Likubin or Huanglungpin, Citrus Virus Disease Closely Related to Tristeza (I)[1]

by

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As already reported by us (1961)(*1*), the disease provisionally call "Likubin" was formerly considered as being due to unfavorable soil conditions such as high moisture or deficiency in essential nutriments (1944)(*2*), but it was now made clear that this disease was caused by virus more closely related to tristeza as will be discussed later. The disease under consideration is now generally called Huanglungpin (yellowing of scions) in Taiwan, because it is said that the similar disease was already observed in China mainland and reported by C. P. CHENG (1943)(*3*) under this name, although no direct comparison has been made up to this time owing to the unavailability of materials necessary for the study. Recently, Rev. Appl. Myc. (1964, 1965)(*4*, *5*) reviewed two papers by K. H. LIN on Citrus yellow shoot occurring in China mainland. In the first (1963)(*4*), the author pointed out that affected plants developed 1st symptoms on spring shoots, new leaves turning greyish green and stiff after maturing normally, and yellowing of midribs of new leaves on late autumn shoots after acquiring normal coloration was also an early diagnostic symptom. The author's preliminary transmission studies showed that an unidentified brown citrus aphid was a probable vector and that there might be seed transmission. In the other (1964)(*5*), it was said that there was as yet no evidence of a relationship between this disease and tristeza or any other known Citurus virus disease. More recently, however, Rev. Appl. Myc. reviewed another paper by H. C. FOAN and C. C. LIU(*6*) which is somewhat contradictory to the aforementioned. According to these authors, Citrus yellow shoot was transmitted by *Toxoptera citricidus* and produced stunting, yellowing, and root-rotting on Eureka lemon and Tankan, and stunting, vein-clearing, and root-rotting of Mexican lime and Meyer lemons. It was suggested by the authors that the virus concerned was related to the Citrus tristeza complex. As will be clear from our data mentioned below, our Citrus virus disease may not be identical with Lin's yellow shoot, but appears to be more closely related to Foan and Liu's Citrus yellow shoot. For this reason, the name "Likubin" will be retained until the matter is made clear.

SYMPTOMS

Although the symptoms of the disease are somewhat different as host varieties differ, the infallible symptoms always associated with the disease concerned are 1) yellowing or mottling of leaves, 2) premature defoliation, 3) die-back of branches,

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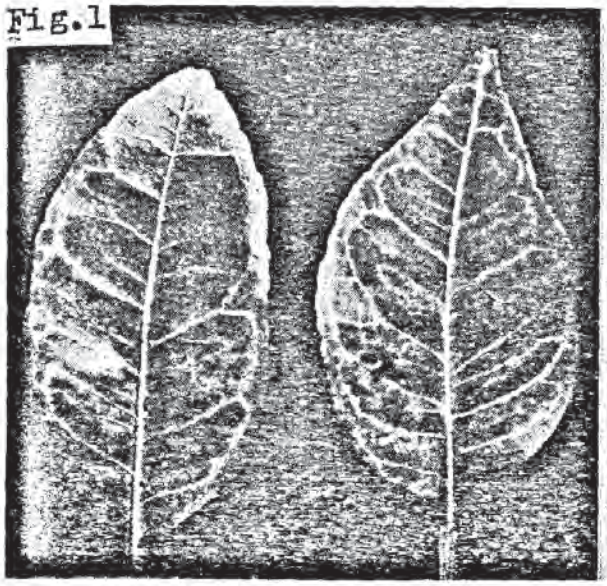
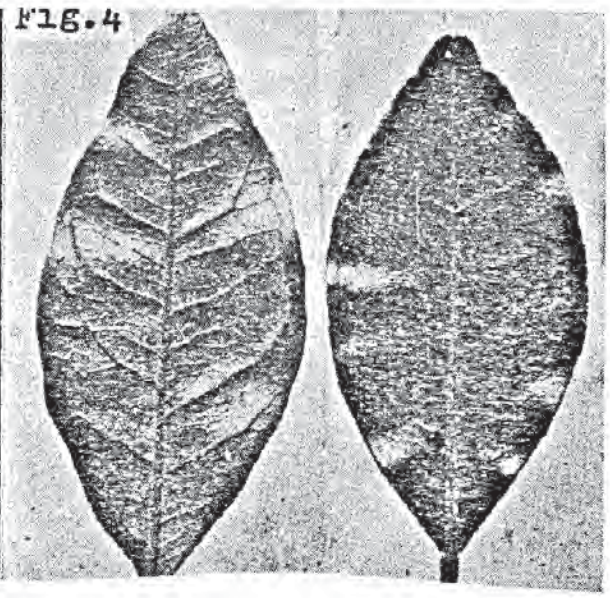
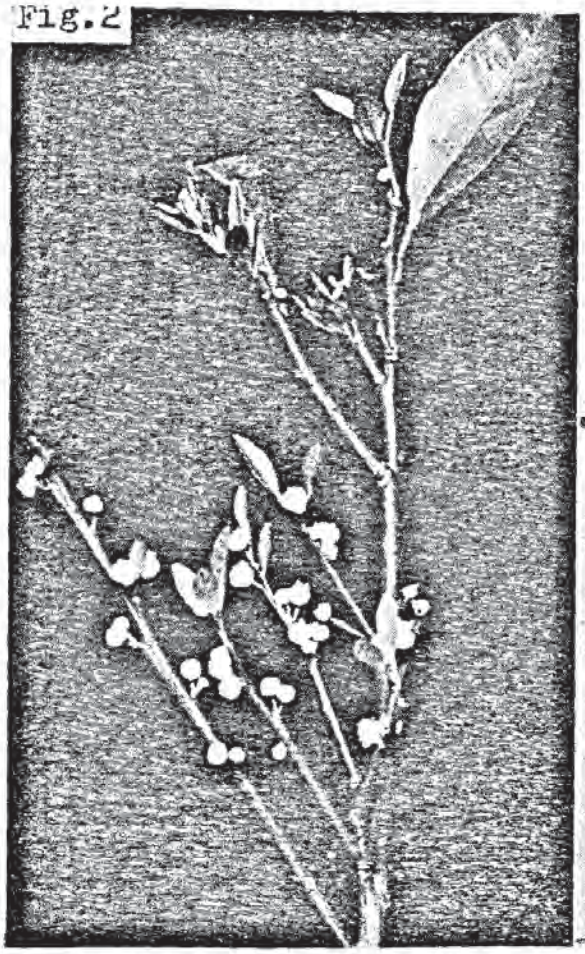






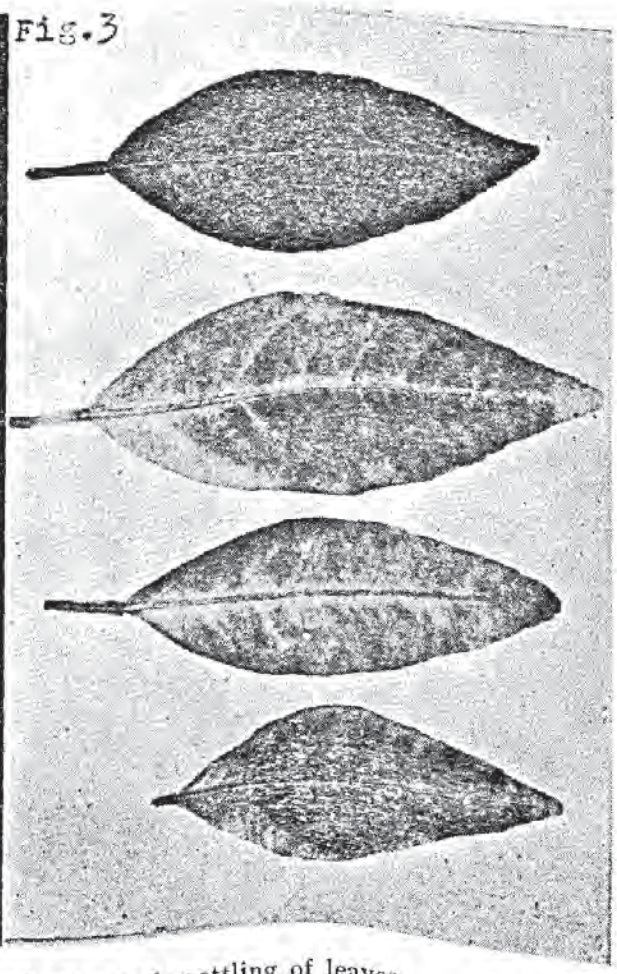
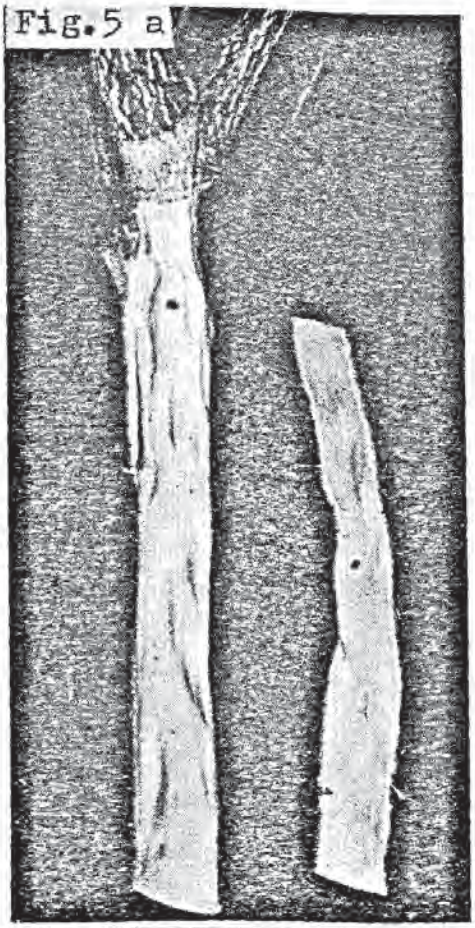
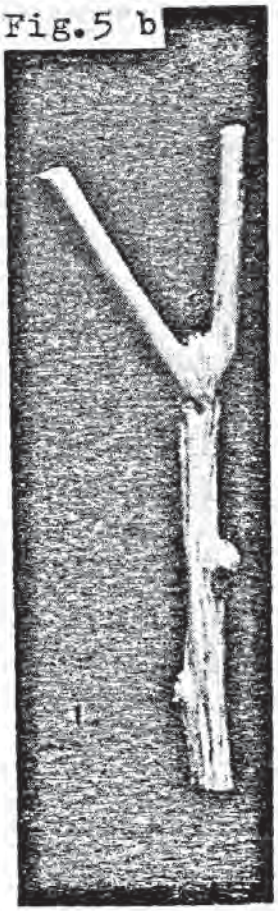
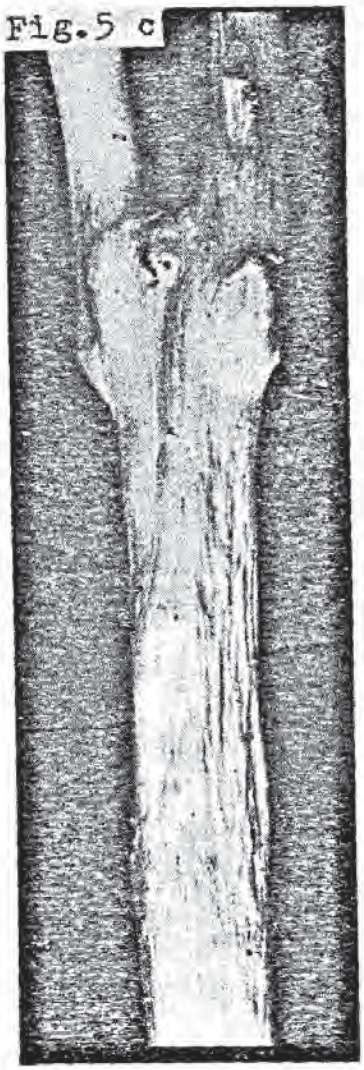


Fig. 1. Diseased Ponkan leaves showing vein yellowing and mottling of leaves.
Fig. 2. Diseased Ponkan branch showing production of multiple, abnormal flowers and reduction in size and color of newly-flushed leaves.
Fig. 3. Diseased Tankan leaves showing yellowing of midrib and interveinal mesophyll tissue (lower 3) and healthy leaf (uppermost).
Fig. 4. Diseased Ponkan leaf (left) showing vein-corking and healthy leaf (right).







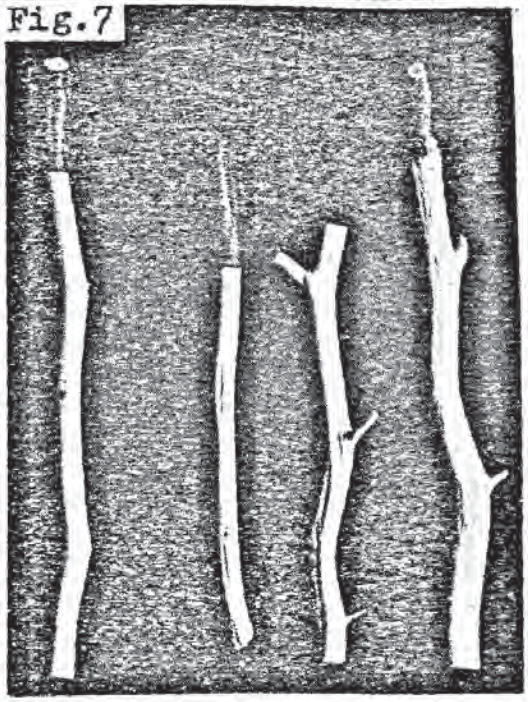


Fig. 5. Stem-pitting on xylem of (a) Washington navel, (b) Tankan, and (c) Rangpur lime infected with Likubin virus.

Fig. 6. Leaves of Mexican lime artificially inoculated showing vein-clearing.

Fig. 7. Stem-pitting on xylem of Mexican lime artificially inoculated and no pitting on xylem of healthy Mexican lime (left one).

4) production of multiple, abnormal flowers, 5) reduction in size and color of newly-flushed leaves, which become elastic and tough later (Fig. 2), 6) root-decay to varied extents. As aforementioned, the yellowing or mottling of leaves is the constant feature of the diseased plant, but the patterns of mottling are somewhat variable. For instance, in the case of Ponkan the leaves first exhibit yellowing of veins and mesophyll tissue surrounding the veins, less frequently yellowing of the mesophyll tissues with veins remaining still green (Fig. 1), while in Tankan leaves, the midrib and a few main veins arising from the former also turn yellow, but at the same time or a little later the interveinal mesophyll tissues are also yellowed, less frequently the apical part of leaves first turning entirely yellow, the tone of the yellow color being not so bright or somewhat pale and dingy as compared with that of the diseased Ponkan leaves (Fig. 3). The leaf symptoms on sweet orange are also more or less variable as varieties differ, but so far as the varieties such as Valencia, Sekkan, Washington navel, Luichen, etc. are concerned, the more prominent feature is yellowing of veins and their immediate vicinity, but the major part of mesophyll remaining green for a considerable length of time.

Besides the yellowing of leaves, vein-corking (Fig. 4) is rather characteristic symptoms associated with the disease following vein-yellowing, and stem-pitting is also occasionally observed in some varieties such as Washington navel, Tankan, Rangpur, lime, etc. (Fig. 5). It is often noticed that the decline symptoms are only confined to the one side of trees and the remainders are apparently normal. Another characteristic feature is the diminution of starch in the roots of stocks and also in the shoot of scions. This can be clearly demonstrated by microchemical tests in the seedling stage.

TRANSMISSION OF DISEASE BY MEANS OF GRAFTING WITH SCIONS OBTAINED FROM DISEASED TREES

Potted Sunki seedlings or several Citrus varieties on Sunki-root stock were grafted with the buds of various kinds of diseased trees growing in different parts of this island, or sometimes inarched to rooted diseased plants *in situ* instead of bud-grafting. The percentage of organic union after grafting was naturally varied as the source differed, being at the range of 30–70 percent or occasionally 100.

Generally, growth of scions was poor or no shoot developed from the scion, though the latter still remained green. Stocks also revealed poor growth, producing a limited number of small pale green leaves born on stunted shoots or no shoot at all. The results of the experiments are tabulated as follows (Table 1).

REACTION OF MEXICAN LIME AS A TEST PLANT

The first trial was made with a diseased Ponkan scions collected from Hsin-pu Aug. 27, 1957. The mother tree of these scions was 7-year old Ponkan grafted on Sunki-root stock, about 6.5 feet high and 5–6 cm wide (measured at the base). According to the proprietor of this orchard, this tree was apparently healthy until

Table 1. Results of transmission studies by grafting

Strains	Inocula from	Plants inoculated	Date of grafting	Date of observation	Results
U. O. 1	Tankan	Sunki-seedling (10)*	Apr. 25, 1956	Oct. 1957	Nine of ten plants exhibited the characteristic symptoms. The leaves on Sunki-stocks also exhibited the symptoms such as chlorosis, corking of vein, etc. However, the remaining one plant did not reveal any typical symptoms and was apparently normal in appearance.
U. O. 3	Ponkan	Sunki-seedling (7)*	Sept. 13, 1956	Oct. 1957	Six of seven plants exhibited the characteristic symptoms, and finally succumbed. The remaining one was still alive, though apparently abnormal in growth.
U. O. 4	Ponkan	Sunki-seedling (11)*	Sept. 13, 1956	Oct. 1957	All the scions died prematurely, though completely united, and three stocks of the eleven plants are still growing, though four of the remainders succumbed and the other four were greatly stunted.
U. O. 6	Valencia	Sunki-seedling (9)*	Sept. 13, 1956	Oct. 1957	All the plants were infected, though three of them were still narrowly surviving.
U. O. 7	Ponkan	Sunki-seedling (7)*	Sept. 13, 1956	Oct. 1957	Five of seven plants exhibited the characterisitic symptoms and were mostly on the verge of death, while two remaineders were somewhat better in appearance.
U. O. 8	Ponkan	Sunki-seedling (3)*	Sept. 13, 1956	Oct. 1957	Two of them already died and the remaining one has already arrested its growth, exhibiting the characteristic chlorotic symptom.
U. O. 9	Temple orange	Sunki-seedling (5)*	Sept. 13, 1956	Oct. 1957	Two plants died shortly after union, but the remaining three were still alive, although some of the leaves on the upper parts of shoots were more or less mottled and apparently abnormal.
YMS 2	Tankan	Sunki-seedling (3)*	June 18, 1956	Dec. 1957	Two plants revealed the characteristic symptoms on both the scion and stock, and the remaining one was also apparently abnormal in growth.
YMS 3	Tankan	Sunki-seedling (3)*	June 18, 1956	Dec. 1957	One plant died after a year; one of the two remainders exhibited the symptoms and the stock of the other was still apparently healthy, though its scion had already died.
YMS 5	Tankan	Sunki-seedling (1)*	June 18, 1956	Dec. 1957	The stock of this grafted plant was then consecutively inarched with two Sunki-seedlings, and the first inarched was further side-grafted with a

Strains	Inocula from	Plants inoculated	Date of grafting	Date of observation	Results
					healthy Valencia scion. Yellowing- and stunting-symptoms were clearly noticed on all the parts grafted and the original scion had already succumbed.
YMS 7	Valencia	Sunki-seedling (9)*	June 18, 1956	Dec. 1957	Four of them already died, and the scions of the remaining five plants exhibited chlorotic-and stunting symptoms.
YMS 10	Ponkan	Sunki-seedling (4)*	Aug. 4, 1956	Dec. 1957	Two plants died and the two remainders were apparently abnormal in vigor, though not greatly stunted.
YMS 15	Ponkan	Sunki-seedling (10)*	Dec. 11, 1956	Dec. 1957	Six plants already died, and four remainders exhibited the characteristic stunting-and chlorotic symptoms.
YMS 15	Ponkan	Trifoliate seedling (5)*	Dec. 11, 1956	Dec. 1957	All the five plants have already died.
SP 1 (B)	Ponkan	Sunki-seedling (2)*	Oct. 22, 1956	Jan. 1958	One of the two plants already died, and the remainder was greatly stunted, the leaves of the stock exhibiting mottling and the scion being arrested in growth.
SP 4 (B)	Ponkan	Sunki-seedling (7)*	Oct. 22, 1956	Jan. 1958	Four of them completely deceased, and the scions of three remainders also died, though their stocks were still alive, but greatly stunted and mottled.
TC 4	Tankan	Sunki-seedling (8)*	Nov. 10, 1956	March, 1958	Two plants already died, and three scions of the remainders also succumbed, but their stocks were still alive, though two of them were stunted and mottled. The other three were still alive, but both the scion and stock were greatly stunted and mottled.
TC 5 (B)	Tankan	Sunki-seedling (6)*	Nov. 10, 1956	March, 1958	Two plants already died, and three scions of the remainders were also killed, but their stocks were still alive, though stunted and mottled. The last one was still alive, though its scion was greatly stunted and mottled.
YL 1	Diseased Ponkan-scion grafted on Sunki-seedling (Apr. 16, 1956)	Sunki-seedlings 5-consecutively grafted by side-grafting	1st: grafted Oct. 17, 1956. 2nd: grafted Oct. 17, 1956. 3rd: grafted Feb. 7, 1957. 4th: grafted Feb. 7, 1957 5th: grafted Feb. 7, 1957.	Aug. 22, 1957	The original grafted tree (i.e. dis. Ponkan-scion grafted on Sunki Apr. 16, 1956) died shortly after the last consecutive grafting (Feb. 7, 1957), but the five successively grafted Sunki-seedlings were still alive. However, these seedlings also exhibited the characteristic leaf-symptoms in turn by the time when the final reading was made (Aug. 22, 1957).

* The parenthesized figure denotes the number of plants completely united.

U. O: Collected from our University orchard; YMS: Yangmingshan; TC: Tour-cheng, Yi-lan Pref.; SP: Hsin-pu; YL: Yuanling.

last spring (March, 1956), but began to show some decline symptoms such as chlorosis, yellowing of veins, vein-corking, defoliation, multiple flowering, die-back of small twigs and limbs, etc., viz., the characteristic symptoms similar to those shown by the diseased trees already used for transmission studies. These scions were side-grafted to Mexican limes, sour oranges and sweet oranges, inasmuch as the latter were still so small that the scions could not be grafted on them in routine way. All the seedlings grew rather slowly, exhibiting no marked symptoms except the sour oranges used for comparision, which showed chlorosis and stunting of leaves a few months after grafting. Later, however, some young leaves of Mexican lime revealed the so-called irregular dash-like chlorosis on some lateral veins, which was detected 8 months after grafting when seen by transmitted light (Fig. 6). It was also evidenced that the sweet oranges tested were tolerant to the disease concerned, whereas sour oranges showed chlorosis and stunting as aforementioned.

In addition, some more tests were made with the use of other sources such as (1) a Mexican lime seedling inarched to the rootstock (Valencia) of a YMS 15-infected Ponkan, (2) a Mexican lime seedling inarched to the rootstock (Sunki) of a TC 5-infected Tankan, (3) a Mexican lime seedling inarched to a graft-inoculated Sunki derived from the diseased Ponkan-scion infected with YL 1 which was collected from Yuanling and 5-consecutively grafted to Sunki seedlings (cf. Table 1). The first experiment: About 4 months after inarching, some newly-flushed leaves of the Maxican lime seedling revealed the characteristic vein-clearing which was identical with the symptom mentioned above. The second experiment: After about 4 months, newly-flushed Mexican lime leaves revealed some mottling accompanied with vein-clearing though limited to a few veinlets. The third experiment: A Mexican lime seedling inarched to the fourth grafted-Sunki (viz., the next to the last) revealed the vein-clearing symptom similar to those described in the first experiment 4 months after grafting. The leaf symptoms mentioned above appear to be very much like the vein-clearing and to tristeza, but it is difficult to diagnose the disease on the basis of leaf symptoms alone. In order to decide the matter conclusively, it is absolutely necessary to examine whether stem-pitting is produced or not. The following table will give the details of the observations made particularly for this purpose (Table 2). As shown in this table, three diseases collected from different localities produced stem-pitting first on the upper young stems of Mexican lime seedlings (Fig. 7), while none was noted on the lower part near union when observation was made 8 months (or more) after inarching. On the other hand, another Mexican lime seedling grafted with a diseased Ponkan-scion collected from our University orchard (U. O. 12) revealed stem-pitting almost all over the surface within a year.

TRANSMISSION BY APHIDS

In parallel with the Mexican lime tests, transmission trials were made with *Toxoptera citricidus* (*Aphis citricidus*) which is very common in Citrus groves in

Table 2. Results of observations on stem-pitting in different parts of virus-infected Mexican lime stems

Parts of stem or branches observed	I. YL A-5			II. SP 1 (B)			III. YMS 5		
	Length of stem (cm)	Width of stem (cm)	Stem-pitting	Length of stem (cm)	Width of stem (cm)	Stem-pitting	Length of stem (cm)	Width of stem (cm)	Stem-pitting
1. The uppermost stem	8.5	0.3	Dec. 10* ++	3.5	0.3	Dec. 12 +	4.0	0.2	Dec. 12 ++
2. Stem next to the above (1)	8.5	0.3	Dec. 11 +	3.5	0.3	Dec. 12 +	3.5	0.2	Dec. 12 +
3. Stem next to the above (2)	7.6	0.4	Dec. 11 +	4.0	0.3	Dec. 12 +	5.0	0.3	Dec. 12 +**
4. Between No. 3 & stem near union	7.7	0.4	?	4.0	0.4	—	6.0	0.5	—
5. The uppermost branch	4.5	0.2	Dec. 11 ++	4.3	0.2	Dec. 12 ++	1.0	0.2	+***
6. Branch under No. 5	5.0	0.2	Dec. 11 ++	3.6	0.2	Dec. 12 ++	6.0	0.2	+***

I. YL A-5: An artificially-infected plant inarched to a Mexican lime (Jan. 31, 1958). The latter exhibited vein-clearing.

II. SP 1 (B): An artificially-infected plant inarched to a Mexican lime seedling (Apr. 9, 1958). The latter exhibited vein-corking and vein-clearing.

III. YMS 5: An artificially-infected plant inarched to a Maxican lime seedling (Apr. 9, 1958). The latter exhibited slight dash-like vein-clearing.

* The experiment was conducted in 1958.

** Mostly confined to the upper part.

*** Stem-pitting was also recognizable on branchlets.

Taiwan and is said to be the principal insect vector of tristeza in South America, South and West Africa and Australia. Some young shoots of healthy Citrus plants infested with the aphids were removed to naturally-infected Citrus trees, and the aphids were allowed to move over voluntarily and feed for 24 hours. After 24-hour feeding the aphids were transferred onto test-plants (Mexican lime) by means of a moistened brush (25–70 aphids per plant). When another 24-hour feeding period terminated, the aphids were brushed off by means of a dry cotton swab and the plants were then sprayed with Malathion solution (0.02%). All these experiments were conducted in the special compartment installed in our glass house, a special care being taken not to make them infested with other insects.

In the first series of experiment, two plants inoculated with U. O. 14-strain and one plant inoculated with YMS 15-strain revealed vein-clearing symptoms on young leaves. These plants were inoculated on Oct. 24 and the symptoms were observed on Dec. 2, accordingly, the incubation period in this particular case is 39 days. Similarly, positive results were also noticed on each one of the plants inoculated with YL 6(A) and YMS 15 seven and eleven days later, respectively. More details are shown in Table 3.

Table 3. Transmission trials by aphids (*Toxoptera citricidus*)

Exp. No.	Strains	Plants inoculated*	Date of inoculation	Date of the first appearance of vein-clearing & incubation period
1	U. O. 14(Valencia)(1)	1	Oct. 24, 1958	Dec. 2 (39 days)
		2		—
		3		Dec. 2 (39 days)
	YL 6(A) (Ponkan)(2)	1	Oct. 24, 1958	—
		2		Dec. 9 (46 days)
		3		—
	YMS 15(Ponkan)(3)	1	Oct. 24, 1958	—
		2		Dec. 13 (50 days)
		3		Dec. 2 (39 days)
2	Sp 6(B) (Tankan)(4)	1	Nov. 9, 1958	—
		2		—
		3		Feb. 2 (84 days)
	U. O. 6(B) (Valencia)(1)	1	Nov. 9, 1958	Jan. 2 (53 days)
	U. O. 14 (Valencia)(1)	1	Nov. 9, 1958	Feb. 5 (87 days)
		2		Jan. 2 (53 days)
3	U. O. 1 (Tankan)(5)	1-2 years old plant	May 16, 1961	Sept. 6 (113 days)
		4 month old plant	June 29, 1961	July 21 (22 days)
		4 month old plant	June 11, 1961	July 6 (25 days)
	YMS 15 (Ponkan)(3)	1-2 years old plant	May 16, 1961	Sept. 6 (113 days)
		4-month old plant	June 29, 1961	July 21 (22 days)
4	New YL 7 (Ponkan)(2)	1	Dec. 30, 1958	—
		2		March 17 (76 days)
5	New SP 1 (6-10-years old Ponkan)(6)	1	March 8, 1959	—
		2		—
		3		Apr. 17 (40 days)
	New SP 2 (6-10-years old Ponkan)(6)	1	March 8, 1959	Apr. 17 (40 days)
		2		—
		3		—
	New SP 3 (6-10-years old Ponkan)(6)	1	March 8, 1959	Apr. 22 (45 days)
		2		Apr. 20 (43 days)
		3		—
	New SP 4 (6-10-years old Ponkan)(6)	1	March 8, 1959	—
		2		—
		3		Apr. 18 (41 days)
	New SP 5 (6-10-years old Ponkan)(6)	1	March 8, 1959	—
		2		Apr. 19 (42 days)
		3		—

* Mexican lime seedling
(1) Valencia grown in our University orchard.
(2) Ponkan collected from Yuanling.
(3) Ponkan collected from Yangmingshan.
(4) Tankan collected from Hsinpu.
(5) Tankan grown in our University orchard.
(6) Ponkan collected from Hsinpu.

The next experiment was made by use of aphids collected from naturally-infected Citrus plants growing in orchards. As shown in the same table, infection was evidenced by transferring the aphids collected from naturally-infected Citrus leaves. It is therefore of no doubt that the disease spreade readily from plants to plants by mean of aphilds feeding naturally on diseased plants. In this connection, it is to note that the incubation period required for the appearance of symptoms is rather variable even in the case of the same virus strain. For instance, as shown in the table, in one instance when 4-month old seedlings were used the first symptom of vein-clearing on Mexican lime was observed 22 days after transfer, but in the case of 1-2 years old plants, it was revealed after 113 days. It seems likely that this may be chiefly due to the condition of plants tested.

DISCUSSION AND SUMMARY

The symptoms of the so-called "Likubin" or "Huanglungpin" are somewhat different between those of Ponkan and Tankan which are widely grown in Taiwan, and are also different according to environmental conditions. Generally speaking, however, in the case of Ponkan, veins and mesophyll tissuses surrounding the veins first turn yellow, and the remaining interveinal mesophyll usually remains green for a while, being somewhat like the symptoms resulting from ringing, whereas on Tankan leaves the midrib and a few main veins arising from the former turn yellow, at almost the same time or a little later interveinal mesophyll tissues are also yellowed, not infrequently the apical part of leaves first turns entirely yellow. The tone of the yellow color is slightly paler or dingy in Tankan. On both the leaves, however, vein corking is noticed, and other symptoms such as die-back, premature defoliation, multiple blossoming, root decay, etc. are almost the same.

The chief object of the present paper is to solve the question on the causation of this disease, i.e. decline of Citrus trees, which has been regarded as a non-parasitic disease for a long time. Therefore, during the first 2 odd years since 1956 when this study was entered upon, we made efforts to take a view of the scene all over Taiwan where Citrus trees were growing and collected the so-called "Likubin"-suffering plants from various parts of this island. At the same time laboratory experiments were undertaken by culturing Ponkan, Tankan, etc. on Sunki-rootstocks under various soil-conditions, and also by grafting the diseased twigs or buds onto the aforementioned healthy plants and Mexican lime seedlings as well as transmitting the diseased sap by aphids. In parallel with these experiments, some trials were made by inarching potted healthy Sunki-seedlings grown under various soil conditions to potted diseased Ponkan or Tankan on Sunki-rootstocks or diseased plants growing in the field consecutively until 5-6 similar such potted Sunki-seedlings were inarched in succession. By these means it was definitely confirmed that the cause of the disease was the virus and by no means abnormal soil conditions, since almost all the plants either grafted or infested by insect-vectors contracted the disease.

In consideration of the fact that our virus is transmitted by the aphid, *Toxoptera*

citricidus, and that the characteristic symptoms such as vein-clearing and stem-pitting could be produced on Mexican lime seedlings when bud-grafted with the diseased plant or inoculated by means of the aphid, it seems likely that the virus concerned is very closely related to tristeza. However, as will be shown in the next paper, our "Linkubin"-virus is not absolutely identical with the aforementioned in the reaction of stock-scion combination to the virus under consideration. As regards the matter, further discussions will be made later in the serial papers to be published hereafter.

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