Geminate Particles Associated with Cotton Leaf Crumple Disease in Arizona

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ABSTRACT

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Cotton leaf crumple (CLC) disease caused a major epidemic in Arizona cotton in 1981. The disease agent was transmitted by *Bemisia tabaci*; and produced CLC symptoms in upland cotton (*Gossypium hirsutum*). Cheeseweed (*Malva parviflora*) and bean (*Phaseolus vulgaris*) were infected after exposure to the CLC agent in transmission tests with *B. tabaci*. Viruslike particles (VLPs) (monomers 17–20 nm in diameter and dimers of ~17–20 × 30–32 nm) were detected by electron microscopy in partially purified preparations of CLC-affected bean plants but not in those of CLC-affected cotton, uninoculated bean or cotton, or crude sap preparations made from CLC-affected bean or cotton. VLPs in concentrated bean extracts resemble the monomeric and dimeric particles observed in extracts of plants infected by geminiviruses. Based on transmission, symptomatology, and characteristic particles in purified preparations, the CLC disease agent is similar to previously described geminiviruses.

Additional key words: cotton virus, whitefly-transmitted virus.

Cotton leaf crumple (CLC), a disease causing floral hypertrophy and severe foliar malformation of cotton (*Gossypium hirsutum* L.), was described in California in 1954 (10) and in Arizona in 1960 (1). Information concerning the transmission (4,11,21), host range (1,4,5,11,13), symptomatology (4,10,12,13), and economic impact (1,12,28,32) has been reported periodically, usually following CLC epidemics associated with sporadic occurrences of high population levels of the sweet potato whitefly (*Bemisia tabaci* Genn.) in cotton fields. The CLC disease is transmitted by grafting (21) but not by seed or sap (10,21). The CLC agent infects *Gossypium* sp. within the Malvaceae (10,21), and the host range now includes bean (*Phaseolus vulgaris* L.) and other malvaceous plants (4,11, and this report). Though CLC is suspected of being incited by a plant virus, no viruslike particles have been associated with infected plants. A CLC epidemic in the southwest in 1981 occurred concomitantly with high populations of *B. tabaci* in a number of field crops (4,5,6,11) and stimulated a renewed interest in the nature of the disease agent.

MATERIALS AND METHODS

Collection and maintenance of CLC source plants. Infected cotton plants exhibiting typical CLC disease symptoms were dug from annual and/or stub (perennial) cotton fields in Phoenix, AZ, September 1981. Cotton plants were pruned, transplanted to plastic pots (30-cm-diameter), and maintained in a greenhouse (25–30 C) as perennials. A balanced (20-20-20, N-P-K), water-soluble fertilizer (30 g/L at 200 ml per pot) was applied monthly. Plants were maintained in a greenhouse separate from other plants used in transmission studies.

Transmission studies. Preliminary mechanical and insect inoculation tests were conducted to confirm transmission of the CLC agent from field-collected cotton and to substantiate previous reports concerning the host range and virus-vector transmission characteristics.

Test plants were grown from seed (three to five seeds per 8-cm-diameter pot) sown in a greenhouse (25–32 C), and fertilized weekly following emergence. Test plants in the first to second true leaf-stage were used for inoculation studies and included: bean (*P. vulgaris* L. 'Red Kidney'), muskmelon (*Cucumis melo* L. 'Imperial 45'), cheeseweed (*Malva parviflora* L.), cotton (*G. hirsutum* L. 'Delta Pine 70'), dock (*Rumex obtusifolia* L.), okra (*Hibiscus esculentus* L. 'Clemson Spineless'), spinach (*Spinacea oleracea* L. 'Bloomsdale'), tobacco (*Nicotiana glutinosa* L.), and zinnia (*Zinnia elegans* Jacq. 'Lilliput'). Uninoculated and inoculated test plants were maintained in separate greenhouses (20,000–28,000 lux), and fumigated (Kelthane-Vapona, Carmel Chem. Corp., Westfield, IN 46074) regularly to control migrant insects.

Sap for mechanical inoculations was prepared by grinding symptomatic CLC-affected cotton leaves with a mortar and pestle in 50 mM phosphate buffer, pH 7.4, containing 0.5% diatomaceous earth, with or without bentonite (2%), cysteine hydrochloride (20 mM), β-mercaptoethanol (1%), sodium diethyldithiocarbamate (0.1%), sodium sulfite (20 mM), or thioglycollic acid (20 mM). Inoculum was applied to upper and lower surfaces of both cotyledons and first true leaves of at least 15 plants (three plants per pot) of each test species for each of three trials. Control plants were mock-inoculated with the stock buffer with or without additives.

Colonies of the sweet potato whitefly (*B. tabaci*) and the green peach aphid (*Myzus persicae* Sulzer) were established and maintained (by periodic transfer) in a greenhouse on cotton (*G. hirsutum* 'DP 70') and pepper (*Capsicum annuum* L. 'California Wonder'), respectively. Stock whitefly and aphid colony starts were obtained from established colonies of either G. D. Butler, Jr. (USDA, Western Cotton Research Center, Phoenix, AZ 85040) or W. J. Kaiser (USDA, Regional Plant Introductions Station, Pullman, WA 99164), respectively. Insects were initially tested by allowing a 3-day inoculation-access feed on indicator cultivar DP 70 cotton seedlings to ensure that the colonies were CLC-disease free. Stock colonies were confined to their respective hosts by fine nylon mesh cages supported by wooden plant stakes and held in place with double rubber bands. To alleviate problems of decreased longevity of colony host plants for *B. tabaci*, and mite and sooty mold infestations related to low light intensities under the cages, a working colony of *B. tabaci* was established on cotton plants housed uncaged in a greenhouse chamber exclusive of all other plants. All stock and working insect colonies were routinely tested to ensure that they remained free of the CLC agent.

Adult whiteflies were transferred from plant to plant by using a hand-held aspirator with a sealed-screen compartment in which whiteflies were confined in the interim. Aphids were transferred

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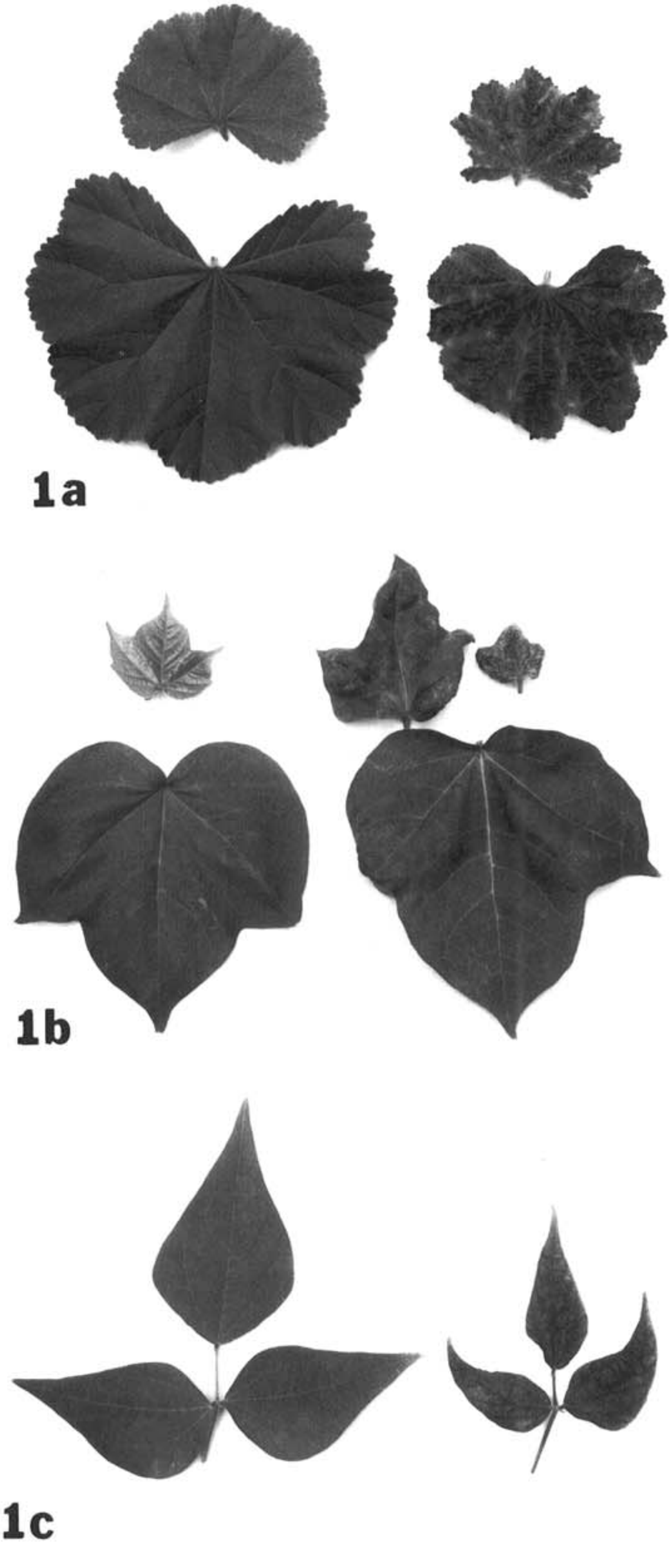
with a moistened, fine-tipped camel's-hair brush. Insects were confined to test plants during inoculations by cages constructed of inverted clear plastic cups (8 × 12 cm). A ventilation hole was cut in the bottom of each cup, and nylon mesh was glued over the opening. Insects were caged on CLC-source plants and allowed either a 10-min or a 24-hr acquisition-access feed. They were then transferred to test plants (15–20 insects per plant), caged, and allowed either 1-hr or 3-day inoculation-access feeds, respectively, relative to the acquisition-access times given above. During inoculations, caged plants were held in growth chambers (23 C, 12 hr day / night cycle, cool white fluorescent illumination, 5,000 lux). Following fumigation with nicotine sulfate (Black Leaf Products Co., Elgin, IL 60120), test plants were transferred to a separate greenhouse (26–32 C) and observed periodically for 4–6 wk.

For back indexing, colony whiteflies were allowed a 24-hr acquisition-access feed on inoculated plants (10–15 per plant) followed by a 3-day inoculation-access feed on cultivar DP 70 indicator cotton seedlings (three plants per pot). Plants were fumigated, transferred to a greenhouse, and observed periodically for 3–4 wk. Cotton cultivar DP 70 was used throughout the study as the indicator host.



1a

1b

1c

Fig. 1. Foliar symptoms of cotton leaf crumple-affected **a,** *Malva parviflora*; **b,** *Gossypium hirsutum* 'DP 70'; and **c,** *Phaseolus vulgaris* 'Red Kidney' (right); and controls mock-inoculated by *Bemisia tabaci* (left).

Purification of viruslike particles. Approximately 200 g of symptomatic cotton leaves (2–3 wk postinoculation or regrowth from CLC perennial source plants) or entire bean plants (2.5–3.0 wk postinoculation) were ground for 30 sec in an electric blender with 3.5 volumes of 500 mM glycine-NaOH buffer, pH 8.2, containing 500 mM α-D-glucose plus 1/10 volume of chloroform:butanol (1:1), strained through four layers of cheesecloth, and the emulsion was broken by centrifugation (700 *g*, 15 min). The upper aqueous phase was removed and strained through one layer of Miracloth®. Extracts were concentrated to 50 ml in an XM300 Diaflo membrane filtration system (Amicon Corp., Scientific Systems Division, Danvers, MA 01923) at 5 C. The concentrate was subjected to one cycle of differential centrifugation (27,000 rpm in a Beckman 30 rotor for 4 hr and 10,000 *g* for 10 min) and high-speed pellets were resuspended by gentle agitation in 5 mM EDTA, pH 7.8, overnight at 5 C.

Final supernatants (5–10 ml) were further concentrated by lyophilization and reconstituted in a few drops of distilled water for electron microscopy.

Electron microscopy. Grids were prepared using crude sap extracts of CLC-affected plants, or from reconstituted, partially purified preparations made from CLC-inoculated cotton or bean plants, and from comparable uninoculated, asymptomatic plants. Crude sap preparations were made by grinding cotton or bean leaves in 20 mM tris buffer, pH 7.8 (1:4, w/v), with a mortar and pestle. Cell debris was allowed to settle, and a small volume of the upper aqueous layer was transferred to a glass test tube. All samples were adjusted to 2% glutaraldehyde (pH 7.0) with an 8% stock in distilled water and fixed for 30 min at room temperature. Carbon-coated grids were floated on drops of fixed preparations for 15 min, transferred directly to a drop of either 2% sodium phosphotungstic acid (PTA), pH 7.0, or 2% uranyl acetate (UA), pH 5.0, adjusted with 1 M sodium acetate, in distilled water. Grids were drained by blotting with filter paper (Whatman No. 1) strips and air-dried. Samples were viewed and photographed with an H-500 Hitachi electron microscope operated at an accelerating voltage of 100 kV.

RESULTS

Transmission. Symptoms did not develop in any of the mechanically inoculated test plants with or without buffer additives. Typical CLC symptoms were observed in 43 of 45 cotton (Fig. 1b) seedlings on which *B. tabaci* (but not *M. persicae*) was allowed long acquisition- and inoculation-access feeding times of 24 hr and 3 days, respectively, but not in any of 45 cotton plants when shorter feedings of 10 min and 1 hr, respectively, were used. Symptoms were also observed in 40 of 45 *B. tabaci*-CLC-inoculated Red Kidney bean (Fig. 1c) and 38 of 45 cheeseweed (Fig. 1a) seedlings but not on any other CLC-inoculated test plants when *B. tabaci* was used as the vector in three different trials. Back indexing of inoculated plants by using transmission tests with *B. tabaci* resulted in typical CLC symptoms in cultivar DP 70 indicator plants for 35 of 45 bean, 36 of 45 cheeseweed, and 38 of 45 cotton plants but not when asymptomatic plants either mechanically inoculated or inoculated with *B. tabaci* or *M. persicae* were back indexed.

Electron microscopy. Viruslike particles (VLPs) 17–20 nm in

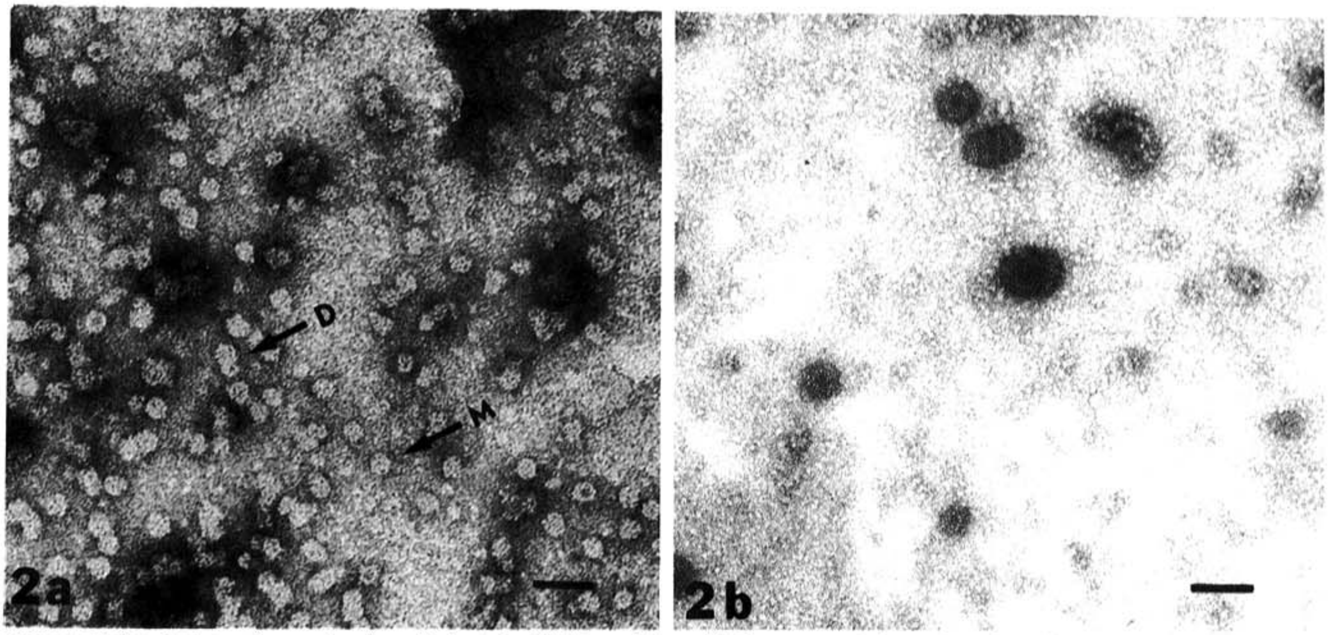


Fig. 2. Transmission electron micrographs of negatively stained (UA), partially purified extracts made from *Phaseolus vulgaris* 'Red Kidney.' **a,** Monomeric (M) and dimeric (D) viruslike particles in cotton leaf crumple-affected bean and **b,** partially purified extract made from healthy, uninoculated bean, magnified approximately ×198,000. Bar = 30 nm.

diameter and dimers 17–20 × 30–32 nm were observed in partially purified extracts (Fig. 2a) of CLC-infected Red Kidney bean plants but not in similarly concentrated extracts of CLC-affected cotton leaves. The particles appeared as monomers and dimers exhibiting the angularity and poorly resolved surface structure that is characteristic of viral nucleoproteins. The ratio of monomers to dimers in partially purified preparations was ~8:1 and was characteristic of this same extraction method in repeated trials with CLC-affected bean plants. Viruslike particles were not observed unless preparations were postfixed with glutaraldehyde and particles were most distinctive when UA instead of PTA was used as the electron-dense stain. Viruslike particles were not observed in similarly prepared partially purified extracts from uninoculated cotton cultivar DP 70 and/or bean cultivar Red Kidney plants (Fig. 2b).

Viruslike particles were not observed in crude sap preparations from uninoculated healthy, field-infected, or greenhouse-inoculated bean, cheeseweed, or cotton plants in glutaraldehyde-fixed, PTA- or UA-stained preparations examined by electron microscopy over a 2-yr period.

DISCUSSION

Based upon similarities in host range, symptomatology in upland cotton, and transmission characteristics (1,10,13,21), the disease occurring in Arizona cotton in 1981 was shown to be the same as the CLC disease described previously (10,13,21). The prevalence of CLC in Arizona cotton appears to coincide with somewhat cyclic, temperature-related field infestations by *B. tabaci* (4,6), the only known natural vector of the disease agent (4,10,21).

The presence of VLPs in concentrated extracts of CLC-affected plants reported here for the first time strongly suggests that the leaf crumple disease agent is a plant virus. The monomeric and dimeric VLPs in partially purified preparations resemble those of plant viruses belonging to the recently established geminivirus group (2,3,16,22). The leaf crumple disease agent exhibits other characteristics shared by some suspected or proven geminivirus group members, including whitefly transmissibility (2,3,9,16,22), lack of sap transmissibility (2,3,9,16), and nuclear alterations (18,29,31). Several lines of evidence reported here and elsewhere, therefore support the view that CLC is incited by a geminivirus.

The identification of two previously unidentified hosts of the CLC agent (4,11, and this report) is significant since the virus was earlier thought to infect only *Gossypium* sp. In addition to cotton, the expanded host range now includes bean as an important agronomic crop, and *M. parviflora*, a weed commonly associated with the overwintering of adult whiteflies in the southwest (15). Until now, the importance of alternate virus hosts has not been considered, because perennially grown (stub) cotton was frequently assumed to be the major overseasoning reservoir of the virus and its whitefly vector. Recent evidence suggests, however, that the disease occurs in most years (with varying degrees of severity, depending on time of infection [4]) even though stub cotton is not routinely cultivated. The role of leguminous, malvaceous, and other weeds in the epidemiology of CLC, therefore, requires further investigation. In addition, the recognition of the expanded host range may aid in the identification of the causal agent of other uncharacterized whitefly-associated diseases previously described in cotton- and bean-growing regions of the world (2,3,8,9,24).

Cotton is known to contain an abundance of polysaccharide and phenolic compounds. Interference by such compounds and/or a low virus titer in cotton plants could explain the inability to visualize or isolate virus particles directly from cotton tissues. The utilization of CLC-infected bean plants as the virus source, however, resulted in the repeated isolation of virus particles.

Viral etiology is suspected for many diseases of cotton (1,7,10–12,14,17,19,20,23–27,30). However, viruslike particles have been associated with only a few to date (17,27), and the viral nature of these diseases remains speculative. Among the reported diseases of cotton, CLC appears in retrospect, to be incited by a virus that is distinct from the others based upon host range and morphological, symptomatological, and transmission characteristics.

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