Cowpea Inoculation Test for Diagnosis of Coconut Wilt Disease in India

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Investigators interested in coconut wilt disease as it occurs in Kerala, India, but working in certain other areas of India and the United States where this disease does not occur, recently began to doubt the validity of the experimental inoculation test of Shanta and Menon [1] that has been used extensively at the Central Coconut Research Station at Kayangulam in Kerala State. The doubt was based on observations of leaf deformations in cowpea (*Vigna sinensis*). These deformations were similar to those produced in the test for wilt disease; however, they occurred spontaneously, without any association with the disease or any experimental inoculation such as extracts from foliage, silt from soil collected near the bases of infected coconut trees, or other obvious sources of infection. Such disturbing observations would be entirely understandable if one could assume that disease agents similar to the tiologic agent of coconut wilt disease may be widespread in nature, perhaps differing radically among themselves in being able to infect and damage coconut trees, but resembling each other in their capacity for soil transmission and the ability to distort the leaves of cowpeas after infection of cowpea roots has taken place through infective soil.

During October and December 1964, the senior author, under the auspices of the Food and Agriculture Organization of the United Nations (FAO), had the opportunity to work with the staff of the Central Coconut Research Station at Kayangulam. In the course of this work, the usefulness of the cowpea test in the study of coconut wilt disease was confirmed and some surprising results in the study of unusual soils and soil mixtures were obtained.

Methods and materials

Cowpea seedlings used in inoculation tests were grown within screened shelters in heat-sterilized soil. They were inoculated on their primary leaves when seven days old. Final counts of seedlings showing leaf distortion in their trifoliate leaves (Figure 1) were made one week after inoculation. However, preliminary indications of infection were progressively more easily observed from about 48 hours after inoculation.

Inocula for tests of soil infectivity were routinely prepared as follows: soil was collected about ⅓ of a meter from the base of each tested tree, at a depth of 15 to 30 centimeters, and the samples were dried overnight. 10 grams of soil were weighed out for each appraisal and this amount was suspended in a 250-milliliter beaker full of distilled water. After a four-minute settling period the supernatant fluid, which was usually distinctly cloudy, was poured off and allowed to settle again in another beaker for a period of one hour. At the end of that time, the nearly clear supernatant fluid was discarded and 2 milliliters of the silt-like sediment were diluted in 2 milliliters of phosphate buffer at pH 7 to form the inoculum for each test.

Inoculation was accomplished by rubbing a cotton swab wet with the inoculum four times over the upper surfaces of the two primary leaves of each seven-day-old cowpea seedling. These leaves had been dusted lightly with 200-mesh grade carborundum powder.

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[1] Shanta, P. and K.P.V. Menon. 1960. Cowpea (*Vigna sinensis* Endl.), an indicator plant for the coconut wilt virus. *Virology* 12: 309-310.

Figure 1. First trifoliate leaves of cowpea showing distortion following inoculation of primary leaves of seedlings. Center leaves are from an uninoculated control plant of the same age.

The possible effects of these procedures may be advantageously borne in mind when certain puzzling findings of the subsequent research are considered.

Validity of the cowpea inoculation test

Attention was first given to testing soil samples from the bases of diseased trees and their healthy neighbors in areas just north of the main wilt disease area in Kerala. In these places, although innumerable coconut trees were still healthy, occasional wilt-affected trees occurred in isolated outposts (Figure 2).

Soil collected from the base of a diseased tree at Muringur furnished inoculum that infected 11 out of 20 cowpea plants to which it was applied. When comparable inoculum from soil collected near the bases of two healthy neighboring trees at Muringur was applied to 20 comparable cowpea plants, no infection was noted.

Similarly, soil collected from the base of an isolated, diseased tree at Arattupuzha Island proved infective to six out of ten inoculated cowpea plants growing in sterile soil, whereas soil from near the bases of the five nearest apparently healthy trees produced no infections in five tests, in each of which nine or ten similarly grown cowpea seedlings were inoculated.

Subsequently, soil from the same samples used in the six preceding tests from Arattupuzha Island were examined for infectivity through the process of inoculation. Five cowpea seedlings grown under unscreened shelters from seeds planted in soil from the infected site but not inoculated showed infection on three of them. On the other hand, seedlings varying from two to five in each of five tests similarly grown in soils collected from near the bases of the five healthy trees showed no infection at all. Thus, the soil-transmission test paralleled and confirmed the results of the inoculation test.

Later, soil from another isolated, diseased tree on Arattupuzha Island furnished inoculum that infected eight out of ten inoculated cowpea plants growing in sterilized soil, whereas inocula prepared from soil samples from the four nearest healthy coconut trees produced no infections in four tests, each involving ten cowpea plants similarly grown and inoculated.

Tests of some unusual soils and soil mixtures

Results obtained by testing some other soil samples, however, showed discrepancies, and further experimentation will be required for their eventual elucidation.

For example, a third isolated, wilt-diseased coconut tree was examined at Arattupuzha Island by a number of individuals who were throughly familiar with the symptomatology of coconut wilt disease. This tree was regarded as definitely and severely diseased by all who examined it. Yet an inoculum derived from soil taken in the usual manner from near its base failed to produce any infection when tested on 24 cowpea seedlings. Samples of soil, taken at the same time from near the bases of four trees growing nearest to the supposedly wilt-affected individual, all proved uninfective in tests with 25 cowpea seedlings in each. These soil samples had been collected 30 centimeters from the base of each tree at a depth of 15 to 30 centimeters, in accordance with routine procedure. It was thought that some other distance or depth might be better suited in this case to demonstrate the infectivity of the soil. Therefore, additional samples of soil were collected from a 30-centimeter depth but

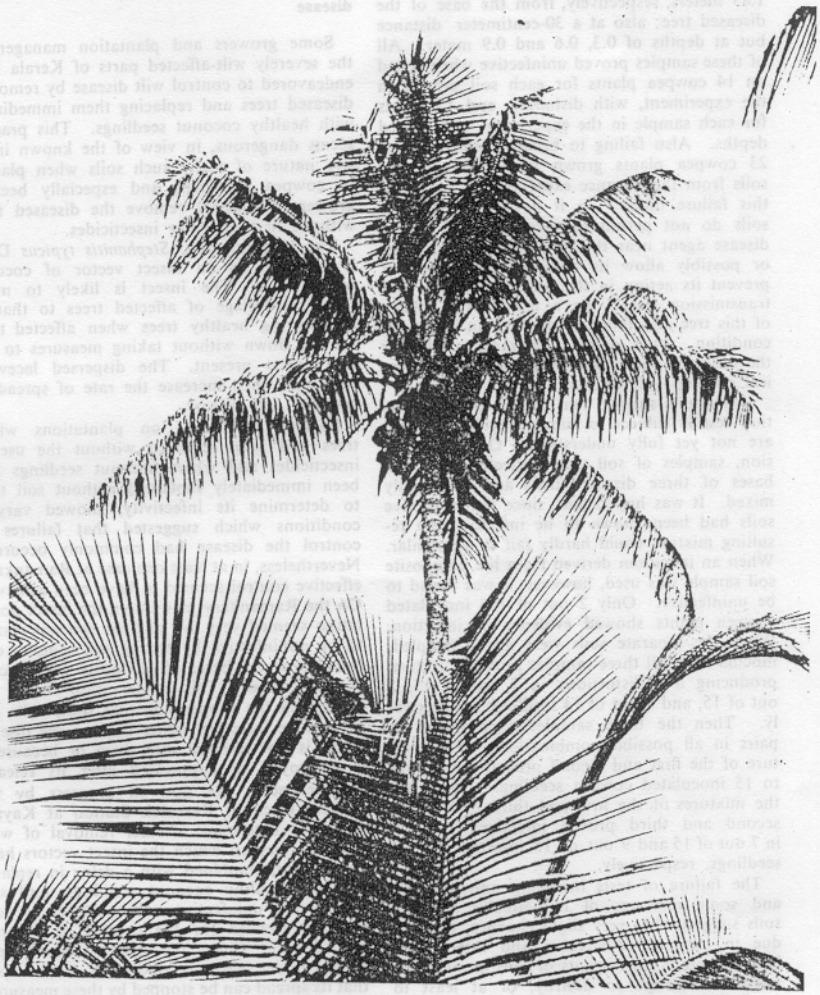

Figure 2. A coconut palm affected by wilt showing typically drooping leaflets over the healthy young palm.

at a distance of 0.3, 0.6, 0.9, 1.22, 1.52 and 1.83 meters, respectively, from the base of the diseased tree; also at a 30-centimeter distance but at depths of 0.3, 0.6 and 0.9 meter. All of these samples proved uninfective when tested on 14 cowpea plants for each soil sample in the experiment, with distances, and 15 plants for each sample in the experiment with varied depths. Also failing to become infected were 23 cowpea plants grown from seeds in the soils from the distance experiment. What did this failure imply? Is it possible that some soils do not permit the accumulation of the disease agent near the roots of diseased trees, or possibly allow its accumulation there but prevent its action in the inoculation and soil-transmission tests? As a test of the foliage of this tree also failed to disclose an infectious condition, some doubt will remain whether the observed symptoms really represented typical coconut wilt disease.

Other tests showed that the cowpea inoculation test is subject to some irregularities that are not yet fully understood. On one occasion, samples of soil were collected from the bases of three diseased trees and thoroughly mixed. It was hoped that, since the respective soils had been shown to be infective, the resulting mixture could hardly fail to be similar. When an inoculum derived from the composite soil sample was used, however, it was found to be uninfective. Only 2 out of 167 inoculated cowpea plants showed evidence of infection. When the separate soils were again sampled, inocula from all three samples proved infective, producing leaf distortions in 7 out of 15, 2 out of 15, and 2 out of 14 seedlings, respectively. Then the three samples were mixed in pairs in all possible combinations. The mixture of the first and second proved uninfective to 15 inoculated cowpea seedlings. However, the mixtures of the first and third and of the second and third proved infective, resulting in 7 out of 15 and 9 out of 15 infected cowpea seedlings, respectively.

The failure of tests involving unusual soils and some mixtures of individually infective soils suggests that very slight changes, possibly due to chemical differences and interactions between minor constituents of soils in mixtures, might be enough to destroy, or at least to suppress, the slight degree of infectivity that can normally be demonstrated in these soils when tested individually.

Relation of findings to control of coconut wilt disease

Some growers and plantation managers in the severely wilt-affected parts of Kerala have endeavored to control wilt disease by removing diseased trees and replacing them immediately with healthy coconut seedlings. This practice seems dangerous, in view of the known infective nature of many such soils when planted to cowpea seedlings, and especially because growers commonly remove the diseased trees without first applying insecticides.

The lacewing bug, *Stephanitis typicus* Dist., is regarded as an insect vector of coconut wilt disease. The insect is likely to move from the foliage of affected trees to that of neighboring healthy trees when affected trees are cut down without taking measures to kill the insects present. The dispersed lacewing bugs may then increase the rate of spread of coconut wilt disease.

Surveys conducted on plantations where trees had been removed without the use of insecticides and where coconut seedlings had been immediately replanted without soil tests to determine its infectivity, showed varying conditions which suggested that failures to control the disease had commonly occurred. Nevertheless, in at least one case at Ramankary, effective control seemed to have been achieved. On the Ramankary plantation, the insect population seemed very low and the soil was shown to be noninfective by testing soil samples collected near the bases of obviously wilt-affected coconut trees. This observation tends to strengthen the supposition that some soils fail to become infective, either because the disease agent is not released into them or because it is destroyed or suppressed after its release.

Experiments are now in progress by the Central Coconut Research Station at Kayangulam to determine whether removal of wilt-affected trees on which the insect vectors have been killed, combined with a delay in replanting with healthy coconut seedlings until soil has become uninfective, will constitute an adequate procedure for the control of coconut wilt disease. Since the disease moves very slowly under natural conditions, it is hoped that its spread can be stopped by these measures.

Experiments are also in progress at the Central Coconut Research Station to determine the length of time during which different types

of infective soils will remain infective after wilt-affected trees are removed from them, and whether these infective soils can be rendered uninfective more promptly by various experimental procedures. such as treatments with chemicals. Preliminary results have indicated that the infectivity of soils, as determined by the cowpea test, can be destroyed or suppressed by a variety of inexpensive treatments, such as mixing of the soil with lime water or with suspensions of coconut leaf ash.

In one such experiment with infective soil treated with lime water, the application of saturated aqueous solution of calcium hydroxide and its dilutions into $^1/_2$, $^1/_4$, $^1/_8$ and $^1/_{16}$ of the strength gave the following rates of infection in cowpeas grown in treated soil: 0 out of 4, 0 out of 5, 0 out of 3, 0 out of 4, and 2 out of 5, respectively.

Similar tests with coconut leaf ash, which has the advantage of being readily available in coconut plantations and furnishes an effective source of alkali as well as some useful potassium for later growth of seedlings, gave similar results in successive dilutions. Beginning with 5 grams of ash in 100 milliliters of water, the record of infections from the progressing twofold dilutions was 0 out of 4, 0 out of 5, 0 out of 4, 0 out of 4, and 1 out of 4.

Further experimentation is needed to show whether the suppression of the disease agent in infected soil samples treated as mentioned above is permanent; as would occur if the agent were actually destroyed, or whether the infective condition will reappear, especially where an extensive root system of a wilt-affected coconut tree remains in the soil after removal of the diseased tree.