Clover Club Leaf Revisited

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In the early 1940s at the branch of the Rockefeller Institute for Medical Research near Princeton, New Jersey, many photographs of plants with clover club leaf were made that were not published. Figure 1 shows leaves of crimson clover (*Trifolium incarnatum* L.) with mild to severe symptoms of clover club leaf; leaflets on the youngest, most severely affected leaves are barely opened so that the leaf looks like a small club. Figure 2 shows flowers of crimson clover with mild to severe symptoms of clover club leaf.

The flowers of periwinkle (*Vinca* spp.) with clover club leaf are dwarfed and deformed and have greenish streaks extending along the midveins. This petal greening and the leafhopper transmission of the pathogen were the original reasons for thinking that clover club leaf was similar to aster yellows, which at that time was commonly called a virus disease. Thus, clover club leaf was thought to be a virus disease, and the pathogen was called clover club leaf virus in the literature. Evidence is that the pathogen is actually a rickettsialike organism (RLO) (5).

Early experiments in which solutions of antibiotics were brushed on clover leaves did not ameliorate symptom development. When inoculated young, potbound clover plants, with a layer of roots holding the soil together, were removed from the pots, placed in an antibiotic solution for a period of time, and then returned to their original pots, a marked reduction was observed in the subsequent development of symptoms in the treated plants as compared with untreated controls. These experiments also demonstrated, incidentally, that the application of an antibiotic, effective against the RLO, may be without effect if the method does not succeed in getting the antibiotic into the plant.

The RLO was first collected in *Agalliopsis novella* captured near woods on the Rockefeller Institute grounds. Several years before this collection was made, a rare crimson clover plant, among the hundreds growing in the greenhouse, developed the characteristic symptoms of club leaf. This was an unusual event, and I thought it to be a genetic mutation of the plant. Subsequently, the leafhopper vector, *A. novella*, was used to transmit the club leaf RLO not only to clover and periwinkle but to plants in five families in addition to the Leguminosae and Apocynaceae, namely, the Compositae, Polygonaceae, Ranunculaceae, Rosaceae, and Umbelliferae. In these tests, no attempts were made to recover the pathogen from plants with symptoms by means of the vector and subsequently to use such vectors to reproduce typical symptoms in crimson clover. Nevertheless, it seems very likely that the symptoms produced in various species were caused by the RLO associated with clover club leaf. Photographs of the symptoms in several species have been placed in the University of Illinois (Urbana) Library archives. In contrast to the wide range of plants susceptible to the pathogen, it could not be transmitted by leafhoppers rather closely related to the vector, namely, *Aceratagallia sanguinolenta, Agallia constricta*, and *Agallia quadripunctata*, all of which feed on clover and were extensively tested with more than 900 plants (1).

Attempts to grow the RLO in culture failed, so Koch's postulates could not be satisfied. An electron microscope study (5) was undertaken to determine the degree to which the presence of RLO bodies was associated with diseased plants. The study was carried out in the laboratory

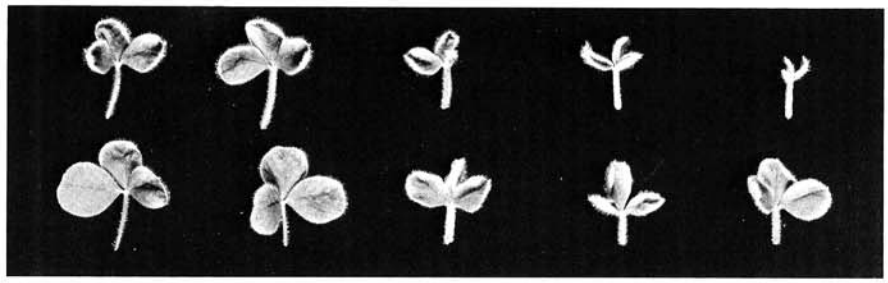

Fig. 1. Crimson clover leaves showing mild to severe symptoms of club leaf.

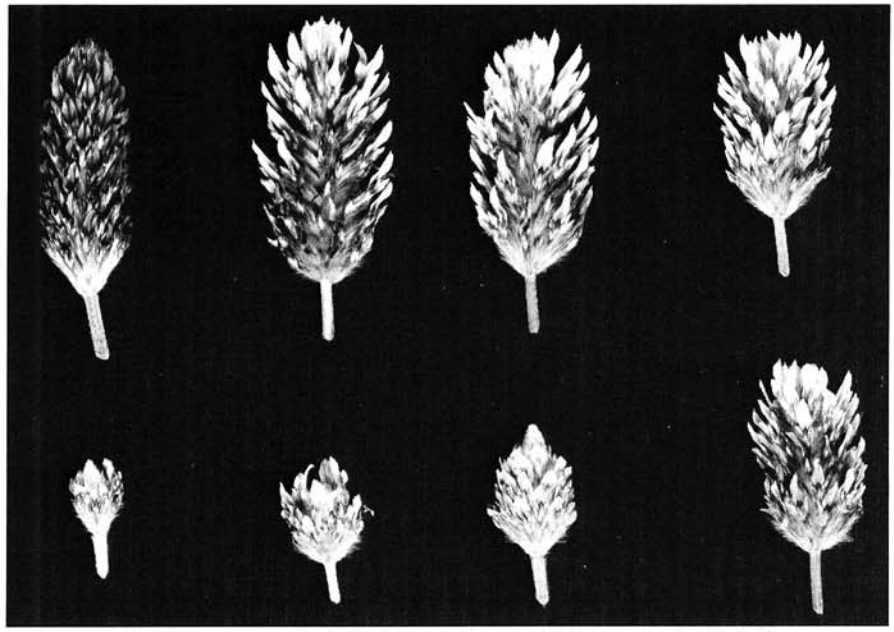

Fig. 2. Healthy influorescence of crimson clover (top row, left) compared with influorescences showing various degrees of dwarfing and branching caused by infection with clover club leaf RLO.

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supervised by Roderick MacLeod at the University of Illinois. The study was a very stressful one for the observer, Isobel Windsor, because healthy and diseased specimens were supplied to her in random order without identification. She was not informed about the correctness of any of her determinations until the entire experiment was completed. These conditions insured that much more study was required of specimens from healthy plants than from diseased plants. Analysis of the final results permitted the conclusion that the association of the RLO bodies with the disease by chance alone was not greater than one in 924.

In the electron microscope study (5), 10 circular bodies approximately 0.2 μm in diameter were found in a single field. This indicated that the bodies were probably cylindrical and that, in this case, a number of them were lying parallel to each other in a phloem cell that had been cut in cross section. The observation suggested that longitudinal sections of the phloem might reveal some of the bodies themselves in longitudinal section. This proved to be so, and the body appeared to be approximately 2.0 μm long. Other thalli appeared to be double or quadruple that length.

The dimensions of the thalli as determined by electron microscopy were confirmed in later studies (3,4) by phase contrast light microscopy, which had been employed by Davis and Worley (2) in work with organisms of comparable diameter. The RLO could not be seen by the use of lenses of the same magnification without phase contrast. By suitable methods, the RLO could be extracted from plants or insects and partially purified by density gradient centrifugations, filtration through coarse Millipore filters, collection on Millipore filters of 0.22-μm pore diameter, and subsequent resuspension in solution for microscopic examination. The bodies could also be detected without such extensive purification in preparations from a small piece of midvein from a leaf or from a single leafhopper adult, nymph, or embryo. The RLO could be identified by its narrow diameter and an apparent undulatory motility that could be best observed in 30% glycerol solutions. It was difficult to be sure that the apparent movement originated within the organism instead of being caused by currents in the suspending medium. However, addition of $HgCl_2$ or penicillin in low concentrations stopped the movement.

What appeared to be large numbers of rickettsias were observed moving very rapidly in some parts of the heads of inoculative leafhoppers. At the time, the specimens were not critically examined for size by comparing them under phase contrast and under the same magnification without phase contrast.

Phase contrast light microscopy made it possible to restudy the association between RLO thalli and the clover club leaf disease (3,4). The observer in this case was H. Y. Liu, and the same rigorous conditions regarding healthy and infected control samples and random sample order were observed as in the electron microscope study. The results (4) demonstrated that an association by chance alone between the RLO thalli and a plant with clover club leaf was about one in a million (actually one in 6.5×10^5).

These observations led us to conclude that the RLO is the cause of the disease.

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