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POSSIBLE ASSOCIATION OF TOBACCO MOSAIC VIRUS WITH THE ROOT (WILT) DISEASE OF COCONUT

P. SHANTA, V. HARIHARASUBRAMANIAN*, AND N. GOPINATHAN PILLAI

Central Plantation Crops Research Institute, Regional Station, Krishnapuram 690 533, Kerala State, India

ABSTRACT

Experiments to check the presence of tobacco mosaic virus have shown that very low amounts of the coconut strain of TMV could be detected when added to coconut root or leaf extract, showing that if the virus is present, infectivity tests should be adequate for testing the presence of the virus in diseased plants. It was also possible to distinguish particles of TMV in negatively stained preparations of coconut root and leaf containing TMV. All infectivity tests and electron microscope observations carried out with diseased coconut material yielded negative results for presence of this strain of the virus.

INTRODUCTION

RECORDED as early as 1882, the root (wilt) disease of coconut found mainly in Kerala State (India), is classified as a disease of unknown etiology (Maramorosch, 1964). The casual agent of the disease is still unknown although information on the symptomatology and transmission of the disease is available.

Summanwar *et al.* (1969) and Summanwar, Raychaudhuri, and Jagadishchandra (1971) reported the presence of a rod like virus believed to be a strain of tobacco mosaic virus (TMV) in concentrated preparations of diseased coconut leaves and roots. The virus was reported to be 320–360 $m\mu$ long and 24–25 $m\mu$ wide. They felt that the virus could not be detected by infectivity tests because of the inhibitory effect of the high tannin in the coconut tissue.

The present paper describes efforts to verify the observations of Summanwar *et al.* (1969) by pathogenicity and serological tests as well as by electron microscopy.

MATERIALS AND METHODS

Antisera used.—Antiserum for the coconut isolate of TMV was prepared by immunisation of young albino rabbits. Two intramuscular injections two weeks apart with 2 ml of purified virus emulsified with an equal volume of Freund's adjuvant were followed a fortnight later by two intravenous injections of 2 ml virus two days apart. Antiserum against the same isolate of the virus was also obtained from the Indian Agricultural Research Institute, New Delhi. Antiserum to the coat protein of U_1 strain of TMV was obtained from Dr. Milton Zaitlin, University of Arizona, Tucson, USA.

Viruses.—The virus isolated by Summanwar *et al.* (1969) from coconut plants affected by the root (wilt) disease and hereafter referred to as the "Coconut isolate of TMV" was multiplied on *Nicotiana tabacum* L. and used in experiments conducted at Kayangulam. Dolichos enation mosaic virus (DEMV), a serotype of TMV (Kassanis and McCarthy, 1967), was used for experiments conducted at the University Botany Laboratory, Madras, India.

Coconut plants.—Leaves and root samples from coconut trees affected by the root (wilt) disease at Kayangulam and healthy trees from Trichur, Kasaragod, and Goa on the West Coast, Madras on the East Coast, and Mohitnagar from northeast of India were studied.

The purification method used was that of Summanwar *et al.* (1969) and Damirdagh and Shepherd (1970).

Serological tests.—Agglutination, precipitin, and ring interphase tests were performed

* Present Address: Department of Biology, Wayne State University, Detroit, Mi. 48202, USA.

according to Matthews (1957). Ouchterlony double diffusion tests were made in 0·9% Ionagar No. 2 (Oxoid Ltd.) in petriplates.

Phenol extraction of nucleic acids was done according to Diener (1965).

Electron microscopy.—Root and leaf samples for electron microscopy were examined in a Siemens electron microscope and a Zeiss 9s electron microscope operated at 60 KV. Samples were examined after negative staining with phosphotungstic acid or uranyl acetate on collodion coated grids.

Results

Infectivity tests.—Tables I and II present the results of tests with diseased coconut tissues ground in three volumes of distilled water to which purified coconut isolate of TMV was added at different concentrations (0·001 to 1·000 μg/g tissue of leaf or root). The virus was detectable in the presence of coconut leaf extracts at very low concentrations of 0·001 μg/g tissue although activity was considerably reduced with incubation. With DEMV, virus was detected at 10^{-4} μg/ml when tested on the systemic host *Dolichos lablab* and at 10^{-3} μg/ml on the local lesion host *Cyamopsis tetragonaloba*.

TABLE I

Infectivity of "coconut isolate of TMV" at different concentrations and incubation periods on N. tabacum *L. var.* xanthi *when the purified virus was added to wilt diseased coconut leaf tissue*

Concentration of virus added per gram tissue	Incubation period in hours					
	0	24	48	72	96	11 days
1·00 μg	130–150*	150–180	84	31	84	2
0·10 μg	150–200	30	53	48	28	1
0·01 μg	60	10	20	1	5	0
0·001 μg	18	2	4	1	2	0

* Lesions per leaf (average of four leaves).

TABLE II

Infectivity of "coconut isolate of TMV" at different concentrations and at different incubation periods on N. tabacum *L. var.* xanthi *and* N. glutinosa *when purified virus was added to diseased coconut root tissue*

Concentration of virus added per gram tissue	Incubation period in hours						
	Virus alone X	0 Coconut root alone X	Coconut root + virus 0 X	24 G	24 G	72 X	120 X
1·00 μg	—	All 436 samples tested yielded negative results	large number coalesced	—	—	76	40
0·10 μg	38		33	40	7	60–80	33
0·01 μg	6		14	3	0	1	1
0·001 μg	3		1	0	0	0	0
0·0001 μg	—		—	2	1	—	—

— Not tested

Numbers indicate lesions per leaf (average of four leaves).

X: *N. tabacum* var. *xanthi*, G: *N. glutinosa*.

Crude extracts and centrifugal fractions of samples of root and leaf of 436 diseased coconut trees were tested for infectivity on these test plants. No infection was obtained in any case indicating absence of any strain of TMV, in the root (wilt) infected coconut trees.

In view of the belief that it is the high tannin content of the coconut tissue which prevents detection of virus in the diseased materials (Summanwar *et al.*, 1969), nucleic acids from diseased leaves were phenol-extracted and tested for infectivity. No infectivity was noticed on *N. tabacum* var. *xanthi*. However, when 2·5 μg coconut isolate of TMV was added to 5 g diseased leaf material and the phenol-extracted nucleic acid was tested, in 1 ml buffer, infectivity could be detected. This shows that the method is sensitive and could detect TMV if it were to be present in the root (wilt) infected coconut tissue.

Serological tests.—Root and leaf extracts of diseased coconut trees were tested against different sera (normal, antiserum to the coat protein of U_1 strain of TMV, CPCRI antisera I and II prepared against the coconut isolate of TMV, and antisera prepared against the same isolate of the virus at the Indian Agricultural Research Institute). Agglutination, ring interphase, precipitin, and Ouchterlony double diffusion tests were conducted.

It was found that agglutination tests were unreliable with coconut leaf and root extracts because of non-specific reactions obtained with normal serum proteins. Clarified 10,000 g supernatants also often gave nonspecific reactions in ring interphase tests and on agglutination slides, and the test as such was found to be unreliable (Table III).

Ouchterlony tests of diseased coconut leaf extracts showed that a non-specific reaction was obtained against all the tested antisera. Presumably, some material in the coconut leaf diffuses and reacts non-specifically with serum proteins.

Electron microscopic observations.—Long tubular particles 1–2 μ long and 19–24 $m\mu$ wide were observed (Fig. 1A) in all the 49 tender, growing, tissue samples (root and leaf) examined from Kayangulam (wilt affected area) as well as from Kasaragod, Goa, Madras, and Mohitnagar (all wilt-free areas). In mature tissues, four out of five root samples and two out of seven leaf samples of healthy areas presented these rods. Three root and three leaf samples (mature) of Kayangulam also did not have these rods. Samples processed according to Summanwar *et al.* (1969) showed particle distribution of different sizes (300–600 $m\mu$; Fig. 1B). This was evidently due to the breaking up of particles during centrifugation (Fig. 1C). This suggests that the very long particles obtained by gentler methods break up during the process of high speed centrifugation. As a further check, purified

TABLE III

Ring interphase tests of four batches of leaves of 10 *diseased plants each, only* 10,000 *g supernatants tested*

Sample	Sera at different dilutions: normal 1/4	normal 1/8	CPCRI I 1/4	CPCRI I 1/8	CPCRI II 1/4	CPCRI II 1/8	IARI 1/4	IARI 1/8	U_1 A 1/4	U_1 A 1/8
Group I	+?	−	−	−	+?	−	−	−	−	−
Group II	+	−	+	−	−	−	+	−	−	−
Group III	+	+	+	−	+	−	Not done		−	−
Group IV	+?	−	−	−	−	−	Not done		−	−
Coconut isolate of TMV+coconut leaf extract	−	−	+	+	++	++	+	+	++	++
Coconut isolate of TMV	−	−	+	+	+	+	+	+	+	+

preparation of the coconut isolate of TMV was added to the coconut fraction and observed under EM. Particles of TMV were clearly distinguishable from the tubular particles of the coconut material in negatively stained preparations, although both had comparable measurements (Fig. 1D).

These tubular particles thus seem to be associated with root and leaf tissue of coconut palms in general and not specifically associated with root (wilt) disease. It was found in large quantities in developing tissues; in more mature tissues, it was either absent or present in low concentration only.

Discussion

Summanwar *et al.* (1971) stated that dilution end point of the coconut isolate of TMV lies between 10^{-6} and $10^{-6.5}$. It was seen here that upto 10^{-4} of this isolate could be detected by infectivity tests on *Nicotiana tabacum* L. var. *xanthi* or *N. glutinosa* when the pure virus was added to coconut leaf or root and the grindates or fractions tested. Yet, in no case was it possible in the experiments reported here to detect this virus in diseased leaf material by infectivity tests. The coconut root or leaf extracts do not seem to exert any great inhibitory effect on the virus if tested immediately after extraction. If the dilution end point of the virus in coconut leaf tissue is 10^{-6}, the amount of virus present in the tissue is very high and is comparable to that present in tobacco. Yet, it was not possible to detect it in the present trials.

Serological tests also showed that testing root or leaf grindates of diseased coconut was unreliable. Tests of clarified leaf and root fractions showed that no virus responsive to the different types of antisera to TMV including the coconut isolate of TMV was present in the root (wilt) diseased plants.

Summanwar *et al.* (1969) showed the presence of rod-like structures in infected trees and also in some of the trees from Kasaragod, hitherto considered as a disease-free tract. They examined preparations, purified by high speed ultracentrifugation, in a Philips EM 100 after shadowcasting. They did not, however, get any comparable particles from samples collected from Calcutta. The evidence presented here shows that long tubular particles were present in large amounts in young root and leaf tissues from diseased as well as disease-free areas. These particles were either absent, or if present, in only very low concentration in mature green leaf or older leaf and root tissues. It has also been shown here that harsh methods of purification involving high speed ultracentrifugation result in breaking up of the long particles into shorter sizes (Fig. 1C). Since shadowcasting does not fully reveal the structure of viruses, it is quite possible that particles of TMV observed by Summanwar *et al.* (1969) and the tubular structures reported here are the same.

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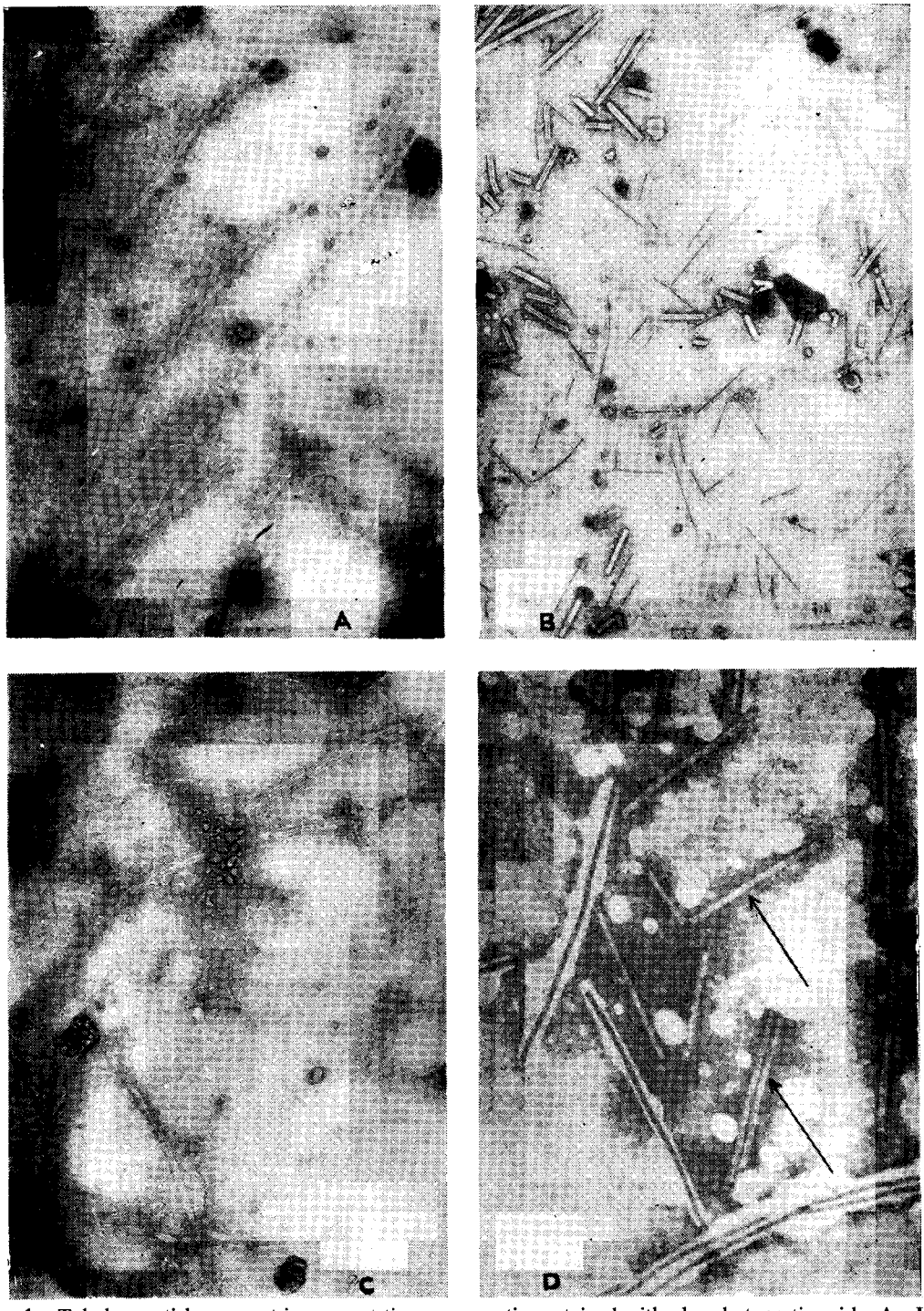

FIG. 1. Tubular particles present in coconut tissue preparations stained with phosphotungstic acid. A. Long unbroken particles from tender leaf, processed by the method of Damirdagh and Shepherd (1970). B. Particles broken up in different lengths when processed by the method of Summanwar *et al.* (1969). C. Breaking of particles under high speed centrifugation. D. Coconut isolate of TMV (arrow) together with the tubular particles of ccoonut leaf preparation. Note the stained central core of the latter when compared to TMV. This central core becomes more prominant on storage of the fractionated coconut material (compare A).