Association of a phytoplasma with witches’ broom, a new disease of acid lime (*Citrus aurantifolia*)


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A new disease of acid lime was observed during surveys conducted in the citrus growing tracts of India. Symptoms of the disease include appearance of small chlorotic leaves, highly proliferating shoots and shortened internodes. Leaves drop prematurely and infected branches have distorted twigs characteristic of witches’ broom symptoms. In advanced stages, infected branches show die-back symptoms. Electron microscopy revealed the presence of phytoplasmas (non-culturable plant mycoplasma) in the sieve tubes of leaf midribs. The disease was successfully transmitted from infected acid lime to periwinkle plants and vice-versa by dodder. A bright yellowish-green fluorescence was observed in the phloem sieve elements of diseased periwinkle stem tissue when stained with DAPI (4′,6-diamidino-2-phenylindole), a DNA binding fluorochrome. The leafhopper, *Hishimonus phycitis*, infesting the acid lime trees failed to transmit the pathogen in glass house tests. This is the first report of natural occurrence of phytoplasma-induced witches’ broom disease of acid lime in India.

Acid lime (*Citrus aurantifolia* (L) Swingle) is one of the most important citrus fruits grown over an area of 0.90 mha and constitute nearly 20% of the total citrus production (0.75 mton) in India. This fruit is rich in vitamins and minerals and largely used for making pickles all over India. During 1995 an unusual type of disease was first observed on a six-year-old acid lime plant in an orchard of Nagpur District in eastern Maharashtra. Since the disease was most conspicuously characterized by the development of witches’ broom symptoms, it was named witches’ broom disease (WBD). Subsequent surveys in 1995–98 revealed the presence of this disease up to 5% in Maharashtra State and in other major acid lime growing states of Andhra Pradesh, Tamilnadu and Karnataka. The present paper deals with the association of phytoplasma (a plant mollicute not available in culture, formerly called mycoplasma-like organism, MLO) with the disease, its detection and transmission.

Bud sticks from infected field plants showing witches’ broom symptoms were grafted on acid lime seedlings grown in an insect-free greenhouse. Buds taken from diseased plants were also grafted on mosambi (*C. sinensis*), rough lemon (*C. jambhiri*), Rangpur lime (*C. limonia*), sour orange (*C. aurantium*), Nagpur mandarin (*C. reticulata*), trifoliate orange (*Poncirus trifoliata*) and Troyer citrange and kept in the greenhouse. Ten plants were kept for each treatment. The grafted plants were regularly observed for symptom development. Dodder (*Cuscuta reflexa* Roxb.) was used to transmit the causal agent of WBD from citrus to periwinkle (*Catharanthus roseus* (L.) G. Don) plants. Dodder seeds were germinated on moist blotter paper and established on healthy periwinkles. After 15 days when the dodder shoots were about 10 cm long, strands were attached to acid lime seedlings showing WBD symptoms. After the dodder had formed haustoria within the infected lime seedlings, the strands between it and the periwinkle were cut. Within one week,
dodder on the infected lime developed new strands which were connected to healthy periwinkle plants. The connections were maintained for 8 to 10 weeks after which the plants were manually freed of dodder and kept under observation for development of symptoms, if any. For vector transmission tests, leafhoppers (Hishimonus phycitis Distant) were captured with an aspirator in acid lime orchards. Batches of ten H. phycitis, each collected from witches’ broom-infected lime tree were transferred to healthy young lime seedlings covered with a net and allowed to feed for one week. The leaf hoppers were then killed by spraying with 0.1% metasystox. The lime seedlings were kept under observation for appearance of symptoms.

To detect phytoplasma by electron microscopy, bits of leaf midribs and petioles (1.5 mm length) from healthy and infected acid lime and periwinkle plants were fixed in 3% glutaraldehyde at 4° C for 12 h and then washed thoroughly in 0.1 M phosphate buffer (pH 7.0). Tissue was then post-fixed in 2% osmium tetroxide for 2 h, washed and dehydrated in a graded acetone series (20, 40, 70, 90 and 100%) and embedded in Spurr’s low viscosity embedding medium. Ultrathin sections were cut with an ultra microtome using glass knives. Sections were picked on 300 mesh copper grids, stained with uranyl acetate followed by lead citrate and examined in a JEM 100S transmission electron microscope. Of late a number of staining techniques have been developed to improve the detection of phytoplasma in plants and to reduce dependency on electron microscope. As of today, DAPI (4¢',6-diamidino-2-phenylindole) staining is the most preferred method for rapid detection of phytoplasma. Samples were taken from petioles of young leaves or from young internode regions of periwinkle plants showing typical symptoms. Free hand transverse sections about 20–30 m m thick were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 4° at 4° C. After rinsing in the same buffer, the sections were stained in a solution (1.0 m g/ml) of DAPI (Sigma Chemicals, USA) for 20 min. The sections were rinsed in buffer solution and mounted in the same buffer on a glass slide. Observations were made.
under a Leica fluorescent microscope with an ultra-high pressure mercury vapour lamp, fitted with blue filter cube H3 containing excitation filter BP 420–490, dichromatic mirror RKP 510 and suppression filter LP 515.

WBD is characterized by the appearance of small, chlorotic leaves and highly proliferating shoots with shortened internodes (Figure 1a). Leaves drop
prematurely and affected branches showed distorted twigs resembling characteristic witches’ broom symptoms on the canopy of infected lime tree (Figure 1 b). In advanced stages, infected plant showed die-back symptom (Figure 1 c). Not all the branches of the affected trees exhibited witches’ broom symptoms. Infected twigs did not bear flowers and fruits. A culture of WBD was maintained on acid lime seedlings in the greenhouse. Within 8–14 months all grafted acid lime seedlings developed symptoms similar to those observed in the field on adult trees (Figure 1 d). Among the other artificially grafted citrus species, the disease was transmissible to Troyer citrange, rough lemon and Rangpur lime but no symptom developed in sweet orange (mosambi), mandarin (Nagpur) and trifoliolate orange till today, i.e. 20 months after graft inoculation (at the time of manuscript preparation). WBD was successfully transmitted to periwinkle plants with dodder. Infected leaves showed slight yellowing, axillary buds of these leaves grew into relatively thin and flexible shoots. Shoots of the plant had shortened internodes, occasionally few flowers appeared on the affected shoots. The disease agent was transmitted from infected periwinkle plants back to a lime seedling in the same manner.

Electron microscopic studies revealed the presence of numerous phytoplasma in sieve tubes of phloem of diseased acid lime and periwinkle leaves but not in healthy leaves (Figure 2). Size of the phytoplasmal bodies varied from 100 to 800 nm in diameter. The bodies were bounded by a poorly defined membrane. Phytoplasmas were particularly abundant in mature sieve tubes. However, in young cells, a large number of developing phytoplasma bodies were observed. When present in low concentrations, phytoplasmas were generally restricted to the periphery of sieve tubes. An intense yellowish-green fluorescence was observed in the phloem elements of WBD infected periwinkle plant when treated with DAPI (Figure 3 a). Yellowish-green fluorescence spots indicated the presence of phytoplasmas. No such fluorescence was detected in the phloem region of healthy samples (Figure 3 b). DAPI is known to bind to AT-rich DNA molecules forming a complex which emits a strong fluorescence under UV exposure. DAPI-stained DNA of WBD phytoplasma, in the present investigation, fluoresces as discrete foci in infected phloem sieve elements. DNA staining of normal plant cellular constituents such as nuclei, mitochondria and plastids were less intense than that of phytoplasma.

The presence of this phytoplasmal disease in different parts of the country suggested the involvement of an insect vector for the natural spread of the disease. However, *Hishimonous phycitis* leafhoppers captured from acid lime orchards in central India failed to transmit the pathogen. In India, *H. phycitis* is known to transmit other phytoplasmal disease (e.g. eggplant little leaf). Since *H. phycitis* failed to transmit the disease, further investigation is needed to determine if other viruliferous leafhopper or any of its biotypes serve as vectors of this disease. Bové *et al.*[^4] reported *H. phycitis* as a possible vector of the disease using molecular diagnostic tools. However, they failed to obtain experimental transmission.
A graft transmissible, multiple sprouting disease in Rangpur lime was observed earlier in India but the etiology was not confirmed\(^5\). There is also a report of phytoplasma infection in mandarin and lemon trees causing rubbery wood disease with highly flexible branches and premature leaf fall\(^6,7\). But, the present observation is the first report of natural occurrence of phytoplasma-induced witches' broom disease of acid lime in India. Phytoplasma witches' broom of lime was however...
reported earlier from Oman and UAE\textsuperscript{8–10}. Even though phytoplasmas are not available in culture, WBD phytoplasma has recently been characterized by molecular techniques and given the \textit{candidatus} name \textit{Phytoplasma aurantifolia}\textsuperscript{11}. Monoclonal antibodies and DNA probes have been obtained for its detection\textsuperscript{4}. It would be interesting to study the relatedness, if any, between the phytoplasma strains reported earlier and the present one in this report.


Received 30 November 1998; revised accepted 20 April 1999