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Electron Microscopical Investigations on Tristeza

CITRUS VIRUSES have been the subject of an increasing number of publications during recent years. So far as the writers know, however, there is no mention of electron microscopically detectable particles representing these viruses, with the exception perhaps of the report that psorosis virus is spherical (10).

Electron microscopical studies on tristeza have been carried out in the Virus Department of the Instituto Agronomico de Campinas for more than two years. This paper reports the finding of a thread-like particle in tristeza-infected citrus plants and evidence that the thread-like particle is tristeza virus. Preliminary results have been published elsewhere (12).

Materials and Methods

In the early attempts, Johnson's exudate method (9), with the aid of an adapted compressed-air sprayer (11), was applied to leaves of healthy or tristeza-infected Galego lime [*Citrus aurantifolia* (Christm.) Swing.] and *Aeglopsis chevalieri* Swing. The exudate was transferred by means of a micropipette to a collodion membrane held on a copper grid and the preparations were metalized with palladium or chromium after drying.

Because of the difficulties encountered in getting exudate from citrus leaves, and also because of the usual low concentration of particles, Brandes' dipping method (3) was tried. Leaf pieces were dipped for about a minute in a drop of distilled water on a collodion membrane

coated grid and the preparation was dried and metalized as mentioned above.

For purposes of measurement, pictures were taken at 5,000 X and the negatives were enlarged optically to a final magnification of 50,000 X. The measured values were then distributed in classes with 25 mμ intervals.

Partially purified preparations were obtained from leaves of healthy and tristeza-infected Galego lime and *A. chevalieri*, as described by Silva *et al.* (13). They were shadow-cast or negatively stained (5) and then observed in the electron microscope. Negatively stained preparations were examined at an instrumental magnification of 40,000 X, using the double condenser.

All electron microscopical work was done in a Siemens Elmiskop I electron microscope.

Results

GREENHOUSE-INFECTED GALEGO LIME AND AEGLOPSIS CHEVALIERI.—Thread-like particles (Fig. 1) were detected in preparations from leaves of tristeza-infected plants but not in those from healthy control plants. Their concentration in exudates was usually low. In dipping preparations, concentration of particles was satisfactory; the dipping technique has, consequently, been used routinely since then.

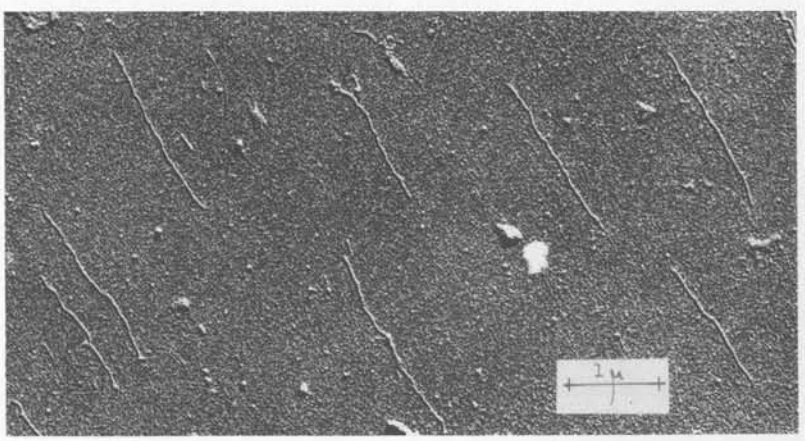

FIGURE 1. *Electron micrograph of the thread-like particles in a dipping preparation made from tristeza-infected Galego lime, shadow-cast with palladium.*

INFECTED FIELD PLANTS.—Observations were subsequently made of tristeza-infected field plants. Preparations from leaves of diseased plants of Galego lime, *A. chevalieri,* and Barão and Pera sweet orange [*C. sinensis* (L.) Osbeck] growing at the Theodureto de Camargo Experiment Station, Campinas, always contained the thread-like particles.

Observations were also made of a large number of citrus types planted at the Limeira Agriculture Experiment Station. The leaf samples were collected in plastic bags and brought to Campinas where they were prepared for electron microscopy. The thread-like particles were detected in practically all tristeza-susceptible citrus species and varieties tested but not in those known to be highly resistant to tristeza or immune from it (6), such as *Poncirus trifoliata* Raf., *Severinia buxifolia* Ten., or Troyer citrange (*C. sinensis* x *P. trifoliata*) (Table 1).

Some preparations made from leaves of side branches growing from a *P. trifoliata* rootstock having a tristeza-infected sweet orange top failed to reveal thread-like particles.

Thread-like particles were also detected in relatively large amounts in preparations from stems of Red Blush grapefruit (*C. paradisi* Macfd.), Pera sweet orange, and Galego lime showing severe pitting, whereas none were seen in those made from stems of healthy grapefruit.

APHID-INOCULATED CITRUS SPECIES.—Additional evidence for association of the thread-like particles with tristeza was obtained in a set of three experiments. Young seedlings of different citrus types (seven in the first experiment; eleven in the second and third), kept under greenhouse conditions, were inoculated by viruliferous aphids (*Toxoptera citricidus* Kirk.). Leaves of these plants were sampled for electron microscopy shortly before inoculation and at about weekly intervals thereafter. At the same time, non-inoculated control plants were sampled. Viruliferous aphids for the inoculation were collected from three different source plants: Barão sweet orange, Galego lime, and Pera sweet orange, for the first, second, and third experiment, respectively. Leaves from the plants were sampled for electron microscopical examination, and were found to contain thread-like particles.

In aphid-inoculated plants, thread-like particles became detectable in from 16 to 28 days after inoculation, except for Troyer citrange. In control non-inoculated plants, such particles were never found (Table 2). The failure to detect thread-like particles in some inoculated plants in the third experiment can be explained on the basis of lack of infection due to poor inoculum.

TABLE 1. RESULTS FROM ELECTRON MICROSCOPICAL EXAMINATIONS OF DIPPING PREPARATIONS TAKEN FROM SEVERAL CITRUS AND RELATED SPECIES[a]

Plants	Number of plants		Plants	Number of plants	
	Examined	With thread-like particles		Examined	With thread-like particles
SWEET ORANGE			LIME		
Hamlin Reserve	4	4	Persian	4	2
Natal Bebedouro	4	2	Galego	5	5
Piralima	3	2	Tahiti	4	4
Caipira	4	4	Rangpur	4	2
Pera coroada	4	3			
Lue Gim Gong	4	0	GRAPEFRUIT		
Baianinha Piracicaba	2	2	Marsh seedless	4	3
Agro dôce	3	2	Red Blush	3	3
			MacCarty	3	2
TANGERINE and MANDARIN			HYBRIDS		
			S. Jacinto Tangelo	4	1
Sunshine	4	4	Sampson Tangelo	4	3
Satsuma Wase	4	1	Orlando "	4	2
Mexerica do Rio	2	1	Thornton "	6	3
Ponkan	4	2	Troyer citrange #6	5	0
Satsuma Owari	2	2	Rusk "	6	0
Italiana	4	4	Norton "	6	1
Campiona	2	2			
Cleopatra	4	0	MISCELLANEOUS		
Mel	4	3	*Citrus ichangensis*	3	2
Mexerica Pará	4	1	*Poncirus trifoliata*	6	0
			Severinia buxifolia	3	0
			Faustine	2	0
LEMON			Microcitrus	4	0
Siciliano	4	4	Hesperethusa	4	0
Lisboa (tetra)	5	0	Comprida Citron	6	6
Vila Franca	2	2	Redonda	3	2
Americano	2	2	Dôce	2	2
			Agrossevilhana	2	1
			Sour orange	3	2

[a]Plants in the citrus collection, Limeira Agriculture Experiment Station.

PARTIALLY PURIFIED PREPARATIONS.—In partially purified preparations, those from diseased plants always contained thread-like particles, not present in control preparations from healthy plants (Fig. 2).

The thread-like particle found in tristeza-infected plants is very flexible, usually appearing to be curled or twisted. Its diameter is about 10-12 mμ. The normal length (1, 14) calculated from the particle length distribution curve (Fig. 3) is about 2,000 mμ. Some ultrastructural de-

TABLE 2. DETECTION OF THREAD-LIKE PARTICLES BY ELECTRON MICROSCOPICAL EXAMINATION OF DIPPING PREPARATIONS TAKEN FROM VARIOUS SPECIES OF INOCULATED PLANTS AND CONTROLS AT THE INDICATED NUMBER OF DAYS AFTER INOCULATION[a]

	Inoculated				Control			
	0[b]	10	16	28	0	10	16	28
Aeglopsis chevalieri	———	———	+——	++	———	———	———	——
Galego lime	———	———	+++	++	———	———	———	——
Rangpur lime	———	———	+——	++	———	———	———	——
Pera sweet orange	———	———	+++	++	———	———	———	——
Cleopatra mandarin	———	———	+——	+—	———	———	———	——
Red Mexican grapefruit	———	———	+——	+—	———	———	———	——
Persian lime	———	———	+——	++	———	———	———	——
Sour orange	——	———	——	++	——	———	———	——
Troyer citrange	——	——	——	——	——	———	———	——
Eureka lemon	——	——	——	+—	——	———	———	——
Florida sweet orange	——	——	——	++	——	———	———	——

[a]Plus sign indicated presence of particles; minus sign indicated their absence.
[b]Sampling made just prior to inoculation by means of aphids.

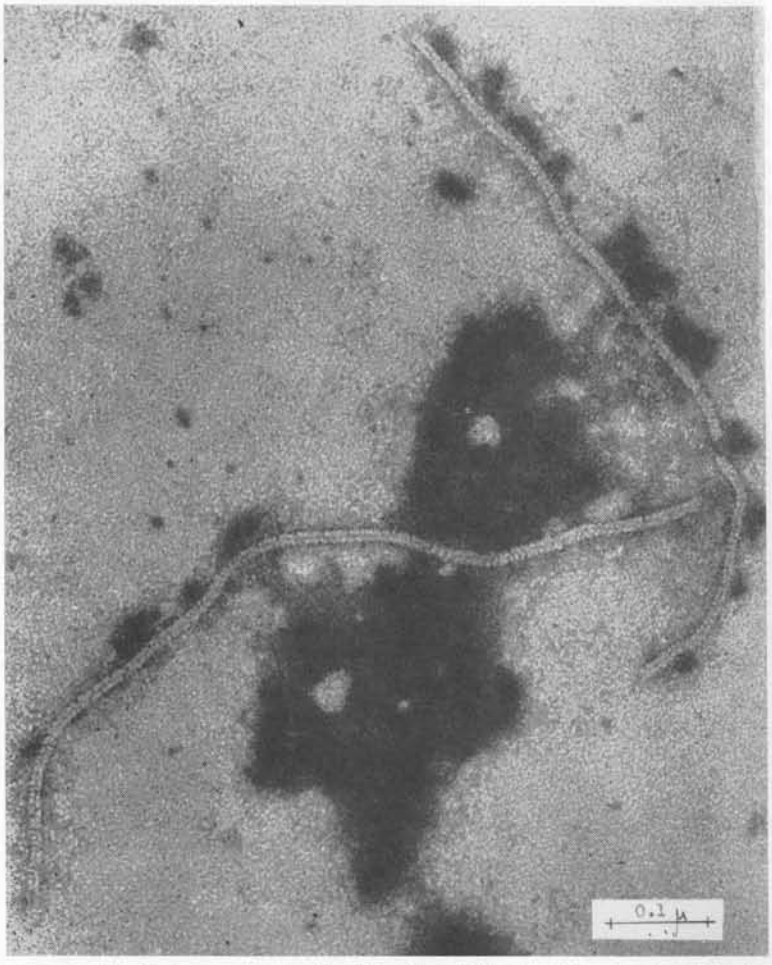

FIGURE 2. *Electron micrograph of the thread-like particles in a partially purified preparation from Galego lime infected with tristeza, negatively stained with phosphotungstic acid.*

tails such as an internal channel, measuring 2-3 mμ in diameter, and signs of a helical arrangement of particle subunits could be seen by negative staining (Fig. 2).

Another kind of particle was found in partially purified preparations from healthy or tristeza-infected plants. It was thinner than the thread-like particle, having a diameter of 6-7 mμ with variable length, and occurred in relatively large amounts. When negatively stained it did not

show any internal detail. In dipping preparations, such particles were seldom found.

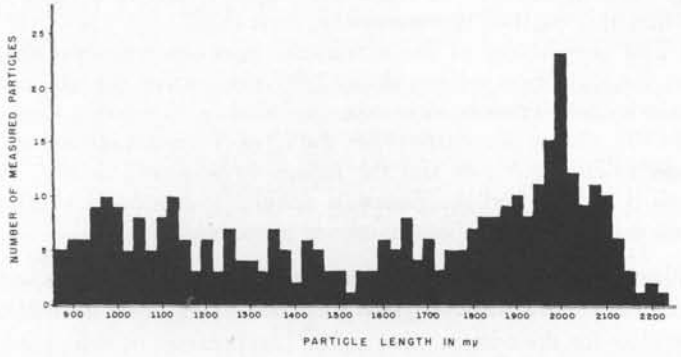

FIGURE 3. *Histogram of distribution of lengths of thread-like particles.*

Discussion

Although infectivity of the preparations containing the thread-like particles was not proved, the bulk of the evidence suggests that the particles represent tristeza virus. The following considerations support this point of view:

1. Thread-like particles were present in preparations from leaves of tristeza-infected plants and not in those from healthy control plants.

2. Practically all tristeza-susceptible plants in a collection belonging to the Limeira Agriculture Experiment Station contained the thread-like particles. Those species known to be highly resistant to tristeza or immune from it, on the contrary, did not contain the particles.

3. The interval in days between the inoculation of young seedlings of tristeza-susceptible species by means of viruliferous aphids and the appearance of thread-like particles coincides with that found necessary to have recoverable virus (7) and also with the onset of the symptoms in the most sensitive hosts. The failure to detect particles in aphid-inoculated Troyer citrange confirms its high degree of resistance to tristeza.

4. The thread-like particles were seen in partially purified preparations from infected plants but not in parallel samples from healthy plants. Chemical analyses (13) indicated that preparations that contained the particles also contained a nucleoprotein.

5. It seems unlikely that an abnormal component resulting from

reactions of the cell to virus infection would have the same morphology in completely different citrus types. The presence of thread-like particles in preparations from some symptomless carriers, such as certain sweet orange and mandarin (*C. reticulata* Blanco) varieties, supports the view that this particle represents the virus itself.

6. The morphology of the thread-like particles resembles that of a known virus, the beet yellows virus (2, 8), except that the tristeza particles are longer. Tristeza virus was included in the genus *Corium* by Klotz (10). On certain citrus types, the virus of tristeza induces thickening and rolling of leaves and the foliage to become leathery. Phloem necrosis is present and the disease is aphid-transmitted. A similar type of disease is induced by beet yellows in some host plants.

Although tristeza and beet yellows viruses present similarities in shape, their difference in normal length is great enough to justify the addition of a new class for the tristeza virus in the classification of elongated plant viruses based on the particle morphology, as proposed by Brandes and Wetter (4).

Regardless of the nature of the thread-like particles associated with tristeza, their presence provides a quick method for identifying infected plants whenever an electron microscope is available. Detection of the particles also provides a useful indicator in the control of purification processes and in solving some basic problems.

The nature of the thin particle found in partially purified preparation is not known yet. It could represent a normal component of the plants used for purification or a latent virus. In the latter case, however, seed transmission to a high extent would have to be postulated. Whatever the nature of the particles, their presence in preparations observed in the electron microscope could lead to a misleading concept of the possible shape of other citrus viruses.

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