Leafhopper Vectors and Plant Disease Agents

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Chapter 8

INTERACTIONS OF MYCOPLASMALIKE ORGANISMS AND VIRUSES IN DUALLY INFECTED LEAFHOPPERS, PLANTHOPPERS AND PLANTS

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8.1 INTRODUCTION

The pioneering efforts of Doi *et al.* (1967) and Ishiie *et al.* (1967) revealed that plants and insects could be infected with tetracycline sensitive, wall-less prokaryotes that differ from viruses or walled bacteria. Since those historic reports, scores of published papers describing pleomorphic microorganisms, bounded by unit membranes, in both plants and insects have appeared. Many reports of attempts to grow these microorganisms in pure culture have been published; however, the first successful attempts have been ascribed to Saglio *et al.* (1971) and

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Fudl-Allah *et al.* (1971) for the cultivation of the causal agent of citrus stubborn disease (Maramorosch, 1974). Cultural work on many of these microorganisms is being carried on in laboratories around the world.

Taxonomically, these plant-and insect-infecting microorganisms have temporarily been associated with the class Mollicutes that contains only one order, Mycoplasmatales. Many authors have anticipated the eventual placement of many of these agents in the order Mycoplasmatales and refer to them as mycoplasma-like organisms (MLO). Since there is a paucity of information on the cultural characteristics of these microorganisms, Maramorosch (1974) has used the abbreviation MLO for mollicute-like organisms, thus leaving the taxonomic issue more flexible. Many reviews of MLO's in insects and plants have been published and a selected summary of these can be found in Maramorosch (1974) and Gibbs and Harrison (1976).

Certain well-characterized plant-infecting viruses and MLO's have extensive and overlapping plant host ranges and are transmitted by the same species of leafhoppers (Cicadellidae) and planthoppers (Fulgoridae). Thus, this suggests that there are ample opportunities for dual infections of plants and/or vectors. To date, the number of reports of dually infected plants or vectors, in which both the virus and the MLO are known plant pathogens, is small. Dual infections of plants and vectors are studied for several reasons; i) dual infections can cause more severe plant disease than either agent alone, II) either agent or both, cause diseases of economically important crop plants, and iii) the dual infections provide model systems for investigating interactions between these two types of pathogens in plants and insect vectors.

While our primary purpose is to review and discuss interactions between plant pathogenic MLO's and viruses in leafhoppers, planthoppers and plants, we have not restricted ourselves solely to proven interactions between these pathogens. Included are a number of interesting reports concerning infections of plants, leafhoppers and planthoppers where one agent is a known plant pathogen while the other is as yet uncharacterized. In addition, we have added sections on MLO-MLO interactions, since these studies were conducted prior to the recognition of the etiologic agents involved and demonstrate several interactions that may be of future interest. Finally, we have included a brief review of interactions in other biological systems, vertebrate cell and tissue cultures and vertebrates, that we feel may provide additional insights into the future study of possible interactions in vector and plant systems.

8.2 INTERACTIONS IN LEAFHOPPERS AND PLANTHOPPERS

Two families, Cicadellidae (leafhoppers) and Fulgoridae (planthoppers) contain numerous vectors of plant viruses and MLO's. Ishihara (1969) and Nielson (1968) listed 65 or more plant viruses with leafhopper or planthopper vectors. By 1970, after the discovery that MLO were the cause of certain yellows diseases (Ishiie *et al.*, 1967; Doi *et al.*, 1967), over 50 diseases of plants, some of which were previously thought to have viral causal agents were now suspected of having MLO

etiology (Maramorosch *et al.*, 1970; Whitcomb and Davis, 1970). With the exception of pear decline MLO that is transmitted by psyllids (Psyllidae), (Jensen *et al.*, 1964; Hibino *et al.*, 1971), to date, all known plant pathogenic MLO have leafhopper or planthopper vectors.

8.2.1 MLO-Virus

Many plants are common hosts for plant-infecting MLO's and viruses as well as feeding hosts for leafhoppers and planthoppers. Therefore, it is not surprising to find reports of these vectors acquiring and transmitting both types of agents. Unfortunately, few detailed interaction studies between MLO's and viruses in these vectors have been made. Reported here are studies in which we are reasonably certain that an MLO and a virus were present in the vectors. Other plant diseases have been reported, in which a leafhopper – or planthopper – transmitted MLO and a virus have been implicated as causal agents, but for which convincing evidence relative to etiology has not been provided. Cases in which etiological evidence and transmission data are not given are reviewed in Section 8.4 of this chapter.

8.2.1.1 Aster yellows MLO and oat blue dwarf virus in Macrosteles fascifrons. Although Koch's postulates have not yet been fulfilled for the aster yellows agent (AY), strong circumstantial evidence points to the fact that this agent is an MLO (Maramorosch *et al.*, 1970). Oat blue dwarf virus (OBDV) has been characterized as a 28-30 nm spherical virus (polyhedron), containing single-stranded RNA (Banttari and Zeyen, 1969; Pring *et al.*, 1973). In North America *M. fascifrons* is the only reported vector of OBDV, and in Sweden *M. laevis* transmits an agent that causes a similar plant disease (Banttari and Moore, 1962; Lindsten *et al.*, 1970). Several species of leafhoppers, including *M. fascifrons* are capable of transmitting AY (Chiykowski, 1962, 1963; Murtomaa, 1966; Severin 1947, 1948, 1950). Both AY and OBDV have been shown to multiply in the aster leafhopper, *M. fascifrons*, (Banttari and Zeyen, 1976; Maramorosch, 1952). Tissues of leafhoppers infected with OBDV have been examined for the presence of the virus using electron optics. Membrane bounded virus inclusions of OBDV have been reported in the neural lamellae of the supraesophageal ganglia and paracrystalline inclusions in fat body cells of infected but not helathy insects (Banttari and Zeyen, 1976). Similar inclusions have also been detected in salivary glands (Chevone and Zeyen, *unpublished data*). The AY agent has been viewed using electron optics in various tissues of the vector, including salivary glands (Maramorosch *et al.*, 1970). Littau and Maramorosch (1956) using light optics, reported cytological abnormalities in fat bodies of *M. fascifrons* and Raatikainen *et al.* (1976) reported aberrant spermatogenesis in AY-infected *M. laevis*. Studies on the presence of both AY and OBDV in individual leafhoppers have not been done, although dual infections have been studied in plants (Banttari and Zeyen, 1972). In spite of the fact that AY and OBDV invade tissues of the vector, neither agent is known to have any deleterious effect on the longevity or reproductive ability when vectors are singly infected.

Dual infections of *M. fascifrons* by the AY and OBD agents, as measured by dual transmission to flax, *Linum usitatissimum* by individual insects, was reported before the identity of either agent was known (Frederiksen, 1961; 1964). Frederiksen (1961) working with 5 day reciprocal acquisition access periods of AY and OBDV, reported that only 3 of 100 insects transmitted both agents when AY was the agent initially acquired and only 1 of 100 insects transmitted both agents when OBDV was the agent initially acquired. He also reported that of more than 1000 insects collected in flax fields and individually assayed on flax over a 4 year period, only 1 leafhopper transmitted both agents. Hsu (1973) conducted experiments to determine if combined acquisition of AY and OBDV was deleterious to the vector. Hsu found no difference in longevity or fecundity in aster leafhoppers acquiring both AY and OBDV when compared to singly infected insects or uninfected control insects. He also found that dual acquisition of AY and OBDV greatly depressed the rate of transmission of either agent when compared to transmission rates of singly infected insects. Hsu's data strongly suggest an interference phenomenon between these agents in *M. fascifrons*, with regard to transmission abilities. Like Frederiksen (1961), Hsu found that only a small percentage of individual insects were capable of transmitting both agents during their lifetime.

8.2.1.2 Rice yellow dwarf MLO and rice tungro virus in Nephotettix impicticeps. Nephotettix impicticeps was shown to acquire and transmit both the rice yellow dwarf MLO (RYD) and rice tungro virus (RTV) (Basu *et al.*, 1974). The RTV is not persistent in *N. impicticeps* (Ling, 1966; Rivera and Ou, 1967) and may be stylet-borne. Rice yellow dwarf (RYD) is persistent in the vector. Basu *et al.* (1974) gave the insects a 5-day acquisition access to RYD-infected plants and then transferred individual vectors serially to rice seedlings. The insects began to transmit the MLO (RYD) during the seventh serial transfer, 21-23 days after acquisition access began. The leafhoppers were then given a 24 hr acquisition access to RTV-infected plants (between days 25 and 26) and they transmitted RTV between the 26th and 28th days but not during later transfers, while RYD was transmitted until the insects died. Leafhoppers could acquire both agents from doubly infected plants and transmit both agents to test plants. Due to the small number of insects studied, interactions between the two agents could not be determined precisely.

8.2.1.3 Corn stunt MLO and rayado fino virus of maize in Dalbulus maides. Simultaneous transmission of corn stunt MLO (CS) and rayado fino virus of maize (RFV) to corn by the leafhopper *D. maides* has been reported (Gámez, 1973). Although RFV is not well characterized, the agent is apparently unaffected by tetracycline antibiotics and is assumed to be a virus. The RFV may multiply in the vector since 100 insects given a 1 day acquisition access period had latent periods ranging from 8-22 days, and 11 insects transmitted virus for up to 46 days. When 18 leafhoppers were allowed a 5 day acquisition access period on RFV-infected plants followed by 5 days on CS-infected plants, 4 insects transmitted both agents while 13 transmitted only CS and 2 transmitted only RFV.

8.2.1.4 Corn stunt MLO and maize Colombian stripe virus in D. maides. Martinez-Lopez *et al.* (1974) described a new disease of corn caused by the maize Colombian stripe virus. The virus, approximately 30 nm in diameter, has a long incubation period in the vector, *D. maides*. Martinez-Lopez (*personal communication*) reported that the vector could acquire both the virus and corn stunt MLO, although specific interactions of these agents in *D. maides* were not reported.

8.2.2 MLO-MLO

Prior to the discovery of MLO's as probable etiologic agents of many 'yellows diseases' in plants (Doi *et al.*, 1967; Ishiie *et al.*, 1967) there was an active interest in the interactions of these agents in plants and leafhoppers. The interaction of particular interest was that of cross protection, defined as a type of interference phenomenon by Loebenstein (1972). Cross protection refers to the phenomenon in which infection by one agent protects the host from subsequent infection by a strain of the original agent or from a closely related agent. Thus, most of what is known about MLO-MLO interactions in leafhoppers is derived from cross protection experiments in which we are reasonably certain that two MLO agents were used.

8.2.2.1 Interaction of strains of the aster yellows MLO in M. fascifrons. Kunkel (1955) made a series of cross protection experiments using aster leafhoppers (*M fascifrons*) and two strains of aster yellows agent, designated as 'ordinary' and 'California' aster yellows. The two strains could be distinguished from each other by symptoms produced on China asters, *Callistephus chinensis*, and several other indicator plants. Use of 2 week acquisition access periods, for either strain, resulted in leafhoppers transmitting only the strain to which they had initial access. Thus, if the insects had a 2 week acquisition access to the 'California' strain followed by a 2 week acquisition access to the 'ordinary' strain, the insects transmitted only the 'California' strain; if they acquired the 'ordinary' strain first they transmitted only the 'ordinary' strain. Cross protection between the strains was reciprocal if the insects had a 2 week acquisition access period for the first agent; however, if the acquisition access period was shortened to 1-2 days, followed by 14 acquisition access days of the opposite strain, then leafhoppers transmitted both strains. Kunkel's results with this combination of MLO's suggested that if adequate acquisition access time were given cross protection would be complete. Later Kunkel (1957) confirmed this hypothesis.

Freitag (1967) working with three strains of aster yellows agent in plantain, *Plantago major*, and the leafhopper vector *M. fascifrons*, reported both reciprocal and unilateral cross protection interactions among these agents in the vector. The leafhoppers used for these experiments were determined to be equally efficient vectors of each of the three strains when infected singly. The three aster yellows strains used were designated as the 'Dwarf,' 'Severe' and 'Tulelake.' Using 2 week acquisition access periods, Freitag reported that leafhoppers feeding first on plantain infected with 'Dwarf' and then on 'Severe' strain trasmitted only the 'Dwarf'

strain. If the 2 week acquisition access periods were reversed for 'Severe' and 'Dwarf' strains, then leafhoppers transmitted only the 'Severe' strain. Thus, the 'Dwarf' and 'Severe' strains exhibited reciprocal cross protection in insects given 2 weeks to acquire the first agent. Using the same acquisition access period, insects acquiring the 'Dwarf' strain followed by the 'Tulelake' strain transmitted only the 'Dwarf' strain. However, if insects acquired the 'Tulelake' followed by the 'Dwarf' strain they would initially transmit the 'Tulelake' strain and later in their lives the 'Dwarf' strain. Thus, cross protection between the 'Dwarf' and 'Tulelake' strains was unilateral.

If Freitag (1967) allowed leafhoppers alternating 2 day acquisition feeding for 20 days on plants infected with the normally cross-protecting 'Severe' and 'Dwarf' strains of aster yellows, cross protection did not occur. Evidently 2 day acquisition access periods did not allow the initial aster yellows strain to thoroughly infect all vectors and protect them from the challenge strain. Even though cross protection was ineffective with these shortened, alternating acquisition access periods, insects rarely transmitted both agents. In these experiments insects usually transmitted one strain exclusively, even if it was not the strain to which the insect had initial access. Only 5% of Freitag's insects ever transmitted both strains to which they had 2 day alternating access periods. Since the ability to transmit the agent(s) was the only criterion for recognizing dual infections in insects, it is possible that more than 5% of the vectors had dual infections but these infections could not be detected by transmission histories.

8.2.2.2 Interaction between corn stunt MLO strains in D. maides. Maramorosch (1958) compared cross protection between two strains of the corn stunt agent (CS), in *D. maides*. Leafhoppers allowed 2 week acquisition access periods on plants infected with the 'Rio Grande' strain of this agent were prevented from transmitting the 'Mesa Central' strain. However, the 'Mesa Central' strain did not give complete cross protection against the 'Rio Grande' strain, because leafhoppers acquiring the 'Mesa Central' strain first could eventually transmit the 'Rio Grande' strain. When these vectors were allowed to acquire both strains simultaneously, the insects tended to transmit the 'Mesa Central' strain early in their transmission histories and the 'Rio Grande' strain later. Maramorosch concluded that cross protection between these strains was unilateral and emphasized the importance of adequate acquisition access periods during the experimentation.

8.3 INTERACTIONS IN PLANTS

Several diseases in plants are caused by MLO-virus interactions, and MLO-MLO interactions have been reported. These diseases are widespread in plants and occur in both monocotyledonous and dicotyledonous species.

8.3.1 MLO-Virus

There are at least six reported diseases of plants in which an MLO and a virus are verified or strongly suspected as the causal agents. Either leafhoppers and planthoppers have been implicated as vectors of these diseases (Banttari and Zeyen, 1972; Basu, 1974; Fedotina, 1974, Gamez, 1973; Martinez-Lopez, *personal communication*; Zummo *et al.*, 1975).

8.3.1 Aster yellows MLO and oat blue dwarf virus in flax. Both AY and OBDV have wide host ranges in monocotyledonous and dicotyledonous plants and the host ranges of the two agents overlap (Banttari and Moore, 1962; Banttari, 1965; Westdal, 1968, Murtomaa, 1966; Chiykowski, 1962, 1963; Halisky *et al.*, 1958; Frazier and Severin, 1945; Severin, 1948, 1950, Severin and Freitag, 1945; Severin and Frazier, 1945). *Macrosteles fascifrons* is the only reported vector of OBDV in North America (Banttari and Moore, 1962) whereas several species of Cicadellids, including *M. fascifrons*, are capable of transmitting the AY-MLO (Severin, 1947, 1948, 1950; Chiykowski, 1962, 1963; Murtomaa, 1966).

In flax, *Linum usitatissimum*, the dual AY-OBDV infection results in symptoms that are more severe than those caused by either pathogen in single infections. Plants, dually infected when young, are severely stunted, and there is swelling, deformation and chlorosis of the stem apex, and veinal enations on leaves and general yellowing of foliage. (Banttari and Zeyen, 1972). Infected plants are usually sterile and die prematurely (Frederiksen, 1964).

Both agents, AY and OBDV, are phloem-restricted in plant hosts, and the opportunity to study the agents in single and dual infections of flax prompted the light and electron optical study by Banttari and Zeyen (1972). In singly infected plant hosts, OBDV apparently multiplied in immature phloem elements having a full complement of cellular organelles (Zeyen and Banttari, 1972). The AY-MLO, in single infections, can also be found abundantly in phloem elements but the condition and stage of phloem maturity necessary for multiplication is unknown. Dually infected plants exhibited extensive hyperplasia of phloem elements and hyperplasia and hypertrophy of fibers and cortical parenchyma in stem sections. The disorganization and destruction of phloem in dual infections was more pronounced than with either agent in singly infected plants. Both AY and OBDV could be located using electron optics in dually infected plants; however, only rarely were both agents observed in the same phloem element (Fig. 1). The OBDV particles were never seen within AY-MLO bodies and there was no evidence of interactions or associations between them at the ultrastructural level. Although no apparent interaction of these agents was observed at the ultrastructural level, Banttari and Zeyen concluded that dual infection accentuated phloem damage. From histological evidence, no statements could be made relative to the presence of one agent affecting the replication of the other.

8.3.1.2 Pupation disease in cereals. Fedotina (1974) reported that the "pupation disease" of cereals, occurring in Siberia and the Far East, is due to a mixed MLO-rhabdovirus infection. Symptoms in dually infected oats, *Avena sativa*, included mosaic in leaves, stunted and bushy growth of plants and a proliferation of the spikes. The bacilliform virus (167 ± 20 x 57 nm) was found in the cytoplasm of epidermal and mesophyll cells as well as in phloem of infected oats. The MLO

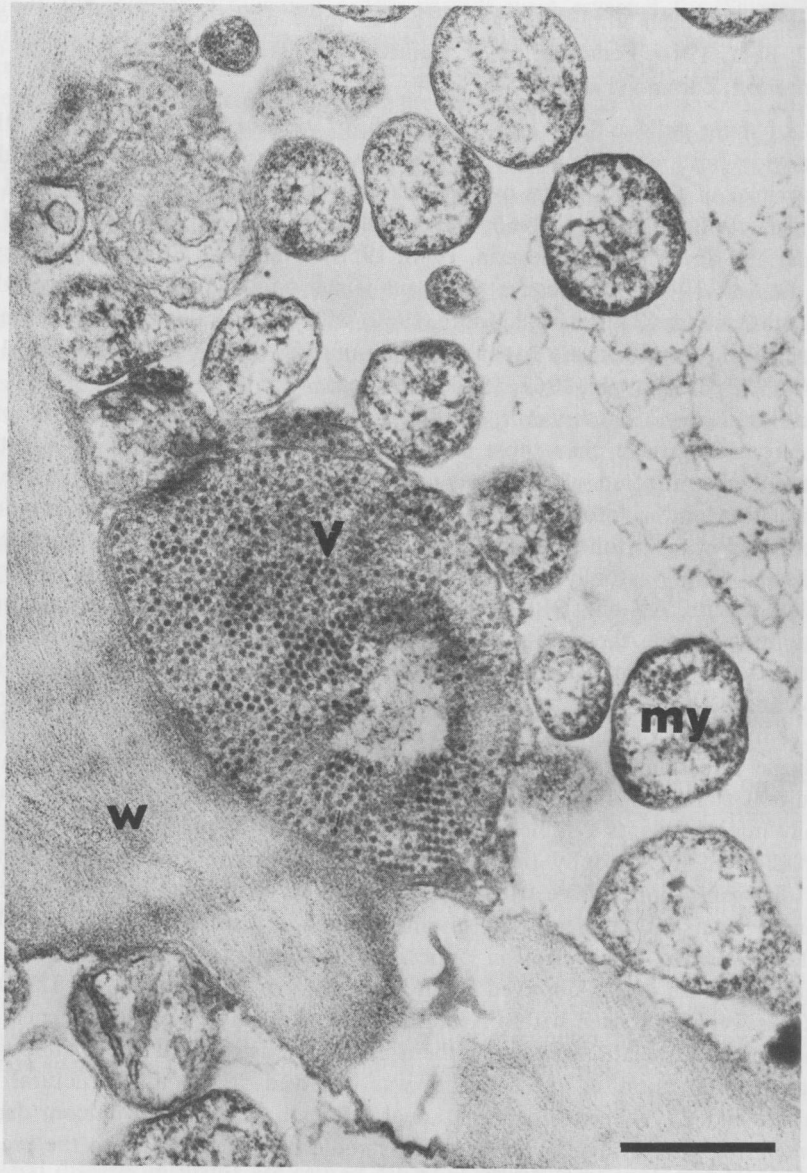

Figure 1. Electron micrograph of a transverse section of an aster yellows-oat blue dwarf virus-infected phloem element. The virus (v) has partially aligned into rows in a membrane-bounded inclusion along the cell wall (w). Mycoplasmalike bodies (my) were dispersed in the lumen of this cell as well as in adjacent cells. Bar = 1 μm (Banttari and Zeyen, 1972, reproduced with permission from Academic Press.)

was described as polymorphic having round, oval and budding bodies 80-800 nm in diameter and was noted only in phloem. Using electron optics, no sections were observed in which the MLO and the virus appeared in the same plant cell. The author attributed the mosaic symptoms to the viral infection and the proliferation of the spike to the MLO. Stunting and bushiness were more severe in dually infected than in singly-infected plants. Amorphous and paracrystalline viral inclusions were also noted in the leafhopper vector *Laodelphax striatellus.*

8.3.1.3 Yellow dwarf MLO and tungro virus in rice. Basu *et al.* (1974) reported rice, *Oryza sativa*, naturally infected with rice tungro virus (RTV) and yellow dwarf MLO (YD) in West Bengal. The principal symptoms, including pale yellowish leaves, dwarfing, and bushiness were suggestive of YD. Stunting in dually infected plants was more pronounced than in those with single infections of RTV. The authors succeeded in transmitting both agents from dually infected plants to rice with the leafhopper *N. impicticeps.* The MLO etiology of YD was demonstrated by Maramorosch *et al.* (1972c) and the viral nature of RTV was confirmed by Ling (1975).

8.3.1.4 Corn stunt MLO and rayado fino virus in maize. Gamez (1973) described simultaneous transmission of rayado fino virus (RFV) and the corn stunt (CS) spiroplasma by *D. maides* to corn. He stated that symptoms of RFV occurred within 8-21 days after inoculation whereas symptoms of corn stunt developed only after 45-60 days. This difference in time of symptom appearance helped to distinguish the diseases in doubly infected plants. Rayado fino virus causes fine chlorotic dots or short stripes on leaves. Gamez did not mention any changes in symptoms and there were no other indications that might suggest an interaction of the pathogens in corn.

8.3.1.5 Corn stunt MLO and maize Colombian stripe virus in maize. Although there are no published reports concerning possible interactions between the pathogens, Martinez-Lopez (*personal communication*) found that the maize Colombian stripe virus (MCSV) and corn stunt (CS) spiroplasma can be transmitted simultaneously to corn by *D. maides*. No description of symptoms of this dual infection were available.

8.3.1.6 Yellow sorghum stunt MLO and maize chlorotic dwarf virus in sweet sorghum. Yellow sorghum stunt, a disease of sweet sorghum, *Sorghum bicolor*, was reported in Alabama, Georgia, Kentucky, Louisiana, Mississippi, Ohio and Texas (Zummo *et al.*, 1975). Affected plants were severely stunted; leaves were rigid, curled adaxially about the blade axis and puckered resulting in undulating margins; and had a yellow-tinged cream color. These plants rarely produced seed heads and any that developed were barren.

Electron microscopy of thin sections of leaves of affected plants revealed mycoplasmalike organisms (MLO) alone in sieve elements of phloem or MLO together with a virus they identified as maize chlorotic dwarf virus (MCDV). MCDV was detected by presence of characteristic dense granular inclusions containing iso-

metric viruslike particles and associated straited sheet inclusions. Symptoms in plants containing only MLO's were not obviously different from those of plants infected with both MLO's and MCDV.

A vector of the MLO's was not found. Transmission tests using *Rhopalosiphum maides, Nasonovia lactucae, Dactynotus ambrosiae, Dalbulus maides* and *Graminella nigrifrons* were negative.

8.3.2 MLO-MLO

Several interactions involving MLO's in plants have been investigated, although some were completed before the causal agents were known to be MLO's. In reviewing and discussing these interactions we have chosen examples for which we are reasonably certain that MLO's were involved, even though the studies may have preceded the pioneering works of Doi *et al.* (1967) and Ishiie *et al.* (1967), implicating MLO's as causal agents of plant disease. Much of the work done on MLO interactions in plants involves an interference phenomenon known as cross protection (Loebenstein, 1972).

8.3.2.1 Interactions between strains of the aster yellows MLO. Kunkel (1955) was the first to demonstrate interactions between two strains of the aster yellows agent in plants. Using strains designated as 'California' and 'ordinary' aster yellows, that could be distinguished from each other by symptoms in plants, Kunkel demonstrated that China aster (*Callistephus chinensis*), *Vinca rosea* and *Nicotiana rustica* plants infected with either agent could not subsequently be infected by a challenge inoculation of the opposite strain. Kunkel stated that cross protection in these plants was complete because there were no symptoms of mixed infection. Furthermore he was unable to recover the challenge strain using the leafhopper vector *M. fascifrons*. Thus, either strain protected plants against its opposite challenge strain and the cross protection was termed reciprocal.

Freitag (1964) working with three California strains of aster yellows in plantain (*P. major*), *Nicotiana rustica* and several other plant species, found reciprocal cross protection between certain strain combinations and unilateral protection between others. Freitag also reported that certain strain combinations in *N. rustica* gave an antagonistic interaction that resulted in the development of symptomless plants.

8.3.2.2 Other MLO interactions in plants. Valenta (1959a, b) used complex plant grafting experiments with several yellows-type agents from Europe and America for interaction studies. Based on symptoms and pathogen recovery from the grafted plants, he reported protection between some agents, dual infections with others and suppression of the original causal agent by the challenge agent in others.

Chiykowski (1971) reported finding unilateral cross protection between clover phyllody and aster yellows MLO in asters. The aster yellows agent protected plants from subsequent infection by the clover phyllody agent but clover phyllody did not protect against subsequent aster yellows infection. Chiykowski's evidence was based on both symptom expression and recovery of the pathogens from asters using *M. fascifrons.*

G.W. Oldfield, (*personal communication*) reported data that indicated possible interference between the citrus stubborn agent, *Spiroplasma citri*, and an unidentified MLO transmitted by *Circulifer tenellus* to *Vinca rosea*.

8.4 ASSOCIATIONS OF VIRUS-LIKE PARTICLES (VLP'S) AND MLO'S IN LEAFHOPPERS, PLANTHOPPERS, AND PLANTS

In contrast to interactions of known viruses and MLO's in plants, leafhoppers or planthoppers in which both disease agents are pathogenic to their host, numerous examples of VLP's have been reported to occur both in plant and insect hosts infected with known pathogenic MLO's. In most, if not all, of these examples there was no demonstrated pathogenicity of the VLP's to the host or no evidence for interactions between the VLP's and MLO's. Therefore, we will describe these examples as associations.

8.4.1 Ultrastructural Observations

Ultrastructural studies of vector and plant tissues infected with plant pathogenic MLO's have revealed VLP's in association with the MLO's. In most of these observations the VLP's have been closely associated with normal appearing or degenerating MLO's.

Because more than 50 Mycoplasmatales viruses have been reported (Gourlay, 1971; Gourlay *et al.*, 1971; Liss and Maniloff, 1971; Maniloff *et al.*, 1977) the association of VLP's with MLO's in vectors and plants has led to speculation that some of the observed VLP's may be pathogens of MLO's. Some of the rod-like structures may prove to be part of the MLO structure itself as it is with certain striated structures in some animal-associated mycoplasmas and in the plant-infecting citrus-stubborn MLO (Rodwell *et al.*, 1973; Cole *et al.*, 1973a). The only report of a virus (bacteriophage type) attacking a cultured plant-infecting MLO occurred in cultures of the citrus stubborn MLO (Cole *et al.*, 1973b). Regardless of the origin or function of the VLP structures we are reviewing, the VLP's reported were not found in healthy vectors or plants and were associated with plant-infecting MLO's *in situ*.

8.4.1 VLP associated with the stolbur MLO in Euscellis plebejus and in plants. Giannotti *et al.* (1973) reported finding rods associated with stolbur MLO in sieve elements of infected *Vinca rosea, Lycopersicon esculentum* and *Cuscuta suaveolens*, and in midgut cells of the vector *E. plebejus*. The straight to slightly curved rods (31 x 160-170 nm) were often aggregated in parallel layers. The rods were observed inside ruptured MLO's and in degenerated masses of MLO's in leafhopper midgut cells. The internal structure of the rods was not like that of typical rod-shaped viruses. In plants the rods were most numerous in sieve elements containing highly pleomorphic MLO's. The authors hypothesized that the particles represented a peculiar form of the stolbur MLO or that they were a type of MLO phage.

8.4.1.2 VLP's associated with clover phyllody MLO in E. lineolatus and in clover. **Gourret** *et al.* (1973) reported finding VLP's associated with clover phyllody MLO in salivary glands of infected *E. lineolatus* and in a few phloem elements in root nodules of clover, *Trifolium repens*. The particles, (27 ± 3 x 50-150 nm)

with rounded ends, occurred in "pockets" between the cytoplasmic membrane and basal lamina. In salivary gland cells the VLP's could be found free in the cytoplasm or were membrane bounded. Sometimes the VLP formed a ring around MLO and were most numerous surrounding apparently degenerate MLO's. The particles were not found inside MLO's but were always associated with them in cells. In plants the VLP's were also found in association with MLO's and were similar in morphology and relationships to those found in leafhopper salivary glands. The authors concluded that the VLP's had characteristic features of viruses and appeared to be associated with MLO's. The VLP's were not normal constituents of plant or vector cells and pathogenicity of the VLP's to the MLO's was not demonstrated. The authors suggest that some of the features of this association may indicate that the VLP's are an MLO virus.

8.4.1.3 VLP's associated with clover dwarf MLO in periwinkle. Virus-like particles were observed in association with MLO's in phloem elements of periwinkle (*Vinca rosea*), infected with the clover dwarf MLO (Ploaie, 1971). These particles (31-33 x 85-88 nm) had an 11 nm central canal and were rounded at either or both ends (bullet-shaped or bacilliform). In some sections of plant tissue the VLP were fixed to the MLO bodies to form a rosette-like structure. The author suggested that the VLP's infected the clover dwarf MLO or were a virus transmitted by the MLO.

8.4.1.4 VLP and aster yellows MLO in asters. Bacilliform VLP's were observed in association with aster yellows MLO in one of eight infected aster plants examined (Allen, 1972). The VLP's (24 x 70 nm), rounded at both ends, occurred singly, in groups, or closely associated with a dense band in phloem elements containing MLO's. Cross sections of particles revealed a 9 nm diameter core with two zones of differing electron density surrounding the core. The viral nature of the VLP's was not confirmed in this report.

8.4.1.5 VLP's associated with yellow dwarf MLO and common dwarf MLO of mulberry. The Virus Research Group, Academia Sinica, Shanghai, and the Disease and Insect Pest Section, Agricultural Research Institute, Hangchow (1974) reported a flexous VLP (11-13 x 600-700 nm) associated with yellow dwarf MLO in mulberry and a VLP (11-13 x 1000 nm) associated with common dwarf MLO in infected mulberry. Both diseases were thought to be caused by an MLO and infected plants responded to tetracycline antibiotic treatment. The role of the VLP's in these diseases was not clear although the authors speculated that the yellow dwarf disease might be caused by an MLO-virus interaction.

8.4.1.6 MLO's and VLP's in grassy stunt disease of rice. The grassy stunt disease of rice affects *O. sativa* and at least 15 other species of *Oryza*, and occurs in the Philippines, Thailand and other east Asian countries. The disease may be caused by a complex of a virus and MLO (IRRI, 1966; IRRI, 1968). The pathogen(s) is transmitted by the brown planthopper *Nilparvata lugens* and 70 nm diameter VLP's were observed in sections of infective vectors (IRRI, 1966); however, MLO's were also found in infected plant tissues (IRRI, 1968). The application of tetracycline antibiotics to diseased plants or to seedlings prior to or after inoculation did not eliminate symptoms (IRRI, 1968). To our knowledge the etiology of grassy stunt disease of rice has not been settled, and whether either or both kinds of pathogens are involved has not been determined.

8.4.1.7 VLP's and MLO's in witches'-broom of pigeon pea. Maramorosch *et al.* (1974) hypothesized that a witches-broom disease of pigeon pea, *Cajanus cajan*, may be caused by the combined effects of a leafhopper toxin, an MLO and a rhabdovirus. Plants with witches'-broom symptoms collected in Puerto Rico were heavily infested with *Empoasca* sp., and when tissues of these plants were examined using the eletron microscope, sieve tube elements contained abundant MLO's as well as rhabdovirus-like particles. Healthy plants were free from both pathogens. Confirmation of the involvement of these disease agents in the eitology of this disease was not completed. The authors stated that this disease resembled a "Proliferation disease" of *C. cajan* in the Dominican Republic reported by Hirumi *et al.* (1973). Hirumi *et al.* (1973), reported finding rhabdovirus-like particles (45-55 x 240-260 nm) as well as MLO's in phloem of naturally infected wild pigeon pea. No comparisons of possible relationships of these diseases were reported and the role of the pathogens in either disease has not been demonstrated.

8.4.1.8 VLP's and MLO's in witches'-broom of Opuntia sp. Rod-shaped VLP's as well as MLO's were observed in phloem elements of witches'-brooms on *O. tuna monstrosa* (Maramorosch *et al.*, 1972a, b). Cuttings of witches'-broom affected plants (monstrosa-type), immersed for 3 hours in 100 ppm solution of tetracycline HCl and then planted in pots, developed normal-appearing branches of the *O. tuna* type. Mycoplasma-like organisms were absent in the recovered *O. tuna*, but the VLP's remained. After about 18 months, 75 percent of the treated plants reverted to the monstrosa-type and contained both MLO's and VLP's. The authors concluded that an MLO was the cause of the witches'-broom disease, but the role of the VLP's was not confirmed.

8.4.1.9 VLP's and MLO's in periwinkle affected with yellowing disease. DeLeeuw (1975) reported finding VLP's associated with MLO's in phloem elements of periwinkle affected with a yellowing disease. The 10 x 15 nm particles, occasionally surrounded by degenerate MLO's, occurred along membranes or free in cell lumina of sieve elements. The role of the VLP's in this disease was not determined.

8.5 INSECT AND PLANT TISSUE CULTURES

Although tissue culture techniques for insects and plants are well developed, there are no studies pertaining to MLO-virus interactions in these systems. However, MLO's and viruses are frequently found in insect cell cultures (Hirumi, 1976), and plant cell cultures have been used for propagating plant viruses for several years. Attempts to infect leafhopper cell lines with plant pathogenic MLO have not been successful, although this has been accomplished with plant viruses. A similar situation exists relative to plant cell cultures (Maramorosch, 1976). Thus, research relative to interactions of plant infecting MLO's and viruses in insect or plant tissue cultures depends on future developments.

8.6 MLO-VIRUS INTERACTIONS IN OTHER BIOLOGICAL SYSTEMS

Investigations into interactions of mycoplasma and viruses in tissue cultures and in leafhoppers and plants are not, in some respects, as advanced as are investigations of these agents in vertebrate cell and tissue cultures or in vertebrates. Since it is our purpose not only to review what has been done with respect to dual infections of leafhopper and plant systems, but to suggest areas that may yield further information and insights, a brief, and by no means comprehensive discussion of dual infections in vertebrate cell and tissue cultures and in vertebrates is included.

8.6.1 Vertebrate Cell and Tissue Cultures

Mycoplasma contamination of vertebrate cell and tissue cultures, with and without cytopathic effects is well known and documented (Barile, 1973; Singer *et al.*, 1973). Most work done with dual infections of mycoplasma and viruses in these systems has been directed toward the effects that mycoplasma have on viral replication and yield. This research emphasis is understandable, since virus yield is of primary interest and the techniques for measuring virus yield are readily available. In terms of virus yield from dual infections, there is evidence indicating that the presence of mycoplasma can enhance, suppress, or have no effect on virus yields. Singer *et al.* (1973) after reviewing the literature on the reported effects of dual infections, put forth explanations for increased and decreased virus yields. Decrease in virus yield due to concomitant mycoplasma infection of cell and tissue cultures may be caused by: i) destruction or partial destruction of cells, resulting in less substrate in which the virus can replicate, ii) lowering of the pH in the culture media making the total system unsuitable for virus replication, and iii) depletion of arginine in the media by mycplasma, so that viruses requiring this amino acid for coat protein synthesis are deprived. Mechanisms postulated for explaining increased virus yield in cell cultures infected with mycoplasma are more complex and involve a multiplicity of factors governing control of virus replication in cells and the timing of mycoplasma introduction into the cultures. One explanation for increased virus yield that is supported by research evidence (Singer *et al.*, 1969), involves decreased interferon concentrations in dually infected cultures which allows for increased virus synthesis. Of the 25 mycoplasma-virus reports reviewed by Singer *et al.* (1973) only 9 reports indicated that dual infections increased virus yield. The remaining 16 reports identified decreased yields; suggesting that mixed infections may often result in reduction of virus yield.

8.6.2 Vertebrate Systems

Vertebrates are common hosts of many mycoplasmas and viruses; therefore, there are many opportunities for interactions between these agents in animals. Kasza *et al.* (1969) simultaneously inoculated gnotobiotic pigs with a swine adenovirus and *Mycoplasma hyopneumoniae* that resulted in more severe pneumonia in dual infections than with either agent alone. The severity of the pneumonia was determined by macroscopic and microscopic evaluation of lung lesions. Serological tests for adenovirus antigens did not indicate any fluctuations in virus titer when dually infected tissue was compared to its appropriate control.

In experiments with 7-week-old turkey pullets inoculated with an avian influenza A virus and *M. gallisepticum*, Ranck *et al.* (1970) noted synergistic effects in terms of air sac lesions when the two agents were inoculated concurrently. Concurrent inoculation of the virus and mycoplasma also resulted in higher virus titers in dually infected than in singly infected pullets, as determined by serological testing. When the experiments were repeated using an avian paramyxovirus and *M. gallisepticum*, no synergistic effect was noted and serological tests indicated lower virus titers in tissues of dually infected turkeys than in tissues of turkeys infected with only the virus. In contrast to situations where dual infections resulted in no reaction or in increased severity of disease, Katzen *et al.* (1969) reported amelioration of an apparent viral disease by the addition of cultured mycoplasma. Katzen *et al.* (1969) reported that intraperitoneal injections of cultured *M. gallisepticum* into chicken pullets suffering from Marek's disease resulted in at least temporary remission of the symptoms of the disease in the individuals tests. Evidence suggested that a cell associated component of the mycoplasma culture induced symptom remission. Thus, a brief review of a few mycoplasma-virus dual infections in vertebrates suggests that varied reactions are not only possible but are entirely probable.

8.7 DISCUSSION

We have largely restricted this review to virus-MLO and MLO-MLO interactions and associations in which plant pathogenic agents were involved. We have further restricted the vector information to examples in which leafhoppers (Cicadellidae) and planthoppers (Fulgoridae) were implicated as vectors of either agent, or were infected with either agent. Thus, several reports of virus-MLO associations in plants were not presented, such as those of Chen *et al.* (1972), Kahn *et al.* (1972), Casper *et al.* (1970), von Wechmar *et al.* (1970), and Lawson *et al.* (1970); however, some of these reports have been discussed in previous reviews (Banttari and Zeyen, 1973; Maramorosch, 1974). With the rapidly expanding literature base of both viruses and MLO's we may have inadvertently neglected other reports that may be of interest. Nevertheless, we think that the reports reviewed are sufficient to explain the state of interaction studies to date.

8.7.1 Leafhoppers and Planthoppers

Many detailed studies with viruses or MLO's in singly infected leafhoppers and planthoppers have been made. With the exception of rice tungro virus in *N. impicticeps* (Ling, 1966), the viruses transmitted by leafhoppers and planthoppers are persistent in the vectors. Several well-characterized viruses have been shown to multiply in their vectors and plant hosts and these have been termed "phytarboviruses" by Whitcomb and Davis (1970). Several phytarboviruses have been studied

in detail, with respect to transmission characteristics, titers, and locations within vectors. Studies of phytarboviruses indicate that the viruses are ingested and spread from the filter chamber and gut, into the haemolymph and other organs, including salivary glands, from which they presumably pass with the saliva into plants. Similar spread and multiplication experiments concerning MLO's in leafhoppers have been made, and the method of internal spread in the vectors results in infection of salivary glands from which the MLO's presumably pass via saliva into plant hosts (Whitcomb and Davis, 1970; Gibbs and Harrison, 1976). Thus, in terms of possible MLO-virus interactions in leafhoppers and planthoppers the "phytarboviruses" and "phytarbomollicutes" (suggested terminology) offer the most intriguing possibilities for study within the vectors and especially in salivary glands. Unfortunately, the only MLO-virus interaction information available for vectors is based solely on transmission studies; nothing is known of the fate of either agent in dually infected vectors.

In transmission experiments using OBDV, a phytarbovirus (Banttari and Zeyen, 1976) and AY MLO, a phytarbomollicute (Maramorosch, 1952), Hsu (1973) demonstrated that individual aster leafhoppers, *M. fascifrons*, given one week acquisition access periods for each agent were most likely to transmit the agent first acquired. Both Hsu (1973) and Frederiksen (1961, 1964) noted that only a small percentage of the aster leafhoppers acquiring both agents were capable of transmitting both during their lifetimes. These studies indicated interference between the agents in the insect. Saturation of multiplication sites or competition for substrates, perhaps in the salivary glands of the vector by the initially acquired agent resulting in exclusion of the challenge agent, is a possible explanation for the transmission data. Since both agents replicate in the phloem, competition for sites or substrates may be possible; such competition has been suggested for MLO-virus interactions in vertebrate tissue cultures (Singer *et al.*, 1973). Another hypothesis to explain the transmission data is that infection by the first agent stimulates a "resistance mechanism(s)" in the insect and leads to the limited replication of the challenge agent. Although insects do not produce antibodies, they do apparently posses some primitive immunological capabilities that resist invading microorganisms (Whitcomb *et al.*, 1974). Whether an MLO would trigger an immunological response effective against a virus, and vice versa, is speculative. Regardless of the fate of the agents in the OBDV-AY dual infection the transmission data strongly suggest an interaction within the insect. Similar transmission results between strains of MLO's in leafhoppers (Kunkel, 1955, 1957; Freitag, 1967; Maramorosch, 1958) suggest that some of the same mechanism(s) may be functioning in these interactions in vectors. However, the unilateral cross protection reaction between certain MLO combinations is suggestive of intrinsically differing replicative capabilities of the MLO strains themselves in the insect vectors.

The question of MLO-virus and MLO-MLO interactions in vectors will probably not be answered until titers of the agents can be determined in insect tissues. Research in this area should attempt to correlate titers of the agents with varying acquisition access periods and should also attempt to correlate both the titers

and acquisition access periods with the transmission histories of individual insects. This type of study would enable investigators to deduce more accurately the meaning of transmission histories relative to the events occurring in the insects.

Certain plant-infecting MLO's are pathogenic to their vectors and cause increased mortality. The MLO causing stubborn disease of citrus, *Spiroplasma citri*, is pathogenic to its vector *Scaphytopius nitridus* as well as to non-vectors such as *M. fascifrons* and *D. maides* when these insects are inoculated with the cultured agent (Whitcomb and Williamson, 1975). In addition, peach western-X MLO in *Colladonus montanus* and corn stunt (CS) spiroplasma in *D. maides* have been shown to be pathogenic and induce early mortality in these vectors (Jensen, 1959; Whitcomb *et al.*, 1968; Granados and Meehan, 1975). None of the reports of dual MLO-virus or MLO-MLO infections of leafhoppers have revealed increased mortality. Increased mortality of dually infected vectors would likely affect transmission data and should be considered when interaction studies are undertaken. For instance, a higher mortality of *D. maides* dually infected with MSCV and CS spiroplasma, or RFV and CS spiroplasma may have been expected although it was not reported by Martinez-Lopez (*personal communication*) or Gamez *et al.* (1973).

The role of VLP's and identified and unidentified MLO structures in leafhopper tissues is an area that invites speculation. The idea that certain VLP's may be MLO phages has been advanced by several investigators. Proof of the phage-like nature of the VLP's awaits the culturing of the MLO involved, however, at least 50 viruses of Mollicutes are now known including a type B bacteriophage of a cultured plant pathogenic MLO, *S. citri*, (Cole *et al.*, 1973b; Gourlay, 1971; Gourlay *et al.*, 1971; Liss and Manilofff, 1971; Maniloff *et al.*, 1977).

It is also possible that some of the VLP's are latent insect viruses because VLP's have been noted in apparently healthy insects (Lee, 1965; Granados, 1969; Herold and Munz, 1967). Numerous MLO's are common saprophytes in vertebrates although they are not intracellular. It is possible that some of the unidentified MLO's in leafhopper tissues are saprophytic or symbiotic and are part of the microflora of the individual insect.

8.7.2 Plants

Dual infection by MLO-virus combinations has been shown to result in symptoms that are more severe than with either agent in single infections (Fredericksen, 1964; Basu, *et al.*, 1974; Banttari and Zeyen, 1972; Fedotina, 1974). These examples are based mainly on visual estimates of symptoms. Certain dual virus-virus infections of plants are synergistic and result in elevated titers of at least one of the viruses (Damirdagh and Ross, 1967; Ross, 1959). Moreover, MLO stimulation of virus multiplicaton in vertebrates and vertebrate cell tissue culture has been demonstrated (Singer *et al.*, 1973; Ranck *et al.*, 1970). There are no published studies of pathogen titers in MLO-virus infected plants, and histopathological evidence has been provided only for the OBDV-AY MLO interaction in flax. Both OBDV and AY are phloem-restricted in plants, so both agents must rely on the same tissue for substrates and multiplication sites. The OBDV has been

found in greatest concentrations in young phloem elements that have not fully differentiated into sieve elements; whereas, the AY agent can be found in high concentrations in mature sieve elements (Zeyen and Banttari, 1972; Banttari and Zeyen, 1971, 1972). When dually infected flax plants were investigated, both agents were found only occasionally in the same phloem element and the two agents were not observed in any conformation that suggested a direct interaction. The authors concluded that the increased symptom severity was due to OBDV affecting the development of young elements whereas AY was capable of affecting all stages of phloem development. Thus, the two agents complemented each other in damage to phloem.

Interactions between MLO's and between MLO strains have not been studied with respect to disease severity but rather have been studied with regard to the cross protection phenomenon. These experiments resulted in demonstrating complete cross protection, unilateral cross protection, and antagonistic interaction between various MLO's and MLO strains (Kunkel, 1955, 1957; Freitag, 1964; Valenta, 1959a, b). As was true for studies of MLO-virus interactions, investigations of MLO-MLO interactions are based on symptom expression and no information on the titers of the different MLO's in dual infections is available. Histological evidence of interactions in plant or vector tissues may be impossible to obtain since the different MLO's are morphologically similar in conventional thin sections used for electron microscopy, even though certain MLO agents have a helical morphology in thicker sections.

The role of VLP's associated with plant pathogenic MLO's remains inconclusive and depends upon further investigation. Virus-like particles are suspected of being involved with an MLO in the etiology of grassy stunt disease of rice, witches' broom and proliferation disease of *C. cajan*, common and yellow dwarf of mulberry, and yellowing disease of *V. rosea* (de Leeuw, 1975; Hirumi *et al.*, 1973; Maramorosch *et al.*, 1974; IRRI 1966, 1968; Virus Research Group, Academia Sinica, Shaghai, and Disease and Insect Pest Section, Agricultural Research Institute, Hangchow, 1974). The role of the rod-shaped VLP's associated with MLO's in witches' broom of *Opuntia* sp. was also not determined (Maramorosch *et al.*, 1972a, b). That such VLP's in one or more of these examples are benign virus infections cannot be ruled out because latent virus infections in plants are not uncommon (Bos, 1970; Smith, 1974). Further work will be necessary to isolate and individually transmit each component of the dual infections to host plants or vectors to prove the pathogenic role of each suspected agent.

Undoubtedly much research is yet to be done on dual infections of insects and plants with MLO's and MLO-virus combinations. We have not attempted to review additional areas that represent interactions in leafhoppers, planthoppers or plants. We did not review virus-virus interactions because the area is too extensive, especially relative to certain plant reactions. Neither have we included rickettsia-like agents transmitted primarily by xylem feeding leafhoppers (sharp-shooters) and spittlebugs and causing diseases such as clover club leaf, peach phony disease and Pierce's disease of grapevine.

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