrathin sections of mid and lateral veins of symptomatic leaves of huanglungbin-infected trees inoculated by citrus psylla (7, 12, 18). The sizes of BLO's measured 50-600 X 170-1600 nm with a double complex membrane measuring 25 nm. The ribosome and fibrillar nuclear materials were visible in these organisms (6, 10, 11). These BLO's resembled citrus greening organisms described elsewhere (15). The progress on the study of citrus huanglungbin has been slow because of the slow growth of citrus, the protracted incubation period, and the uneven distribution and low titre of the huanglungbin agent in the affected citrus plants (8, 9). Therefore a search for an alternative host appeared to be appropriate. After the report of successful transmission of citrus greening agent from citrus to citrus (16) and from citrus to periwinkle by *Cuscuta campestris* (8), we have also succeeded in transmitting the huanglungbin agent from citrus to periwinkle by dodder. The report summarizes the experiments conducted from 1981-1985.

MATERIALS AND METHODS

Plant materials. Test plants including Tankan and Fuzhou tangerine, Ponkan mandarin, periwinkle, orange jessamine, cabbages (*Brassica chinensis*, *B. oleracea* and *Brassica* sp.), cucumber, tobacco (*Nicotiana tabacum* and *N. glutinosa*) and *Solanum nigrum* were grown in sterilized soil in a greenhouse at 25-30 C. The huanglungbin agent was maintained in the seedlings of Tankan and Fuzhou mandarin that had been inoculated by the citrus psyllid, *Diaphorina citri* (Kuw.). Tissues of

all source plants used in the study were examined for the presence of huanglungbin agents by electron microscopy.

Transmission by *Diaphorina citri.* Healthy *D. citri* was reared on orange jessamine plants in a greenhouse at 25-30 C. After a 1- month acquisition access period on the infected citrus plants, a group of 10-20 *D. citri* adults was transferred and caged on one of the following test plants: Ponkan mandarin, periwinkle, *B. chinensis, B. oleracea, Brassica* sp., cucumber, *N. tabacum N. glutinosa* and *S. nigrum.* Ten plants from each species were used for *D. citri* inoculation trials. After a 20-day inoculation feeding period, the test plants were sprayed and kept in an insect proof greenhouse for symptom development. An equal number of healthy *D. citri* was placed on healthy plants as a control and the same number of healthy plants without exposure to the insects was also used as a control.

Dodder transmission. Seeds of *C. campestris* and *C. japonica* were germinated on moist paper and transferred onto healthy periwinkle and Ponkan mandarin plants for continued growth. Healthy strands of dodder were cut and attached to the Ponkan mandarin plants carrying the huanglungbin agent. After the haustoria were formed, and the newly developed strands were about 15 cm long, connections to the test periwinkle plants were made. The strands between the infected citrus and periwinkle plants were cut after 6 weeks. The periwinkle plants were then kept free of dodder strands and maintained in an insect proof greenhouse for observation. Healthy dodder strands were connected onto healthy periwinkle plants as a control. Inoculations from infected periwinkle to healthy citrus seedlings were made by the same procedure.

Graft transmission. Healthy 8-week-old periwinkle plants were top and base-grafted with infected shoots ca. 3-4 cm long. After grafting, the plants were covered with plastic bags for 7-10 days to maintain high humidity.

Electron microscope examination. Tissue samples used for the EM study were collected from experimentally inoculated periwinkle and healthy control plants as well as from strands of dodder from infected plants. Leaf pieces (1 x 2 mm^2) containing the lateral veins and dodder strands (ca. 2 mm^2) near the haustoria were excised. Tissue specimens were fixed for 15 hr in 0.1 M 4% glutaraldehyde and postfixed in 2% osmium tetroxide. After dehydration in a graded ethanol series, the specimens were embedded in Epon 812, and sectioned with an LKD-V ultramicrotome. Thin sections 60-100 nm were collected on Formar-coated grids and examined with JEM-100 CXII electron microscope.

RESULTS

Transmission by *D. citri.* Three experiments were conducted from May 1981 to December 1982. None of the ten test plants from each of the eight noncitrus species inoculated by *D. citri* developed huanglungbin symptoms after 5 months observation. The survival of *D. citri* on the test plants ranged from 3 to 10 days. However, four of the ten Ponkan mandarin seedlings inoculated by *D. citri* developed huanglungbin symptoms. All control plants remained healthy throughout the experiments.

Dodder transmission. Twelve periwinkle plants inoculated by *C. japonica* and five control plants remained symptomless after 6 months of observation. Of the 25 periwinkle plants inoculated by *C. campestris* 24 plants developed huanglungbin symptoms. The initial symptom on periwinkle leaves was a localized yellowing around the secondary veins followed by yellowing of leaf margins. Eventually the entire leaves became yellow. Other leaves on the same branch also progressively developed

Fig. 1. Healthy periwinkle.

symptoms. New leaves that developed from affected branches were also small and yellowed (figs. 1, 2, 3). The incubation period prior to symptom development in the periwinkle plants ranged from 3-6 months. No control plants developed symptoms.

Graft transmission. After 1½ to 2 months, symptoms developed in 70 (71.4%) of the periwinkle plants that were topgrafted with infected shoots from periwinkles previously infected with huanglungbin agent through dodder. The topgrafted plants also developed multiple branches below the union. Twenty-six of the 28 base-grafted periwinkle plants also developed symptoms after 1 to 2 months.

Back inoculation. Attempts were made during 1984-1985 to transmit the huanglungbin agent from periwinkles that were previously infected through dodder to Ponkan mandarin seedlings via *C. campestris*. None of the 15 Ponkan mandarin seedlings developed symptoms after 6 months.

Electron microscope examination. Thin sections of leaf tissue of *D. citri*-inoculated Ponkan mandarin

Fig. 2. Huanglungbin affected periwinkle showing early localized yellowing symptoms along the secondary veins.

Fig. 3. Huanglungbin affected periwinkle showing late generalized yellowing symptoms on old leaves.

seedlings and periwinkles infected using *C. campestris*, as well as infected *C. campestris* strands, were examined by electron microscopy. Pleomorphic BLO's were present in the sieve tubes of these samples but not in those of control plants. BLO's had an envelope comprising three zones: an electron-absorbing inner membrane; a dark outer membrane;

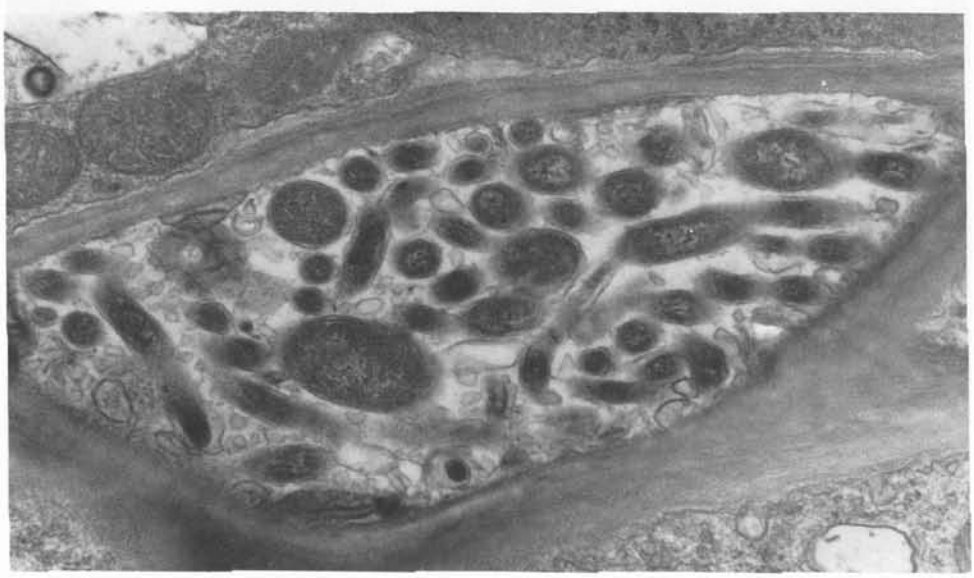

Fig. 4. Ultrathin section from a huanglungbin affected periwinkle leaf showing one sieve tube cell packed with the bacteria-like organisms. (28,600X).

and a transparent intermediate layer. The thickness of the envelope measured 20-25 nm (figs. 4, 5). Of all sieve tubes of the infected periwinkles examined, 50% contained BLO's. Their titres assessed by visual observation under EM were always higher than that of Ponkan mandarin leaves and *C. campestris* strands.

DISCUSSION

Obviously, *D. citri* was able to transmit huanglungbin agent from citrus to citrus as reported by other researchers (12), but not to periwinkle. Although *D. citri* survived on the periwinkle for 5-6 days, the number of pathogen-carrying insects was probably not enough to inoculate the plants successfully. More experiments are in progress to answer this question. Our data have demonstrated that *C. campestris* is an efficient vector for transmitting huanglungbin agent from citrus to periwinkle. *Cuscuta campestris* may be ideally suited for separating the mixed infections in the field (10). However, we have failed to transmit the agent from periwinkle to citrus using *C. campestris*. The reason for this failure is unknown. Further experiments to answer this question are in progress. Although *C. japonica* survived well on both infected citrus and periwinkle, it did not transmit the huanglungbin agent. The failure to do so could be due to different vector specificity. The BLO's introduced into periwinkle plants through *C. campestris* have characteristics similar to the greening agent in periwinkles described by Garnier and Bove (8). The organism is sieve-tube limited and is enveloped by three zones: a dark and electron-absorbing cytoplasmic membrane; an intermediate, electron-transparent zone; and an outer membrane that resembles the cell wall of

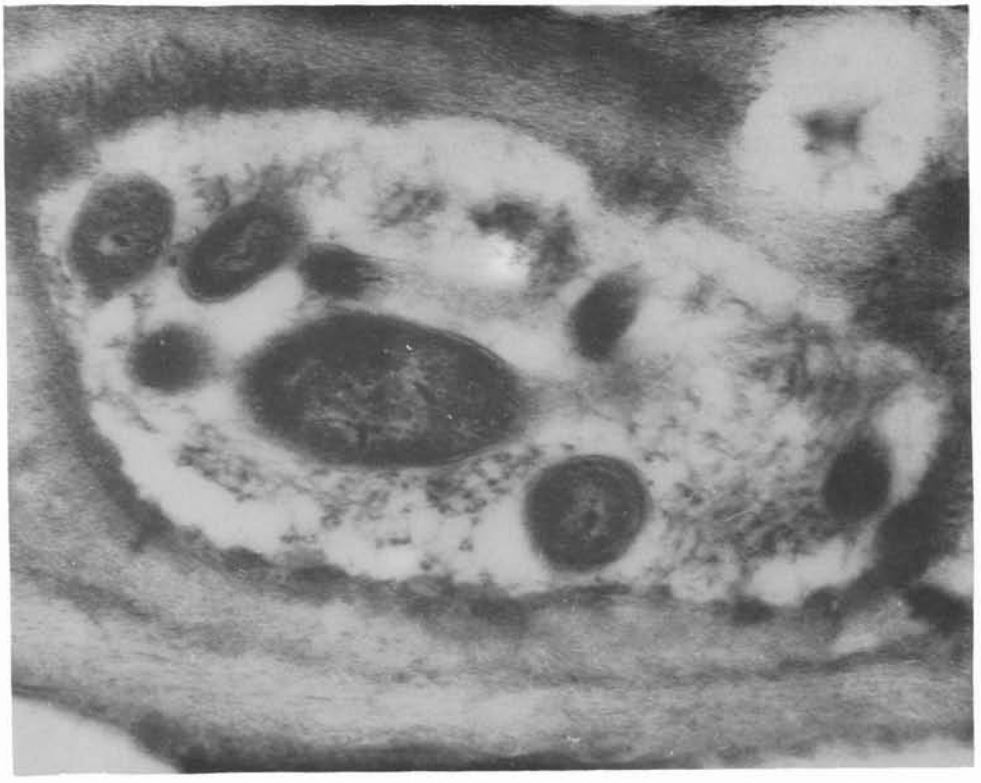

Fig. 5. Ultrathin section from a huanglungbin affected Ponkan mandarin leaf showing one sieve tube cell with the bacteria-like organism. (39,600X).

the Gram-negative bacteria. The symptoms observed on the infected periwinkle were similar to those of periwinkle infected with Indian greening (Poona strain) (8), but were different from the symptoms on periwinkle induced by *Spiroplasma citri* (3) and mycoplasma-like organisms (5). Our results also demonstrated that periwinkle is a more convenient host for studying the huanglungbin agent than citrus as it had more infected sieve tubes and higher pathogen titre based on visual observation. Additionally the infected periwinkles may provide an ideal alternative system for isolation, culture, and serological study of the huanglungbin agent.

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