CHARACTERIZATION OF ASTER YELLOWS IN OKLAHOMA

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PREFACE

Aster yellows (AY), caused by mycoplasma-like organisms (MLOs), was investigated in this study. Natural AY incidence in carrot and lettuce was recorded in three different locations in Oklahoma. MLO isolates from carrot, lettuce and daisy fleabane were collected and characterized. A trispecific polyclonal serum, produced against an AY MLO isolate from Oklahoma carrot, was used in serological detection of AY in different host sources. Selected wheat and peanut cultivars were screened for susceptibility to Oklahoma AY MLO.

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CHAPTER I

GENERAL INTRODUCTION

Aster yellows (AY), an extensively studied plant disease of mycoplasmal etiology, affects a large number of plant species. Approximately 350 species of plants in 54 plant families have been recorded as hosts of the AY mycoplasma-like organism (MLO), of its vector, the aster leafhopper Macrosteles fascifrons (Stal), or both (Peterson 1973). AY causes devastating economic losses in vegetable, horticultural and agronomic crops. All the known vectors of these infectious agents are leafhoppers.

Chiykowski and Chapman (1965) and Drake and Chapman (1965) found that aster leafhoppers which become infective in the south central United States carry the yellows MLO into the upper midwest and lower mid-Canada during the annual northward flight of the leafhopper in early spring. Rye (Secale cereale L.), bromegrass (Bromus sp.), bluegrass (Poa pratensis L.), and timothygrass (Phleum pratense L.) and other hosts provide sites for overwintering eggs and nymphs, and serve as early spring hosts for aster leafhoppers which disperse northward. Small grains are important breeding hosts of the leafhoppers during early summer, although later in the season the leafhoppers may

shift from small grains to vegetable crops and ornamentals. Preferred host plants for these leafhoppers include lettuce (Lactuca sativa L.), china aster (Callistephus chinensis L.), oats (Avena sativa L.), barley (Hordeum vulgare L.), and wheat (Triticum aestivum L.). Certain plants including potatoes (Solanum tuberosum L.), tomatoes (Lycopersicon esculentum L.), radishes (Raphanus sativus L.), cabbage (Brassica oleracia L.) and onion (Illium sepa L.) are feeding hosts but are not favorable for breeding. Some winter annuals, biennials, and perennials may serve as overwintering reservoirs for the AY MLO. Aster leafhoppers prefer AY diseased carrots (Dacus carotus L.) , horseweed (Leptilon canadence L.) , marigold (Tagetis erecta L.) and sowthistle (Sonchus oleraceus L.) to healthy plants for both feeding and oviposition. Selective feeding on diseased plants may increase the percentage of infective leafhoppers in the population (Peterson 1973).

Yellows diseases are usually differentiated from one another on the basis of host range, symptomatology and vector species. Such identification criteria, because they are not specific, have resulted in confusion. The causal agents of yellows diseases were long believed to be viruses. However, in 1967 Doi et al. reported the presence of MLO in sieve elements of plants affected with yellows diseases.

Extensive work on AY was carried out from 1902 to 1970. Comparatively few publications on AY have appeared since. This review concentrates on the period

prior to 1970. However, all pertinent literature on AY since 1970 has also been reviewed.

Causal Agent of Aster Yellows

Mistaken Virus Etiology

A disease with AY-like symptoms was first recognized and described by Sturgis in Connecticut in 1893. Calendula, marigold, zinnia, and aster were found to be affected, but observation of root galls on aster suggested a nematode etiology. Smith (1902) reported a malady of China aster in Massachusetts and mentioned the similarity of symptoms in diseased plants to those described by Sturgis (1889). Root galls, however, were never found. Further investigation by Smith eliminated known identifiable pathogens such as bacteria and fungi, plant treatments, and environmental factors as possible causes of the disease. The pathological conditions were attributed to inherent constitutional weakness or "misjudgement of the plant". Kunkel (1924, 1925, 1926a, 1926b), was the first to characterize AY as a virus disease, and to demonstrate its transmission by the aster leafhopper, M. fascifrons. He was able to transmit the pathogen by bud grafting, but not by mechanical means such as needle jabbing or rubbing of infectious sap into the leaves of healthy plants. Because of its graft transmissibility, filterability (Black 1943), similarity to known insect transmitted viruses, and lack of association with identifiable pathogens, aster yellows was

assumed for years to be a virus disease. Use of agar gel filtration (Steere 1967) and differential centrifugation techniques (Lee and Chiykowski 1963) failed to isolate a virus.

Evidence of Mycoplasmal Etiology

Electron Microscopy. Doi et al. (1967) published electron micrographs showing structures resembling mammalian mycoplasmas within phloem elements of plants infected with mulberry dwarf, Japanese aster yellows, and potato witches' broom. The stuctures were pleomorphic, spherical or ellipsoidal, 80-800 nm in diameter, and surrounded by a unit membrane. Subsequent EM studies added more information on morphology of these bodies in both plants and insects infected with AY (Maramorosch et al. 1968a, Shikata and Maramorosch 1969, Worley 1969, 1970, Ploaie and Maramorosch 1969, Hirumi and Maramorosch 1972, 1973a, 1973b). Ovoid, pleomorphic MLO 30-760 nm in size were found in asters infected by the Tulelake isolate of AY. Morphologically the spherical structures were bound by a unit membrane about 6-7 nm thick, lacked a nuclear envelope and contained ribosome-like granules and DNA-like strands. During early infection MLO in infected plants showed morphological characteristics indicating binary fission (Hemmati and McClean 1980). Whitcomb and Davis (1970a) pointed to the similarity of MLO structure in plants and insect vectors to that of Mycoplasma spp. and L-forms

of bacteria.

Chemotherapy. The mycoplasma etiology of AY was substantiated with antibiotic chemotherapy experiments. Ishiie et al. (1967) reported suppression of disease symptoms in mulberry dwarf diseased plants treated with tetracycline antibiotics which are also effective against mammalian and avian forms of Mycoplasma (Hayflick and Chanock 1965, Newnham and Chu 1965). Remission of AY symptoms was observed in annual chrysanthemum treated with tetracycline, chlortetracycline, or chloramphenicol, but not with penicillin. The ineffectiveness of penicillin and vancomycin suggests etiology of a mycoplasma rather than that of a walled bacterium (Davis and Whitcomb 1969). The symptoms did not appear in infected plants that were treated by immersing washed roots into antibiotic solutions containing 1000 parts per million of the compound, and then spraying the plant foliage every three days with the same antibiotic. Acquisition of the AY MLO by insects was greatly reduced when they were fed upon AY MLO-infected plants treated with antibiotics. Simultaneous injection of chlortetracycline and the AY MLO into the leafhoppers blocked transmission of the AY MLO by these vectors (Davis et al. 1968a, 1968b). When infective leafhoppers were fed on aster treated with antibiotics, either the transmission of the disease was blocked or symptoms were delayed for 2 to 3 weeks, and the incubation period in the vectors was prolonged. Symptom development in plants and

transmission of the AY MLO by infective insects were suppressed by chlortetracycline, oxytetracycline, tetracycline and meglacycline but not by spectinomycin, oleandomycin or kanamycin. The later three are effective against mammalian and avian mycoplasmas. This indicates differences between avian and mammalian mycoplasmas and plant and insect MLOs (Davis and Whitcomb 1969). Oxytetracycline hydrochloride and tetracycline hydrochloride prolonged the life of AY-diseased barley plants when applied as a soil treatment, and delayed onset of symptoms when applied as foliar spray to the plants before symptom expression (Timian and Alm 1977). Chang and Donaldson (1985) reported the remission of phyllody symptoms in AY MLO infected periwinkle by a kinetin spray.

Properties of the AY MLO

Properties of Sedimentation. Highly infectious sediments from infective leafhoppers centrifuged at 5,000 rpm for 1 min suggested that the AY MLO was larger than known plant viruses (Black 1943). Whitcomb and Davis (1969) reported reduced infectivity of supernatant liquid by centrifugation at 8,000 x g for 10 min and complete elimination in 10 min at 25,000 x g.

Stability in Vitro. The AY MLO is unstable in vitro. The infectivity of the AY MLO was reduced within two hours at room temperature (Black 1943). Lee and Chiykowski (1963) retained high infectivity of

supernatant fractions containing 0.01 M MgCl and 0.85% NaCl in 0.01 M phosphate buffer, pH 7, for 24 hrs. Filtered extracts of AY MLO lost infectivity within 3 hrs (Davis et al. 1970). Whitcomb and Davis (1969) showed sensitivity of infectious fractions to ether, chloroform, phenol, and freezing in liquid nitrogen, which indicated that the AY MLO is more fragile than known plant and insect viruses.

Effect of High Temperature. Kunkel (1941) showed that plants of China aster and Nicotiana rustica L. infected with AY and subjected to 35 C for as long as eight days were not cured, but the disease symptoms were reduced. However, periwinkles infected with the same strain exposed to hot room temperatures of 38 to 42 C for two weeks, or immersed in hot water baths at 40 to 45 C for several hours, completely recovered from the disease. Leafhoppers kept at 31 to 32 C for 12 days (Kunkel 1937), 36 C for 10 days (Chapman 1949), or 40 C for 7 days (Granados 1965), permanently lost their ability to transmit the yellows agent. The above results indicate that the high temperatures over a prolonged period of time have a detrimental effect on the aster yellows agent in both plants and insects.

Taxonomy of AY

Minimal standards for the description of new species of mollicutes set by the International Committee

on Systemic Bacteriology, Subcommittee on the the Taxonomy of Mollicutes, are as follows: 1.) electron microscopic examination should show the absence of a full cell wall and that the microorganism is bound by a unit membrane; 2.) the organism must be filterable; 3.) the organism must be resistant to penicillin; 4.) characteristic fried egg shaped colonies should be established on solid media; 5.) there should be no reversion of the organism to a bacterial form for at least five consecutive subcultures on medium lacking penicillin or other bacteriostatic agents; 6.) the organism should be susceptible to growth inhibition by antibodies (Hayflick and Arai 1973, Subcommittee on the Taxonomy of Mycoplasmatales 1979). These standards distinguish mycoplasmas from other prokaryotes. Sterol requirement is of major importance in taxonomic divisions of mollicutes. Other criteria include genome size and localization of the enzyme NADH oxidase. The Class Mollicutes is subdivided into three families: Mycoplasmataceae (which requires sterol for growth, has a genome size of about 5.0×10^8 daltons, and localizes NADH oxidase in the cytoplasm), Acholeplasmataceae (which does not require sterol for growth, has genome size of about 1.0×10^9 daltons, and localizes NADH oxidase in membranes) and Spiroplasmataceae (which requires sterol for growth, has helical morphology during some phase of growth, has a genome size of about 1.0×10^9 daltons, and localizes NADH oxidase in the cytoplasm) within the order Mycoplasmatales (Tully, 1978).

Mollicutes were included in the approved lists of bacterial names in 1967 (Bergey's Manual of Systematic Bacteriology 1984).

The taxonomy of the culturable mycoplasmas has advanced significantly within the past 30 years, but very little progress has been made with plant mycoplasmas or MLO. These MLO are wall-less, polymorphic, 0.2 to 0.8 μm diameter, nonculturable organisms, morphologically resembling mollicutes. Most workers currently believe that there is a discrete group represented by the "yellows agents" (Kunkel 1926a, McCoy 1979). Although symptom expression has proved to be an inaccurate indicator of the taxon of causal agents in the past, the similarilty of the yellows syndrome in many plant hosts suggests that these classical agents may represent a single taxon. Without confirmation of identity through cultural techniques, these agents are assigned to the uncertain aggregation of organisms termed MLO (McCoy 1984).

Culturing of the AY MLO

To establish a mycoplasma as the causal agent of AY, the organism must be cultured, characterized in cell-free media and have Koch's postulates completed for proof of pathogenicity. So far the AY MLO have resisted culture attempts. Hirumi and Maramorosch (1969) were able to maintain inocula from AY infected leafhopper extracts for 6 hrs at room temperature in a medium utilized in

leafhopper cultures and supplemented with 20% fetal bovine serum. Attempts to inoculate periwinkle plants with the above presumptive MLO were unsuccessful. Infectivity of AY MLO rapidly declined at 22 C in media of known composition (Davis and Whitcomb 1969). Davis et al. (1970) detected infectivity after 6 hrs but not after 12 hrs at 22 C in filtered extracts from AY infected plants and leafhoppers in a medium containing amino acids, vitamins, inorganic salts, sucrose, and cholesterol. The MLO survived up to 24 hrs when the medium was supplemented with 5% horse serum. Hayflick and Arai (1973) tried unsuccessfully to culture AY from diseased aster and infective leafhoppers in 16 different media including aster plant extract and lobster hemolymph. Skripal and Malinoviskaya (1984) reported the isolation and culturing of phytopathogenic mycoplasmas on SM IMV 72 medium. But this claim was unsubstantiated after several attemps of other researchers to repeat the culturing of MLO (Davis, personal communication). Different media with plant, soil, insect extracts, amino acids, different sera, and antibiotics have failed to stimulate in vitro growth of the AY MLO.

Partial Purification and Serology of AY MLO

Serological studies of plant and insect mycoplasmas have been employed in identification and classification. But lack of availability of highly specific antisera has hampered progress in this area. Sinha (1979a) purified the

AY MLO from infected aster plants and produced polyclonal antiserum (PA) against it. He also studied the reactions of cytoplasmic and membrane antigens of the MLO in agar double diffusion tests. Sinha and Benhamou (1983) detected the AY MLO by enzyme linked immunosorbent assay (ELISA), and immunosorbent electron microscopy (IEM) in preparations partially purified from AY-infected asters. Antiserum against a European aster yellows was developed by Clark and Davies in 1984. Because of the non-culturable nature of the AY MLO sufficient quantities of pure AY MLO have never been obtained for use as an antigen. PA produced against partially purified antigen showed nonspecific reaction with the healthy controls. Even after cross-absorption with healthy plant material, the PA reacted to plant material.

Lin and Chen (1985) prepared monoclonal antibodies (MAB) to the AY MLO by using a partially purified salivary gland preparation from infected leafhoppers. With these MAB the AY MLO in diseased lettuce, periwinkle and inoculative insects was specifically detected by ELISA. Lin and Chen (1986) compared the MAB produced against the AY MLO and PA from serum collected after splenectomy for the detection of the AY agent. They suggested that MAB reacted specifically to AY MLO antigen and discriminated between the AY and other MLO specifically. But PA reacted with plant antigens in AY infected samples and thus were unsuitable for distinguishing different MLO. Cross and

longitudinal sections of leaf midribs of healthy and AY infected plants were stained with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG. MAB bound specifically to AY MLO in the sieve tubes of diseased but not healthy plants, while the PA treatment showed fluorescence in both healthy and diseased plants suggesting nonspecific binding of the antibody to plant cells. Jhiang and Chen (1987) reported a method for partially purifying AY MLO from infected lettuce plants based on centrifugation of concentrated extracts in a discontinuous percoll density gradient. The resultant MLO structures reportedly were well preserved.

Clark et al. (1989) reported a method for obtaining a partially purified preparation of MLO which is suitable for use as immunogen for PA and MAB production and for coating ELISA plates. Satisfactory yields of the MLO were obtained from AY MLO infected leaf and soft vascular tissue from recently grafted periwinkle plants. They compared MAB and PA to primula yellows MLO with PA to European aster yellows MLO to detect MLO in infected plants. Primula yellows MLO MAB and PA, and European aster yellows MLO PA, appear to recognize only a single major antigen of approximately 22,400 daltons.

DNA of the AY MLO was cloned and is being used as a specific hybridization probe for detection of the MLO in plant and insect extracts (Lee and Davis, personal communication). These hybridization techniques using DNA

probes will facilitate detection, quantification and differentiation of non-culturable MLO. DNA probes allow direct analysis and comparison of MLO genomes, and the identification and sequencing of evolutionarily conserved regions of MLO genome may help identify phylogenetic relationships between MLO and other culturable or nonculturable prokaryotes (Kirkpatrick et al 1987). Also these techniques may help in identifying the genes involved in plant pathogenesis and insect transmission.

AY Disease Syndrome

AY MLO are known to affect a large number of host species, but the symptoms caused in the diverse host range were not historically considered as signs of infection by a single disease agent. For this reason, initial descriptions of the disease refer to the syndrome induced in China aster (Callistephus chinensis), a popular ornamental plant in the early part of the twentieth century. This discussion of general symptomatology will be limited to the effects of yellows in aster as described by Kunkel (1926a).

The first observable symptom is slight yellowing along the veins (vein clearing) in the whole or in a part of a single young leaf, followed by chlorosis of most terminal leaves. The leaves that are mature at the time of inoculation remain normal in color and shape. Leaf petioles often become etiolated and assume an upright condition

compared to the lateral growth of a healthy petiole. One side of the leaf or the plant becomes chlorotic before the remaining portion is affected. Lateral shoots sometimes show disease before symptoms appear in the terminal portion of the stem. The MLO causes chlorosis in all green portions of the plants. In contrast, petals become green in color (virescence). One of the most striking symptoms of AY is proliferation or abnormal growth of the secondary shoots. The affected shoots are thin and etiolated. Although Granados (1965) observed lengthening of internodes in aster infected with the Eastern strain of AY during early stages of disease development, Stunting and shortening of internodes occur in advanced stages of disease, As the disease progresses, stem necrosis usually occurs at a point below the apical bud. Flower buds may show partial symptoms but more frequently become stunted and chlorotic. Diseased flowers from distorted plants are often much larger than those of the healthy plants, but can be dwarfed. Flower heads and individual flowers may develop into vegetative branches. Trichomes on diseased flowers frequently develop into leaf-like structures. Some AY strains produce phyllody, proliferation, and virescence of floral organs (Frietag 1964). In aster, the disease eventually leads to the death of the plant. However, this is not the case in all host plants. AY persists almost indefinitely in periwinkle and in some instances plants seem to recover from disease (Lee 1961, Granados 1965).

Strains of AY

Eastern and Western Strains. Smith (1902) first described the Eastern strain of AY MLO in Massachusetts. Kunkel (1924, 1925, 1926a) characterized a New York isolate as AY virus disease and demonstrated its transmission by the aster leafhopper, M. fascifrons. Similar yellows-type diseases were reported on celery (Apium graveolens L.) in California (Severin 1929). The California isolate was transmitted by the aster leafhopper and showed symptoms similar to those of a New York AY isolate. However, Kunkel (1932) reported that the two isolates differed in infecting certain plant species. California yellows infected celery and zinnia (Zinnia elegance L), whereas New York yellows either was not transmitted to celery and zinnia, or transmission was achieved with great difficulty. Symptom differences between the two strains on China aster, Indian tobacco (N. rustica L.), periwinkle (Catharanthus roseus L.), and zinnia were reported. California yellows produced severe stunting or short swollen side shoots in the plants mentioned above, whereas New York yellows did not retard the growth of infected plants but produced the elongated, spindly side shoots known as witches' broom. The Western strain (California yellows) requires a longer incubation period in the leafhopper than does the Eastern strain (New York yellows), whereas the reverse is true for mean

incubation period in the plants (Granados 1965). In cross-protection experiments, Kunkel (1955) demonstrated that Eastern and Western strains protected against each other in plants and the vector, and for the first time referred to them as strains instead of separate diseases.

Lee in 1961 obtained two yellows isolates in aster from a field in Wisconsin. One isolate, designated CAYV, readily infected celery and was placed in the Western group. The other isolate, designated as NACY, failed to infect celery and was considered to be an Eastern strain. The symptoms on periwinkle were similar to those in the description given by Kunkel for the Eastern strain (1955).

Other Strains. Some strains were reported to lose the ability to be transmitted by a leafhopper vector or to infect certain plant species. Granados (1965) reported that one of the celery-infecting Western strains lost its ability to infect celery under greenhouse conditions. This isolate was classified as an Eastern strain. Another report indicated the occurrence of both isolates in the same infected plant (Granados 1965). Kunkel (1955) reported a strain that lost its ability to be transmitted by insect vectors.

Frietag (1958, 1964) differentiated three celery-infecting Western strains of AY in California according to symptoms produced on common plantain, aster, and periwinkle, and designated these as severe, dwarf and Tulelake strains.

AY in the Family Gramineae

The first report of AY in the family Gramineae appeared in 1960. Banttari and Moore (1960) first demonstrated that the "leafroll dwarf" of barley Hordeum vulgare L. cv Vantage was incited by the AY MLO and was transmitted by the aster leafhopper, M. fascifrons. Oat, Avena sativa, was susceptible to the Western strain (Freitag 1964). A celery-infecting strain of AY from Canada was transmitted by the leafhopper Endria inimica from barley cv Vintage to plants of the same cultivar and to wheat, Triticum vulgare Desf. cv Ramsey. Several varieties of different commercial grains also were found susceptible (Banttari 1965, 1966; Chiykowski 1965, 1967; Richardson 1967; Gill and Westdal 1966; Gill et al. 1969; Westdal and Richardson 1969; Westdal 1969). An AY type disease was reported in wheat, rye, and barley and in Poa anua L. in Finland (Murtomaa 1966). Yellows infection was obtained in various grasses (Banttari 1966), and in wild oats, Avena fatua L. (Westdal and Richardson 1969). Wheat, oats and barley were susceptible to both Eastern and Western strains (Chiykowski 1965, 1967; Westdal and Richardson 1967). Only the Eastern strain was known to infect Triticale and rye. Three isolates of AY were collected from naturally infected barley in Manitoba. Two non-celery infecting isolates readily infected common wheat cv Selkirk, whereas a celery-infecting isolate could

not be transmitted to wheat (Westdal and Richardson 1969).

European and Japanese Aster Yellows

An AY type disease transmitted by M. laevis was reported in Germany by Heinz and Kunz (1955). But Maramorosch (1958a) failed to obtain transmission of American AY by M. laevis. In Finland, M. laevis collected from the field readily transmitted an AY type disease to oats and barley, but only rarely to aster (Murtomaa 1966).

In Japan, AY was observed in carrots in the vicinity of Sapporo and Hokkaido. The causal agent was transmitted by a leafhopper, (Scleroracus flavipictus L.) from infected aster to carrot and also from diseased carrot back to aster. Electron micrographs of infected carrot and aster showed MLOs in the phloem cells. The authors conclude that the AY in carrot was identical to American AY (Nemato et al. 1974a). Nemato et al. (1974b) observed AY in the celery fields in Sapparo and suggested that this isolate is an Eastern (celery-infecting) strain.

Since MLO cannot be cultured, much of the designation of strains and even of AY MLO as a distinct organism is based on indirect evidence. Koch's postulates cannot be completed becuse of the nonculturable nature of AY MLO. Because there is no method of precise identification of the AY MLO we must be extremely careful in interpreting our work as well as that of others. Most of the previously

characterized AY isolates were not preserved for further reference. However, Chiykowski (1977) reported long-term storage of Canada AY by cryopreservation.

The Leafhopper Vector

Taxonomy and Distribution

The vector of the AY MLO is the aster leafhopper, Macrosteles fascifrons Stal. (order Homoptera and family Cicadellidae), also referred to as the six-spotted leafhopper. In 1967 the Committee on Common Names for Insects approved "aster leafhopper" as the accepted common name for the insect (Blinkenstaff 1967). Bierne (1952) established the species M. fascifrons as a complex comprised of a number of local and geographical forms which vary considerably in morphological characters. The complex ranges from Mexico and Puerto Rico to Alaska and the boundaries of the Atlantic tundra. M. fascifrons inhabits open grasslands and meadows of all kinds ranging from sea level to an elevation of 2,850 meters.

Description of Adult and Life Cycle

The adult aster leafhopper is light greenish yellow in color and 4 mm long. Three pairs of markings occur on the vertex; one pair of round spots situated on the posterior aspect, a second pair of transverse dashes on the center of the sclerite, and another pair of transverse dashes located at the juncture of the vertex

with the frons. The frons itself is marked by five pairs of dark transverse arcs (Osborn 1916).

Eggs are deposited under both the upper and lower epidermis of leaves, in leaf petioles, and sometimes in branches and main stems. Eggs usually are placed between the lower epidermis and the mesophyll of leaves. A gray waxy substance plugs the hole made to deposit the eggs in the leaf (Kunkel 1926a). The eggs are elongate, about 0.8 long x 0.16 mm wide, slightly curved and narrow at both ends (Osborn 1916, Hagel and Landis 1967). Incubation period of leafhopper eggs is about 10-11 days under greenhouse conditions.

There are 5 nymphal stages of development before the adult leafhopper is formed (Osborn 1916, Hagel and Landis 1967). The first instar nymph is 0.6 to 0.7 mm long and dusty brown in color. Later instars are yellow with some individuals mottled with brown. The fifth instar is 3 mm in length (Osborne 1916). The time between molts ranges from 3 to 6 days for the first four instars and 5 to 7 days for the fifth instar nymph (Osborn 1916, Hagel and Landis 1967). Depending upon temperature and rearing conditions, the aster leafhopper completes one generation in about three to six and a half weeks (Osborn 1916, Kunkel 1926a). Adult females live longer than males. There are four to six generations per year. However, Kunkel (1926a) stated that adult females have long life spans (eight to 10 weeks) and lay eggs over a long period of time

those of one generation will still be ovipositing when the females of the next generation begin depositing eggs. Thus he could not find any distinct broods. In Midwest and Central Canada this overlapping of generations is further complicated by the presence of both migratory and local populations whose oviposition periods are asynchronous (Chapman, cited by Shultz 1979).

Additional Vectors of AY

Leafhoppers belonging to the order Homoptera, class Cicadellidae are known vectors of AY MLO. In North America, M. fascifrons is the only vector of the Eastern strain, whereas 25 species including M. fascifrons were reported to trasmit the Western strain of AY (Kunkel 1926a, Severin 1947a).

Host Plant-Leafhopper-AY MLO and Environment Interactions

Survival and perpetuation of the AY MLO in nature depends upon two diverse hosts, plants and insects. AY is known to affect more than 350 plant species (Kunkel 1953) but its insect host range is limited to 25 leafhopper species belonging to the order Homoptera and class Cicadellidae (Wallis 1960, Chiykowski 1963a, Chiykowski and Sinha 1969). The initial reports of Kunkel (1926a) suggested that the causal agent was predominantly transmitted by the leafhopper vector and to some extent by

budding but not by mechanical means. Thus an important link must be maintained for the survival of the pathogen.

There are four steps involved in the plant-AY MLO and the vector relationship (Shultz 1979). First, a susceptible leafhopper species must acquire the AY MLO by feeding on a diseased plant for a minimum period of time (acquisition access period, or AAP). Second, the yellows agent must multiply within the body of the vector and invade the salivary glands in order to render the leafhopper infective (incubation period in the leafhopper, or IPL). Third, an infective leafhopper must feed for a minimum length of time on a susceptible plant before successfully inoculating it with the AY MLO (inoculation access period or IAP). Fourth, the AY MLO must undergo a second incubation period in the plant, during which it multiplies, before it can be acquired once again by a non-infective leafhopper (incubation period in the plant, or IPP).

Factors Affecting Acquistion Access Period

Length of Acquisition Access Period. The length of time required for an aster leafhopper to acquire the AY MLO ranged from two hours to two weeks in a study by Kunkel (1954). Only a few leafhoppers became infective when held on yellows infected plants for two hours. Transmission efficiency increased with the increase of AAP. About two weeks of feeding was required before all individuals in

large colonies were rendered infective (Kunkel 1954).

Effect of Light and Temperature. Light had no effect on acquisition of the AY agent by the aster leafhopper (Chapman 1949). Maramorosch (1953) concluded that low temperature (10 C) did not inhibit the ability of the leafhopper to acquire the AY MLO but high temperature (>25 C) had an adverse effect on the acquisition (Lee 1961).

Effect of the Age of the Infected Plant. Kunkel (1926a) reported that the leafhoppers could acquire the AY agent from both old and young leaves. Maramorosch (1952a) reported that only 3% of non-infective leafhoppers acquired the AY MLO from old, symptomless leaves. He also showed that the leafhopper could acquire the AY MLO at any time ranging from 2 days before symptom appearance in the plant until the death of the plant. Chapman (1949) reported that leafhoppers became infective when fed on the symptomatic plant portion, but rarely became infective when fed on asymptomatic leaves.

Incubation Period in the Vector

Kunkel (1926a) found that both adults and nymphs could transmit the AY disease. He was the first to recognize and describe the process of the disease agent's incubation within the insect vector. Kunkel reported a ten-day minimum incubation period in the vector, whereas the findings of other researchers showed a requirement of 14

to 19 days of incubation period in the vector (Chapman 1949, Strong and Rawlins 1958a). For a celery-infecting strain the longest incubation period was 24 to 30 days (Chiykowski 1958).

Incubation Period in the Plant

Upon inoculation, AY must undergo an incubation period in the plants, during which time the AY MLO multiplies and spreads throughout the phloem tissue. This incubation period is defined as the time between inoculation of a susceptible host and the appearance of first symptoms (Maramorosch 1953). Kunkel (1926a) showed an average incubation period of 18 days in China aster infected with the Eastern strain. The longest time required for visible symptom development was 39 days, more than twice the average incubation period. The incubation period in aster infected with the Western strain ranged from 10 days to 4 weeks. Chiykowski (1958) reported the incubation period in China aster infected with the Eastern strain to range from 13 to 40 days.

Evidence of Multiplication of AY in its Leafhopper Vector

Multiplication of AY in its leafhopper vector was demonstrated by partial inactivation of the AY agent by heat treatment (Kunkel 1937a). Kunkel concluded that holding infective leafhoppers at a high constant temperature of 42 C for 12 or more days rendered the leafhoppers

permanently noninfective. His experiments showed that long heat treatments lengthened the amount of time required for the leafhoppers to regain their transmission ability and insects thus treated were as efficient in infecting plants as untreated ones. Kunkel concluded that the pathogen must multiply in the body of the vector. At various intervals the AY MLO from the infected leafhoppers was injected into healthy leafhoppers. The results showed the multiplication of the AY agent at least 100 fold in the source insects between the second and twelfth day of a 17-day incubation period (Black 1943). Maramorosch (1952a, 1952b) determined by serial passage of AY from insect to insect that the AY MLO multiplied in the vector, and was successfully carried through 10 serial transmissions.

Factors Affecting Inoculation of AY by Vectors

Length of Inoculation Access Feeding Period. The aster leafhopper transmits AY plants by sustained feeding on the host over an extended period of time. Chapman (1949) concluded that a feeding period between 4 and 16 hrs is required for an infective leafhopper to consistently transmit AY. A minimum of a 7.5 min inoculation period is required to transmit the celery-infecting strain of AY to aster (Chiykowski 1958). Lee (1961) reported that the percentage of infection ranged from 3.4% at feeds of 7.5 min to 72.9% at 32 hrs, when single leafhoppers were used. Granados (1965) showed that the transmission occurred

efficiently with IAPS between 0.5 to 2 hrs. The Eastern strain was transmitted with greater frequency (99%) than the Western strain (53%) at 32 hrs IAP. Fasting of the insects before inoculation did not enhance the transmission efficiency (Chapman 1949).

Plant-AY MLO Interaction

The absence of vector transmission of the AY MLO over a long period of time may result in the inability of the pathogen to be transmitted by its vector. Granados (1965) reported the loss of transmissibility of a readily transmissible Wisconsin AY strain by the leafhopper vector to celery. Severin (1934a) reported that AY strains from Idaho and Maine were transmitted from infected carrot to carrot but not from carrot to aster. Kunkel (1945) was unable to transmit an AY isolate infecting carrot. Westdal and Richardson (1969) reported a low percentage of recovery of AY from summer wheat and oats.

Effect of High Temperature. High temperature adversely affect the transmission of the Eastern AY strain by M. fascifrons. Infective colonies of leafhoppers, when exposed to a constant temperature of 31 C or 38 C for 12 days or longer, permanently lost their ability to transmit AY (Kunkel 1937a). Colonies held at 25 C transmitted with high efficiency (Granados 1965).

Movement and Location of AY MLO in Plants

AY produces systemic infection in diseased plants. Kunkel (1926a) observed that AY does not produce chlorosis in mature leaves, but chlorosis was seen in leaves one half to two thirds fully grown. Symptoms of the disease may develop strongly on one side of an infected leaf for some time before the remaining plant becomes infected. This suggested that rapid spread along the stem of the plant may not take place. Symptoms occurred in new growth both in terminal and axial leaves. Movement of AY in the plants was traced in transmission experiments by several researchers (Chapman 1949, Maramorosch 1962a, Kunkel 1926a, Frederiksen 1964).

In electron microscopic investigation, MLO were observed in the phloem regions of diseased flower stalks, distorted flowers, and veins of leaves of infected aster, but not in the healthy control (Shikata and Maramorosch 1969). Worley (1969, 1970) found MLO in phloem of N. rustica and aster. MLO were also found in sieve tubes of infected symptomless leaves.

Proposal Justification

Ivanoff and Ewart (1944) reported that AY was an economic problem on carrot in the Winter Garden area of Texas. Up to 85% AY incidence was recorded in some commercial fields. In western New York state AY disease was

a threat to carrot production (Harvey and Shroeder 1947). Carrot also was severely affected, with as much as 95% incidence in Wisconsin (Drake and Chapman 1965, Chiykowski and Chapman 1965). In Minnesota, complete losses in carrot fields occurred during the devastating epidemic of 1957 (Meade and Peterson 1964). Chiykowski and Chapman (1958) considered lettuce to be the crop most susceptible to AY in Wisconsin, with 100% losses occurring in commercial fields in some years.

AY was reported in daisy fleabane, Erigeron stigosis in Payne County, and in lettuce, Lactuca sativa, in Tulsa County of Oklahoma as early as 1945 (Preston 1945). In the fall of 1984 AY was observed in carrot trap plots at Bixby, OK, and in spring 1985 it occurred both at Bixby and Stillwater (Errampalli et al. 1985). During the summer of 1985, lettuce plots at Bixby showed up to 80% AY incidence. Aster leafhoppers are common in the state (Don Arnold, OSU Extension Entomology, Stillwater, personal communication). Recent emphasis on growing vegetable crops in southeastern Oklahoma and knowledge of the presence of AY and its vector in the state led us to investigate the natural disease incidence and to characterize the AY pathogen in vegetable crops of Oklahoma.

Canadian cultivars of wheat, T. aestivum, (both hard red and soft white varieties) and T. turgidum were shown to be susceptible to aster yellows (Chiykowski 1973). Although damage was erratic, losses to individual growers in given

years were sometimes severe. The lack of symptoms in some cases, and the similarity of symptoms to those of virus diseases in other cases, resulted in the disease being undetected or misdiagnosed for several years (S. Haber, Agriculture, Canada, personal communication). As wheat is a predominant crop in Oklahoma, we also investigated the potential threat of AY to selected wheat cultivars grown in Oklahoma.

This dissertation is presented in the Journal style format outlined in the Oklahoma State University Graduate College Style Manual. The dissertation is composed of three manuscripts (Chapters) written according to the format style established by the American Phytopathological Society for manuscripts submitted to the professional journals Phytopathology and Plant Disease. The General Introduction (Chapter I) describing literature review of AY and proposal justification, and the Appendices describing the three experiments, are not presented in manuscript style.

Chapter II, entitled "Natural Incidence of Aster Yellows (AY) in Carrot and Lettuce plots and Characterization of Aster Yellows Mycoplasma-like organism Isolates in Oklahoma," describes the symptomatology, natural disease incidence of aster yellows in carrot and lettuce trap plots in Bixby, Lane and Stillwater, Oklahoma, and characterization of the collected AY isolates using traditional methods. This manuscript will be submitted to

Plant Disease.

Chapter III, entitled "Production of Monospecific Polyclonal Antibodies Against the Aster Yellows Mycoplasma-like Organisms," describes the production and purification of antibodies specific to AY MLO. This manuscript will be submitted to Phytopathology.

Chapter IV, entitled " Screening of Selected Wheat and Peanut cultivars for Susceptibility to the Oklahoma Aster Yellows Mycoplasma-like Organisms(OK AY MLOs) ," describes the transmission, staining, and electron microscopy studies conducted to evaluate the reaction of selected Oklahoma wheat and peanut varieties to the Oklahoma aster yellows mycoplasma-like organism. This manuscript will be submitted to the Proceedings of the Oklahoma Academy of Science.

The Appendix describes experiments dealing with isolate collection and comparison of detection methods for aster yellows in different host plants.

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CHAPTER II

NATURAL INCIDENCE OF ASTER YELLOWS (AY) IN CARROT AND LETTUCE PLOTS AND CHARACTERIZATION OF AY MYCOPLASMA-LIKE ORGANISM ISOLATES IN OKLAHOMA

Abstract

Incidence of aster yellows (AY) was greater in lettuce than in carrot at three different field locations in Oklahoma. Incidence was greater at the Bixby and Lane locations than at Stillwater, Oklahoma. Eight isolates of mycoplasma-like organisms (MLOs) were collected from carrot, lettuce and daisy fleabane. Four isolates, two from carrot and one each from lettuce and daisy fleabane, were distinctly different from each other based on symptomatology on aster. All the isolates were characterized as "Western strains" based on the symptomatology on celery. MLOs were detected by electron microscopy in representative plants infected with each isolate. Western blotting with antiserum against isolate OK AY MLO obtained from carrot was positive with one carrot isolate and the lettuce isolate, but negative with the second carrot and the daisy fleabane isolate.

Introduction

Aster yellows (AY), an intensively studied plant disease of mycoplasmal etiology, affects approximately 350 different plant species belonging to 54 plant families (16). Kunkel (11) demonstrated that the AY mycoplasma-like organism (MLO) agent was transmitted by the six spotted aster leafhopper, Macrosteles fascifrons Stal., and suggested that the AY MLO might multiply inside the vector. Smith (18) and Kunkel in 1926 gave comprehensive descriptions of the disease in China aster, Callistephus chinensis L (11). The first symptoms to appear on aster are vein clearing in immature leaves followed by reduced leaf size, chlorosis, and stunting of the plant (11). In mature flowering asters, virescence and phyllody are predominant symptoms. In periwinkle, older leaves become yellow and as new growth develops proliferation occurs in axillary shoots. Flower size is reduced and all the floral parts become leaf-like (11). Doi et al. in 1967 found MLOs in phloem elements of yellows infected plants (3). MLOs are wall-less, pleomorphic, 80-800nm diameter bodies, containing ribosome-like granules and a prokaryotic genome. MLOs are sensitive to tetracycline and chlortetracycline and remission of symptoms occurs in AY MLO infected plants (10).

Because of the non-culturable nature of the pathogen,

the AY MLOisolates have traditionally been characterized on the basis of symptomatology (11). Two strains of AY MLO are differentiated on the basis of host range and vector specificity (12). The California or Western strain readily infects celery with symptoms appearing 40 days after inoculation, and is transmitted by 25 leafhopper species (12, 13). The Eastern strain produces symptoms 115 days after inoculation in celery, and is transmitted by only one leafhopper species (12). Both strains are transmitted by M. fascifrons (1).

AY was first reported in carrot by Ivanoff and Ewart in 1944 in Texas (9). Linn in 1940 reported an average of 5 to 6% incidence of "yellows" in lettuce in New York state with some fields suffering 70 to 80% incidence (15). Lettuce was considered to be the crop most susceptible to AY in Wisconsin, with 100% losses occurring in commercial fields in some years (15).

AY was reported in daisy fleabane and lettuce in Oklahoma in 1945 (17). In 1985 Errampalli et al. reported AY MLO in carrot in two locations in Oklahoma (6). The aster leafhopper, M. fascifrons, a common insect in the state, is the major vector of AY. Recent emphasis on growing vegetable crops in Oklahoma and knowledge of the presence of AY and its vector in the state led us to investigate the natural incidence of AY in vegetable crops of Oklahoma and to characterize the AY MLO isolates collected from

Oklahoma vegetable crops.

Materials and Methods

Field Studies. In 1985 three cultivars of carrot (Daucus carotus L.) (cvs. `Danvers', `Imperator'and `Spartan Bonus') were planted at the Oklahoma State University (OSU) Vegetable Reseach Station (VRS) at Bixby and OSU Plant Pathology Farm (PPF) at Stillwater (Fig. 1). The plots were arranged in a randomized complete block design with 4 replications of a 10 m row. Planting dates were April 15, 1985 and August 23, 1985. Incidence of AY MLO disease was recorded at approximately 100 days after planting (DAP). The disease is herein referred to as AY, although our findings indicate the possibility of more than one MLO being involved.

In 1986-1987 the same carrot cultivars plus two cultivars of lettuce (Lactuca sativa L.) (cv. `Great Lakes' and `Ithaca') were planted at the OSU VRB at Bixby, at the OSU PPF at Stillwater, and at the Wes Watkins Agriculture Research and Extension Center (WWAREC) at Lane (Fig. 1). Each carrot cultivar was planted in four 10m rows and each lettuce cultivar was planted in six 10m rows. Planting was arranged in a randimized block design. All cultivars were planted in 4 replications. The plots were planted in April 30 and August 15 of 1986, April 25 and August 10 of 1987. MLO disease incidence was recorded

at approximately 100 DAP. Representative leaf samples from MLO diseased carrot and lettuce leaves were collected and checked by Dienes' stain (2), which is specific for MLOs in bright field microscopy. A limited number of leaf samples were prepared for observation by electron microscopy (7).

Analysis of variance (ANOVA) of the 1985 disease incidence data was performed. The 1986 and 1987 data were analyzed as a split-plot with locations, years and seasons in the main plots, and cultivars in the subplots in a randomized block design. F-tests from the ANOVA procedure were used to determine interactions and main effects. ANOVA was performed on carrot and lettuce data separately. Comparisons of cultivars and seasons within each year and location, and comparisons of locations and cultivars within each year and season were determined by Duncan's Multiple Range test at the P=0.05 level.

Isolate Collection. Representative diseased carrot and lettuce plants from the trap plots, potted into 15 cm dia. plastic pots, were sprayed with Malathion and transferred to the greenhouse for further transmission studies. Three days after the insecticide spray, 30 third and fourth instar nymphs of the leafhopper *M. fascifrons* were allowed to feed on each infected plant in a cylindrical cage. After a two week incubation period, the leafhoppers were allowed to feed on aster (a short term maintanance

host) or periwinkle (a long term maintanance host). The isolates were characterized based on the symptom expression on aster and celery. Later transmission experiments involving isolates of interest were carried out using these AY MLO infected asters as source plants.

Transmission experiments.

Insect rearing. The leafhopper vector of AY MLO, M. fascifrons, was reared at 25-35 C in large cages (60 x 60 x 60 cm) in the greenhouse. The insects were cultured on barley, (Hordeum vulgare L. cv `Post'). Colonies were monitored frequently for AY MLO contamination by allowing leafhoppers from the healthy colonies to feed on asters and observing those asters for symptom development.

AY MLO-inoculative insects were maintained on asters and barley. For transmissions, healthy insects placed on AY diseased plants were allowed to feed for 3-7 days. Leafhoppers were transferred in groups to barley (a non-host for AY MLO) for a two-week incubation period. Infected leafhoppers were placed on test plants such as asters or celery for 3-7 days. The inoculated plants were sprayed with Malathion and observed for symptom development.

The success of transmission of AY MLO to celery and aster was confirmed by Dienes' staining and by back-inoculation to asters. Back-inoculation tests consisted

of allowing healthy leafhoppers to feed on celery and asters showing symptoms after inoculation with AY MLO, and then transferring them to healthy asters to confirm infection.

Results

Field data:

Incidence of AY in carrots

Considering three planting locations, there was a trend of greatest incidence of AY in carrots planted at Bixby, followed by those at Lane and then those at Stillwater. Bixby showed higher ($P = 0.05$) AY incidence than Stillwater during spring and fall of 1985 and 1986 and spring of 1987 (Tables 1,2). In spring and fall 1986, incidence of AY in carrots was highest ($P = 0.05$) at Bixby, followed by Lane ($P = 0.05$) and then at Stillwater (Table 2). The general trend however, was not seen in spring 1987, when carrots showed highest average incidence of AY at Stillwater followed by Bixby and then Lane. Also in fall 1987 carrots at Bixby showed the highest incidence of AY followed by carrots at Stillwater and then those at Lane (Table 2).

A seasonal difference in incidence of AY was recorded in carrot plots during all three study years, with the fall crops generally sustaining greater disease than spring crops. Carrot in plots at Bixby during fall 1985 and at

Bixby and Lane during fall 1986 and 1987, showed higher (P = 0.05) incidence of AY than during spring 1985, 1986 and 1987 at the same locations. However, the trend was reversed at Stillwater when carrots showed higher (P = 0.05) incidence of in spring 1985 than in fall 1985. Also, no significant difference was observed between seasons in 1986 at Stillwater (Table 2).

There were few differences in incidence of AY among carrot cultivars. No differences in incidence of AY were observed among carrot cultivars in either season at Stillwater during 1985 and 1986, at Bixby during 1986, or at Lane during 1986 and 1987 (Tables 1,2). However, variability in incidence of AY within the cultivars was observed at Bixby and Stillwater in 1987. For example, during spring 1987, cv Danvers at Stillwater showed higher (P = 0.05) incidence of AY than cvs Imperator and Spartan Bonus. In the fall of the same year cv Danvers showed the lowest incidence of AY (Table 2). Other cultivar differences were noted in Bixby during both seasons of 1987 (Table 2).

Incidence of AY in lettuce

The incidence of AY in lettuce was different at different locations. The incidence of AY was generally greater (P = 0.05) at Bixby and Lane than at Stillwater. In 1986 in lettuce plots at Bixby and Lane there was

46.8% and 46.7% disease incidence, respectively, while in lettuce in Stillwater there was less than 5%. However, during spring 1987, the highest (P=0.05) incidence of AY in lettuce was at Stillwater, Bixby and at Lane no AY was found. In fall 1987, the highest average incidence of AY in lettuce was at Lane, followed by Stillwater and then Bixby. However, these difference were not significant.

Seasonal differences in incidence of AY were inconsistent. Lettuce showed higher (P=0.05) incidence of AY at all three locations during spring 1986 than during fall 1986. But this seasonal trend was reversed in 1987 with higher (P=0.05) incidence of AY at all three locations in fall than in spring 1987 (Table 3).

There was no difference among lettuce cultivars in 1986 or 1987 in any of the three locations (Table 3).

Characterization of isolates on aster, celery, and periwinkle

Aster yellows from Oklahoma carrot 1 (AYOC 1): An isolate of AY obtained from carrot was the most prevalent of the isolates collected in our study. Three other AY isolates, designated AYOC 3, AYOC 4 and AYOC 5, collected over a period of 4 years (1985-1989) showed symptoms similar to AYOC 1 on aster and periwinkle. AYOC 1 was maintained on aster and periwinkle in the greenhouse. In aster, AYOC 1 caused vein clearing in immature leaves

followed by vein clearing in other developing leaves, chlorosis of the leaves, elongation of the petioles and internodes, and proliferation of lateral shoots from the axils of mature leaves. Severe stunting was observed in aster infected in the first three weeks of germination. Flowers showed phyllody and virescence. Several infected plants showed proliferation from the flowers (sepals and petals became green and stigma, style and ovary were replaced by shoots) (Fig. 2). Necrosis of the axillary shoots was followed by eventual death of the plant. Symptoms on periwinkle included yellowing, leaf size reduction, proliferation, phyllody and virescence. Also observed were elongation of internodes, twisting and intertwining of the stems, elongation of flower stalks, reduction of flower size and flower necrosis. In several cases proliferation from virescent flowers occurred. The symptoms of AYOC 1 in celery were vein clearing, yellowing of the leaves and stunting of the plant. AY symptom appearance in celery at 45 days after inoculation characterized AYOC 1 as a "Western isolate".

Aster yellows from Oklahoma Carrot # 2 (AYOC 2) : AYOC 2 was distinct from the other carrot isolates (AYOC 1, AYOC 3, AYOC 4 and AYOC 5). The symptoms on asters were mild vein clearing, slight yellowing (less than AYOC 1), elongation of petioles, proliferation, virescence and phyllody (Fig. 3). Less stunting of early-infected plants was seen than with AYOC 1. Periwinkle infected with AYOC 2

showed slight reduction of leaf and flower size, and yellowing (but less pronounced than with AYOC 1). The AYOC 2-infected celery showed vein clearing, yellowing and stunting. Symptom appearance between 40-45 days after inoculation indicated that this isolate was also a "Western strain."

Aster yellows from Oklahoma lettuce (AYOL): The isolate collected from lettuce was similar to AYOC 1 on aster, but showed etiolation and elongation of petioles not seen with AYOC 1. The petioles assumed an upright position as compared to the lateral growth of healthy asters. Phyllody, virescence, and stunting of the plant were observed (Fig. 4). Infected periwinkle showed reduced leaf size (more pronounced than with AYOC 1 and AYOC 2) and yellowing (but less than with the carrot isolates). The flowers showed phyllody and virescence, and in severe cases proliferation of shoots arose from virescent flowers. Sometimes flowers were absent.

MLO from Oklahoma daisy fleabane (AYOF): This isolate from daisy fleabane was distinctly different from all other OK MLO isolates. The symptoms of this isolate on aster showed neither vein clearing nor chlorosis on leaves, but leaves became narrow and tapered. Elongation of internodes and virescence and phyllody of floral structures was observed, but no proliferation was seen (Fig. 5). AYOF showed less yellowing and stunting in

periwinkles than did the other MLO isolates. Neither virescence nor phyllody was observed although flowers were slightly smaller than those of healthy controls. On celery, AYOF caused vein clearing and stunting. Production of symptoms at 43 days after inoculation grouped this isolate with the " Western strains".

Discussion

The high incidence of MLO disease in carrot and lettuce plots at Bixby and Lane during spring and fall of 1986 and in Bixby carrot in 1985 may be due to high primary inoculum in weed and vegetable hosts surrounding the trap plots at those locations. Bixby and Lane are predominantly vegetable-growing areas, while Stillwater is not. Volunteer lettuce and carrot and some dicotyledonous weeds adjacent to the trap plots may be serving as sources of inoculum. Another possibile source of AY at Bixby and Lane is the strong wind currents which may carry AY inoculative leafhoppers to these areas from southern United States during spring and summer months. Early in spring, the leafhoppers migrate from the southern United States to the northern United States and Canada (1,4). These two eastern Oklahoma locations are in the path of leafhopper spring migration, while Stillwater lies to the west. Other sources of AY inoculum may come from overwintering nymphs and leafhoppers eggs, since the AY MLO overwinters in nymphs during severe winters and is

disseminated in spring months through active nymphs and leafhoppers (1). The prevalence of AYOC 1 and AYOL isolates in OK suggests that these isolates may overwinter in leafhopper nymphs in those areas. AY has caused higher yield losses and epidemics in vegetables than in cereals (4). Stillwater, located in a wheat-growing region, may not have as much primary inoculum. Also, fewer numbers of leafhoppers were observed at Stillwater than at the other two locations (unpublished observation).

Patterns of disease incidence of AY in lettuce and carrot in all three locations were different in 1987 than in the other study years. At Bixby and Lane, heavy spring rains washed out the carrot and lettuce trap plots. The replanted plots had poor germination and were in the field for only two months instead of the usual 3.5 months. Low rainfall during the summer of 1987 might have caused death of leafhoppers and/or weed plants serving as inoculum sources. These weather conditions might have caused the lower AY incidence at Bixby and Lane in 1987. The situation in Stillwater, however, did not reflect these conditions. During both seasons of 1987, carrot and lettuce at Stillwater showed slightly higher incidence of AY than in the previous two years. At Stillwater during fall 1987, the trap plots surrounded by irrigated winter wheat might have served as a shelter and breeding host for aster leafhoppers during the dry summer months of the

particular year. The trap plots planted in spring and fall of 1985 and 1986, and spring 87 might have helped in increase of primary inoculum in the weeds adjacent to the OSU PPF, Stillwater.

The seasonal differences in AY incidence in all three locations may be due to low numbers of leafhoppers and low primary inoculum in the spring months. Most of the AY infected weed hosts that serve as primary inoculum for the trap plots in spring season are annuals and die during the winter. This would result in lower primary inoculum in spring, whereas by the fall season high primary inoculum may have built up in weeds and leafhoppers. Leafhopper numbers are also affected by winter conditions. Lower leafhopper populations observed in the trap plots during spring as compared to fall (unpublished) corresponds with the AY incidence. The 1985 and 1986 data suggest that under normal rainfall conditions in a given year the carrots and lettuce at Bixby showed the highest AY incidence followed by Lane and Stillwater.

Lack of differences between cultivars reflects our choice of cultivars which were highly susceptible to AY, since our primary objective was to record the natural incidence of AY.

When comparing combinations of factors in the experimental design, several 2-factor, 3-factor, and 4-factor interactions were significant, while others were

not. For example, a nonsignificant 4-factor (location x year x season x cultivar) interaction in the carrot data indicates high homogeneity of percent AY infection, reflecting the consistent differences in means of AY incidence between certain cultivars from year to year. But 2-factor and 3-factor significant interactions suggest extreme heterogeneity of the data, which complicates its interpretation. From a given 2-factor significant interaction, one can find certain factor combinations nonsignificant and others significant. For example, during 1986 and 1987, a year x cultivar (2-factor) interaction ($P = 0.001$) was observed for percent incidence of AY in carrots (Table 2). When a total of six year x cultivar individual factor combinations (year x cultivar) were examined at each location and season, the following two combinations were found significant. 1) Carrots at Bixby showed higher ($P = 0.05$) incidence of AY in fall 86 than in Spring 1987. 2) Carrots at Stillwater during fall 1986 showed lower ($P = 0.05$) incidence of AY than in Fall 1987. Statements based on this analysis must be made with caution. Hence Duncan's Multiple Range test was used to compared individual factor combinations.

In this study, a total of eight MLO isolates were collected and characterized from three geographical locations in Oklahoma. One isolate from carrot, AYOC 2, was different from other carrot isolates. Two isolates

from lettuce, AYOL 1 and AYOL 2 (AYOL group) were different from both carrot groups and from a daisy fleabane isolate (AYOF). The AYOC 1 group and AYOL group isolates were collected during 1985-89 from all three geographical areas suggesting prevalence of these two isolates in these locations in Oklahoma. However, AYOC 1 group isolates were found more frequently than AYOL isolates. AYOC 2 was collected only from Bixby. AYOF was collected only from the Stillwater area.

It is possible that multiple infections could occur with more than one type of MLO co-existing in the same plant. For example, AYOC 1 and AYOC 2 were collected from the same infected carrot plant. All four groups of isolates (AYOC 1, AYOC 2, AYOF and AYOL) were transmitted by aster leafhopper, and all produced symptoms in aster, celery and periwinkle. Carrots and lettuce that had symptoms as AY may have been carrying one, two or more different MLOs. The Deines' stain and electron microscopy did not distinguish between MLOs. Serological tests using anti-AYOC 1 serum were positive with AYOC 1 and AYOL (7). AYOC 2 and AYOF, though aster leafhopper transmissible, did not react with anti-AYOC1 serum. The possibility that these isolates are different MLOs was also suggested by AY-DNA probe testing (14).

Our experiments indicated that AY can be devastating in lettuce and carrot crops in Oklahoma. The collection

of more than one AY isolate in the state suggests the presence of different strains of AY and/or other MLO. These MLO have a potential to occur in high frequencies in carrot and lettuce crops and may cause economic damage. The occurrence and severity of AY disease should be considered in future plans for development of vegetable production areas in the state.

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Table 1. Incidence of aster yellows in carrot in trap plots at Bixby, and Stillwater, Oklahoma during 1985.

Season	Cultivar	Location	
		Bixby[x]	Stillwater[x]
Spring	Danvers	34.2a	5.1a
	Imperator	17.4a	4.4a
	Spartan Bonus	21.9a	4.4a
Fall	Danvers	17.6a	1.2a
	Imperator	36.9a	1.2a
	Spartan Bonus	26.3a	1.8a
Interactions			
Location x season		NS[y]	
Location x Cultivar		NS	
Season x cultivar		*[z]	
Location x season x cultivar		*	

[x] values are the means of four replications and when followed by the same letter within the column and season are nonsignificant (P =0.05) according to Duncans' Multiple Range Test.

[y] Nonsignificant

[z] Significant at (P=0.05)

Table 2. Incidence of aster yellows in carrot in trap plots at Bixby, Lane and Stillwater, Oklahoma during 1986-87.

Season	Cultivar	1986			1987		
		Bixby[x]	Lane[x]	Stl[wx]	Bixby[x]	Lane[x]	Stl[wx]
Spring	Danvers	15.0a	4.1a	0.7a	0.0a	0.0a	4.0a
	Imperator	11.8a	3.5a	0.9a	0.7a	0.0a	3.6ab
	Spartan Bonus	11.7a	4.7a	1.2a	4.8a	0.0a	0.8b
Fall	Danvers	24.3a	10.8a	1.0a	2.4b	4.8b	1.2b
	Imperator	24.3a	8.1a	0.9a	10.9a	8.1a	9.7a
	Spartan Bonus	20.16a	7.9a	1.1a	10.6a	8.0a	12.7a

Interactions

Location x cultivar	NS[y]
Location x year	***[z]
Location x season	***
Year x cultivar	***
Year x season	NS
Season x cultivar	**
Location x year x season	***
Location x year x cultivar	*
Location x season x cultivar	*
Year x season x cultivar	**
Location x year x season x cultivar	NS

[w] Stl = Stillwater

[x] Values are the means of four replications and, when followed by the same letter within the column and season, are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

[y] Nonsignificant

[z] *, **, *** significant at the P= 0.05, P = 0.001, P=0.0001 respectively.

Table 3. Incidence of aster yellows in lettuce in trap plots at Bixby, Lane and Stillwater, Oklahoma during 1986-87.

Season	cultivar	1986			1987		
		Bixby[V]	Lane[V]	Stl[WV]	Bixby[V]	Lane[V]	Stl[WV]
Spring	GL[X]	58.8a	62.2a	4.9a	0.0a	0.0a	7.9a
	Ithaca	62.8a	48.5a	4.7a	4.1a	0.0a	6.6a
Fall	GL	48.2a	49.7a	2.8a	36.3a	56.3a	46.9a
	Ithaca	45.5a	43.6a	2.1a	39.2a	56.3a	72.0a

Interactions

Location x cultivar	*[Y]
Location x year	***
Location x season	**
Year x season	***
Year x cultivar	NS[Z]
Seasion x cultivar	NS
Location x season x cultivar	NS
Location x year x season	NS
Location x year x cultivar	NS
Year x season x cultivar	NS
Location x year x season x cultivar	*

[V] Values are the means of four replications and when followed by the same letter within the column and season are not significantly different according to Duncan's Multiple Range Test (P=0.05).

[W] Stl = Stillwater, [X] GL = Great Lakes

[Y] *, **, *** significant at the P= 0.05, P=0.0015, P=0.0001 respectively.

[Z] Nonsignificant

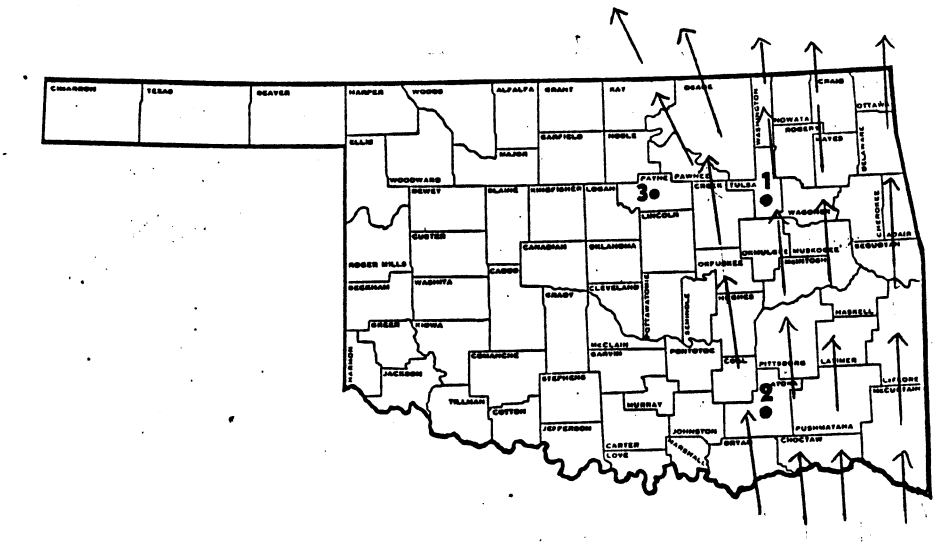

Fig. 1. Map of Oklahoma showing the locations of carrot and lettuce trap plots. Arrows indicate the migration pattern of aster leafhoppers during spring season. (1 = Bixby, 2 = Lane, 3 = Stillwater).

Fig. 2. Aster (Callistephus chinensis L.) infected with an aster yellows mycoplasma-like organism isolate from carrot (AYOC 1). Symptoms include yellowing, stunting, phyllody and virescence. A healthy aster with normal flowers is on the right.

Fig. 3. Aster infected with an mycoplasma-like organism isolate from carrot (AYOC 2). Symptoms include yellowing, stunting, phyllody, and proliferation.

Fig. 5. Aster infected with an mycoplasma-like organism isolate from daisy fleabane (AYOF). Symptoms include no yellowing, but with tapered and narrow leaves, elongated internodes and virescent flowers.

CHAPTER III

PRODUCTION OF MONOSPECIFIC POLYCLONAL ANTIBODIES AGAINST THE ASTER YELLOWS MYCOPLASMA-LIKE ORGANISMS

Abstract

Mycoplasma-like organisms (MLO) associated with aster yellows (AY) were detected by Western blotting in partially purified preparations from AY-infected plants. Polyclonal antiserum (PA) produced against AY MLO reacted with AY proteins in infected plants, but also reacted with healthy plants. The antiserum recognized three AY MLO proteins of 23 Kd, 53 Kd, and 70 Kd in AY infected plants which were not in healthy controls. Cross-absorption of the PA with plant extracts did not completely eliminate the contaminants. Antibodies specific for the three AY MLO proteins were purified from AY MLO PA yielding monospecific polyclonal AY MLO antibodies. To accomplish this, AY MLO proteins were obtained by electrophoresis of infected plant crude extracts and transferred onto nitrocellulose membrane. Specific AY MLO protein bands were excised from the blot. Specific antibodies from AY MLO PA were isolated by trapping with each protein band and then eluted. The resultant monospecific antibodies, pooled together into a trispecific serum,

tested positively with AY MLO in infected plants but not with healthy controls. The trispecific antiserum tested positively with one carrot isolate and one lettuce isolate, but was negative against isolates from another carrot and from daisy fleabane.

Introduction

Aster yellows (AY) is widely distributed and affects more than 350 plant species (14). The disease was reported to cause devastating economic losses in vegetable, horticultural and agronomic crops (14). The causal agent of AY, a non-culturable mycoplasma-like organism (MLO), is transmitted by the aster leafhopper, Macrosteles fascifrons (Stal) (10). MLO-associated diseases are usually differentiated by symptomatology, host range and vector-pathogen relationships. The presence of MLOs in the phloem tissue of infected plants can be demonstrated by fluorochromic DNA stains (3,8), Dienes' stain (4) and electron microscopy. However, none of these methods discriminate among MLOs, or are suitable for epidemiological studies. Electron microscopy, in addition, is time-consuming and involves expensive equipment and reagents. Immunological methods for detecting MLOs have potential to be highly specific and are valuable in detection (16). Polyclonal antisera (PA) have been produced against different strains of AY MLO in several laboratories (9,12,16). All such antisera contained

anti-plant antibodies resulting in cross-reactivity with plant material. Monoclonal antibodies (Mab) are highly specific, but the production of suitable Mab is tedious and expensive. Lin and Chen in 1986 produced PA and Mab against AY and compared both sera for detection of AY MLO (12). They suggested that Mab reacted specifically to AY MLO antigen and discriminated between the AY and other MLO. The PA was unsuitable for specifically distinguishing different MLO because it cross-reacted with plant antigen in AY MLO infected samples.

AY was recently reported in 80% of lettuce plants and 28% of carrot plants in trap plots in Oklahoma (7). This paper reports the production of PA to an AY MLO from OK and the preparation from the PA of AY MLO monospecific polyclonal antisera using an immunoelectroblotting technique. Detection of AY MLO-specific antigens in Western blots of partially purified preparations from infected plants with AY trispecific polyclonal antiserum is also reported.

Materials and Methods

Infected Leafhoppers as Source of AY Antigen

An isolate of AY MLO from Oklahoma carrot (AYOC 1), was maintained on asters by serial transfers through the aster leafhopper, M. fascifrons (5). Late instar nymphs of aster leafhoppers were allowed to feed on AYOC 1-infected asters

for seven days and then on barley for two weeks. Representative leafhoppers from each group were caged on healthy asters to confirm their infectivity. The remainder were frozen (-20 C) in batches of 100. The leafhoppers from those groups which showed positive inoculations to asters were selected for further purification.

Purification of AY MLO from Infected Leafhoppers

Infected leafhoppers were used as the source of OK AY MLO. Partial purification of AY MLO was by a modified method described by Kirkpatrick (1) for Western X-MLO. All purification procedures were carried out at 5 C. One thousand AY MLO infected aster leafhopper heads (combined weight approximately 0.75 g) were plasmolyzed for 10-20 min by mixing 1:4 (w/v) in extraction buffer (0.01 M K_2HPO , 0.01 M KH_2PO , 0.001 M $CaNO_3$, 0.002 M $MgCl_2$, 12% sucrose, 0.8% fructose, 0.05% 2-mercaptoethanol, pH 7.3). The tissue, in extraction buffer, was disrupted with 3-4 strokes of a pestle. The homogenized material was centrifuged (1,100 x g, 5 min) and the pellet was discarded. The supernatant was subjected to high speed centrifugation (15,000 x g, 30 min), and the pellet containing MLO was resuspended in 1 ml of extraction buffer without 2-mercaptoethanol. This suspension was clarified (2,500 x g, 3 min), and the resulting supernatant was filtered through a 1.2 um membrane filter. Aliquots of the filtrate were frozen at -20 C. The procedure was

completed within one day to minimize loss of antigen activity.

The presence of antigen was monitored after each step of the purification by protein-A sandwich-enzyme linked immunosorbent assay (PAS ELISA) (5). The microtiter plates, pre-coated with protein A in NaCO buffer, pH 9.6, were incubated for 2 hr at 25 C. The plates were washed 4 times (5 min each) with PBST [1:1000 in phosphate buffered saline-Tween (PBST- 0.02 M potassium phosphate, 0.015 M NaCl, pH 7.4, 0.5% Tween 20)], and coated with anti-AY PA (coating antiserum) (1:800 in PBST) and incubated for 2 h at 25 C. The plates were washed as above in PBST and the extracts of AY MLO infected periwinkles or healthy periwinkles (1:100 w/v in PBST) were added and incubated for 18 h at 4 C. After 4 washes (5 min each with PBST), the plates were incubated with different serial dilutions of anti-AY MLO PA (as probing antiserum) for 2 hrs at 25 C. The bound antibody was detected by protein A-alkaline phosphotase (1:1000 v/v PBST) (Sigma Chemical Co., St. Louis, MO) for 2 hrs at 25 C. The plates were washed in PBST as before and enzyme substrate [Sigma 104 phosphatase substrate (1 mg/ml in diethanolamine buffer, 200 µl/well)] was added and plates were incubated at 25 C until color developed. The reaction was stopped by adding 3M NaOH (50 µl/well). The plates were read in an EIA reader, model EL-307 (Bio-Tek Instruments Inc., Winooski, VT) at 405 nm.

Preparation of Polyclonal Antiserum

Antisera against partially purified AY MLO preparations were produced in two New Zealand white rabbits following an inoculation schedule similar to that used by Kirkpatrick (1) for Western-X disease. One ml of freshly purified OK AY MLO from infected aster leafhoppers in 0.01 M potassium phosphate buffer, pH 7.3, was emulsified with an equal volume of Freund's incomplete adjuvant and administered intramuscularly into the hind legs, and subcutaneously near the neck of each of two rabbits. Six such injections were given, the first three at one week intervals and the remaining three at bi-weekly intervals. Three weeks after the final injection, the blood was collected and the serum fraction was clarified by low speed centifugation. Aliquots of serum were stored at -20 C. This PA reacted positively with AY infected plants in PAS ELISA and Western blots.

Test Antigens

MLO isolates were transmitted from carrot, lettuce and daisy fleabane with "yellows" type symptoms to aster and periwinkle by aster leafhoppers in the greenhouse. Plant extracts from field-collected, diseased carrot, lettuce and daisy fleabane, and from greenhouse infected aster and periwinkle were partially purified by the AY MLO

purification method described above. In these experiments the leaves and stems of infected plants were homogenized in extraction buffer in a Waring blendor instead of with the mortar and pestle. The resultant homogenate was squeezed through 2 layers of cheesecloth and then subjected to differential centrifugation as described above.

Separation and Electroblotting of AY MLO Proteins

The procedure for protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was based on the method of Laemmeli (10). Partially purified samples (400 ul) were diluted 1:1 in sample buffer [0.5 M Tris HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.1% (w/v) bromophenol blue], and boiled for 3 min. A volume of 40 µl/lane was loaded onto each of 20 lanes of a 14 cm long and 3 mm thick 3%/10% discontinuous polyacrylamide gel cast in a Hoefer SE 400 apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA). The gel was run overnight at 18 18 mA/gel at room temperature.

For Western blotting (17), electrophoresed proteins were transferred to pre-wetted nitrocellulose in a Transphor apparatus (Hoefer Scientific Instuments, San Francisco, CA, USA) for 2 hrs at 100 V in pre-chilled transfer buffer [0.192 M glycine, 0.025 M Tris, pH 8.3, 20% (v/v) methanol]. The molecular weights of the proteins were determined by comparison with pre-stained low-range molecular weight

protein standards (Diversified Biotech, Newton Center, MA). Immediately following the transfer, the membranes were blocked overnight with 5% nonfat dry milk in TBS (0.05 M Tris and 0.2 M NaCl, pH 7.5). After four washes (2 min each) in TBS the membranes were probed for 3 hrs or more with anti-AY MLO polyclonal serum diluted 1:60 in TBS. The nitrocellulose membrane was washed four times in TBS as before and incubated for 1 hr with protein A-peroxidase (Sigma Chemical Co, St. Louis, MO) diluted 1:1000 in TBS. The immunoblots were developed in substrate [1 vol 4-chloro-1-naphthol (Sigma Chemical Co. St. Louis, MO)] at 3 mg/ml in methanol, 5 vol of 50mM Tris/200 mM NaCl pH 7.4, and 1/2000 vol of 3% H_2O_2 (0.015% final concentration). After maximum color development, the blots were rinsed in water to stop the reaction, and air-dried.

Cross-Absorption of PA

Proteins of healthy periwinkle plant material subjected to the partial purification methods above were separated by SDS PAGE and transblotted to nitrocellulose membrane. The nitrocellulose containing healthy periwinkle extract was incubated for 3 hrs with anti-AY MLO serum diluted 1:40 in TBS. One ml of the serum was removed and stored at -20 C for later testing. The cross absorption of the remaining serum was repeated 5 times. To evaluate the effect of cross-absorption, a preparation from healthy

periwinkle was electrophoresed and transferred to nitrocellulose membrane as described. Then 3 mm-wide vertical strips were excised and probed with sera from the six consecutive absorptions or with nonabsorbed serum. The strips were developed as before.

Production of AY MLO Trispecific Polyclonal Antiserum

The procedure for antibody purification was modified from that of Rybicki for plant viruses (14). Following electrophoresis and protein transfer to nitrocellulose membrane, vertical strips corresponding to lanes 1 and 20 (pre-stained standards), lanes 2 and 19 (healthy plant extract) and lanes 3 and 18 (AY MLO-infected plant extract) were excised. The alignment strips (lanes 3 and 18) were blocked overnight and developed as described above. The developed strips were aligned with the remaining piece of the blot of AY MLO-infected plant material (lanes 4-17), and three horizontal strips 5 mm wide corresponding to AY MLO specific proteins, 23 Kd, 53 Kd, and 70 Kd, present in the infected samples, but not in healthy controls, were excised. A region of the blot with no polypeptides was also excised as a control strip. These horizontal strips (preparative strips) were each incubated separately with 40 ml of a 1:40 dilution of anti-AY MLO serum in TBS for 3 hrs at 21 C and then washed 3 times in TBS buffer. The preparative strip of each individual protein with its bound antibodies was agitated in 7.5 ml

of 0.1M glycine-HCL buffer, pH 2.9, for 10 min to elute the specific antibodies. The contents were decanted and immediately neutralized by adding 1.4 ml of 0.1 M NaOH. The resultant solution was designated as the "first eluate." A "second eluate" was collected by repeating the above process. The 1st and 2nd individual eluates of each protein were pooled together to yield 3 different monospecific polyclonal antisera. The three monospecific antisera were then combined to give a single trispecific polyclonal antiserum. This was concentrated by lyophilyzation, and resuspended in 1300 ul of TBS. The trispecific polyclonal antiserum was evaluated using Western blotting and PAS ELISA of AY-infected plants and healthy controls.

The anti-AY MLO trispecific polyclonal serum and non-purified AY MLO PA controls were tested for their anti-AY MLO reactivity by PAS ELISA. The non-purified AY MLO PA (1:800 dilution in PBST) was used as coating antiserum. Serial 2-fold dilutions (1:4, 1:8, and 1:16) of anti-AY MLO trispecific polyclonal serum were used as probing sera. The absorbance values after addition of the substrate were determined at 405 nm.

Results

Partially purified preparations of AY MLO from infected periwinkles, and healthy periwinkle controls, were used to evaluate the specificity of the antisera. The Western blots developed with anti-AY MLO PA showed the

presence of AY MLO-specific proteins of 23 Kd, 53 Kd, and 70 Kd in infected periwinkle but not in healthy controls (Fig. 6). The 23 Kd protein band stained more strongly than did those of 53 Kd and 70 Kd. In addition to the 3 AY MLO specific proteins, other bands appeared in the blots of both infected and healthy material. This demonstrated that the serum contained antibodies to plant proteins which led to the non-specific reaction in both healthy and infected plant extracts.

The specificity of the PA to AY MLO in the infected and healthy plants was evaluated by PAS ELISA. In this test AY antiserum (provided by Bruce Kirkpatrick, Univ. of California at Davis, Davis, CA) diluted 1:800 in PBST was used as coating antiserum and four-fold serial dilutions of antiserum produced against the Oklahoma AY isolate were used as probing antisera. The dilution end point for the anti-AY MLO serum was 1:800. Thereafter, in all PAS ELISA testing, anti-OK AY MLO was used as both coating and probing antisera.

After binding to the nitrocellulose strip containing a corresponding AY specific protein, the monospecific antibodies for each of the 23 Kd, 53 Kd, and 70 Kd AY MLO specific proteins were eluted by acid treatment as described. The acidic solution with antibodies was neutralized and the ten (5 blots with 2 eluates/blot) antibody eluates from each of the 23 Kd, 50 Kd, and 70Kd proteins were pooled together to give a single anti-AY MLO

trispecific polyclonal antiserum.

The "first eluate" from each of the three AY protein strips had a higher yield of antibodies than the "second eluate". First and second eluates were pooled to maximize the amount of the antibodies to each specific protein. To determine completeness of the antibody elution, the blots, after the elution of the antibodies, were incubated with protein A-peroxidase and then with the substrate. The colorometric reaction in blots after the 1st elution was darker than in the blots after the second elution. The faint reaction indicated that most of the antibody was removed after two elutions (data not shown).

The results of comparison of the anti-AY MLO trispecific polyclonal serum and non-purified AY MLO PA controls are shown in Table 1. The absorbance values at 405 nm indicated that the trispecific polyclonal antibodies did not distinguish between healthy and infected plant material in PAS ELISA.

SDS polyacrylamide gels of healthy and infected plant preparations (40 µl/lane) were electroblotted, and vertical lanes (4.0 cm x 0.3 cm) of healthy and infected plant protein samples were excised. Alternating pre-stained standards with samples in lanes helped in locating a particular sample region. The excised strips were probed with non-purified AY MLO antiserum, anti-AY MLO trispecific polyclonal serum or eluate from a control blot (presumably containing no antibodies), respectively. The blots were

developed as above. The results are shown in Figure 7.

In Western blots, non-purified PA reacted with infected plant extracts and showed protein bands of 23 Kd, 53 Kd, and 70 Kd but non-specific activity was observed with plant proteins in both infected and healthy extracts. AY MLO specific proteins were not present in healthy extracts.

The trispecific polyclonal antiserum reacted only with AY MLO specific 23 Kd, 53 Kd, and 70 Kd proteins in the extracts of plants infected with a carrot isolate (AYOC 1) and a lettuce isolate (AYOL), while no reaction was observed in the extracts of the plants infected with isolates from carrot (AYOC 2) or daisy fleabane (AYOF), or in healthy plant extracts. Cross-reactivity was observed neither in healthy nor in infected plant extracts.

The eluate from the control strips (blot with no antibodies) did not react with any of the proteins in either infected or healthy plant extracts.

Discussion

MLO diseases are usually differentiated from one another by host range, symptomatology, and vector-plant relationships. These methods are time-consuming and inconclusive. Serological methods are an alternate approach in which antigens are detected by specific antibodies. The current inability to culture AY MLO hinders the production of truly specific PA to MLO in partially purified preparations from infected plants (2). The first report

of AY MLO purification and its detection in infected plants by ELISA and immunosorbent electron microscopy appeared in 1979 (15). These procedures distinguished AY from peach and clover yellow edge MLO but not from clover phyllody MLO. A report on partial purification of AY from infected plants suggested the need for highly purified antigens for antiserum production (17).

The results described here indicate that a PA produced against AY MLO was highly contaminated with anti-plant antibodies which interfered with AY MLO detection in infected plants by PAS ELISA and Western blotting. Three AY MLO specific proteins of 23 Kd, 53 Kd, and 70 Kd were detected by non-purified PA, along with other plant proteins. Laborious cross-absorption of the antiserum with healthy material was not satisfactory.

AY MLO-specific antibodies were then purified from PA, using a method developed for detection of low-molecular-weight virus proteins that are present in low titers (15). This method is a modification of the "Western blotting" technique, and is based on affinity absorption techniques that use a highly immobilized antigen to remove unwanted antibodies from serum or to purify monospecific antibodies (13). We adapted and modified the method to purify three antibodies that were specific for AY MLO proteins. The trispecific serum tested positively with AY infected plants but not with healthy control plants. Though we were not able to produce PA free

of contaminants, purification of monospecific antisera by immunoelectroblotting from PA further eliminated anti-plant antibody contaminants.

A recent report (2) on comparison of PA and Mab made against primula yellows MLO-associated antigens showed that those antisera detected a single major antigen of 22.4 Kd in primula yellows and European aster yellows. The results of our experiments indicated that a total of three proteins, of 23 Kd, 53 Kd, and 70 Kd, were recognized by the PA produced against a United States AY MLO. The 23 Kd protein may be similar to the 22.4 Kd protein previously reported but the presence of two other detectable antigens in the Oklahoma AY isolate suggests that it may be different from European aster yellows.

Western blotting of SDS PAGE-separated AY MLO proteins was more sensitive than PAS ELISA. A high level of cross reactivity was observed with plant proteins of both healthy and infected plants in PAS ELISA using AY MLO PA as probing serum. All three AY MLO specific bands, 23 Kd, 53 Kd, and 70Kd, were detected with trispecific polyclonal antiserum in Western blotting of infected plant preparations, but not in the healthy controls. But no significant difference was detected between infected and healthy plant material with trispecific polyclonal antiserum in PAS ELISA tests. This may be due to the low titer of the antigen in the test plants or to a low concentration of purified trispecific polyclonal antiserum.

Electron micrographs of the plants infected with AY isolates AYOC 1 and AYOC 2, from carrot, AYOL from lettuce and AYOF from daisy fleabane, showed the presence of mycoplasma-like organisms (6). The Western blotting using AY MLO trispecific polyclonal antiserum showed positive reactions with AYOC 1 and AYOL but not with the other two isolates. This suggests that AYOC 2 and AYOF isolates may be MLO other than AY or that AY MLO was present at an undetectable titer.

The production of monospecific antibodies is time consuming and may not be suitable for large-scale purifications.Very few micrograms of specific antibodies were obtained from the electroblots of many gels. This method is useful in conducting experiments with Western blotting and ELISA that require very small amounts of antisera. These purified antibodies can further help in the study of specific AY MLO proteins and their receptors in the plant and insect hosts .

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Table 4. Evaluation of aster yellows mycoplasma-like organism (AY MLO) polyclonal antiserum and trispecific polyclonal antiserum by Protein A-Sandwich enzyme-liked immunosorbant assay (ELISA).

Sample*	Dilution	A_{405} nm values		
		Healthy plant	AY Infected plant	Buffer control
PI	1:4	0.120	0.119	0.118
	1:8	0.129	0.121	0.120
	1:16	0.118	0.113	0.125
PA	1:4	0.405	1.237	0.062
	1:8	0.370	0.975	0.059
	1:16	0.278	0.853	0.067
TPA	1:4	0.139	0.198	0.062
	1:8	0.137	0.188	0.063
	1:16	0.109	0.144	0.053
Control	1:4	0.078	0.097	0.063
	1:8	0.091	0.073	0.067
	1:16	0.099	0.096	0.054

*PI- pre immune serum, PA = nonpurified AY MLO polyclonal antiserum, TPA = trispecific polyclonal antiserum, Control = the eluate from the blot with no antibodies.

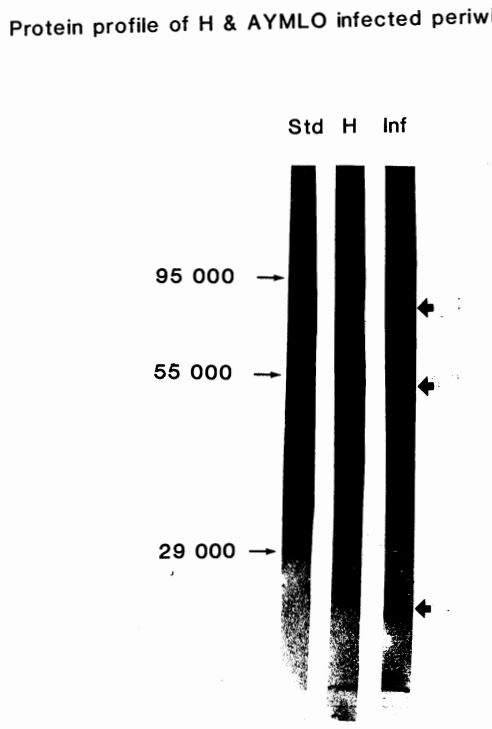

Fig. 6. Protein profile of healthy and aster yellows mycoplasma-like organism (AY MLO) infected periwinkles Western blotted with non- purified AY MLO polyclonal antiserum. Arrows indicate protein bands at 23 Kd, 53 Kd, and 70 Kd.

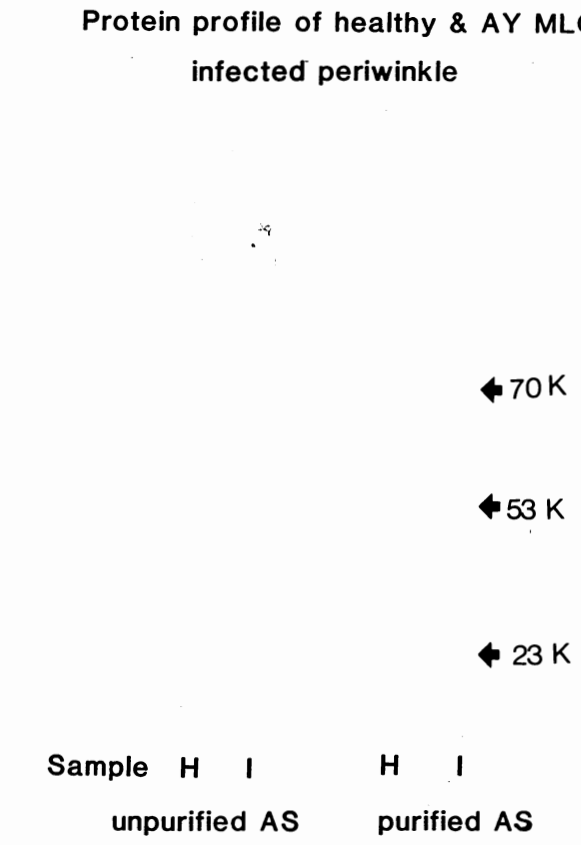

Fig. 7. Comparison of non-purified polyclonal serum and trispecific polyclonal antibodies on proteins of healthy and aster yellows mycoplasma-like organism infected periwinkles.

CHAPTER IV

SCREENING OF SELECTED WHEAT AND PEANUT CULTIVARS FOR SUSCEPTIBILITY TO ASTER YELLOWS MYCOPLASMA-LIKE ORGANISMS (AY MLO)

Abstract

Five cultivars each of wheat and peanut commomnly grown in Oklahoma (OK) and wheat grown in Canada were screened for susceptibility to OK aster yellows (AY) mycoplasma-like organism (MLO). None of the cultivars tested were found susceptible to the OK AY MLO through aster leafhopper transmissions. The failure of OK AY MLO to infect wheat cultivars from Canada, which are natural hosts of AY in Canada, suggests the possibility of different geographical strains of AY or different leafhopper biotypes.

Introduction

Aster yellows (AY), an intensively studied plant disease of mycoplasmal origin, which can be transmitted by more than 25 leafhopper species, affects more than 350 species in 54 plant families (15). AY was reported to cause devastating economic losses in agronomic and horticultural crops (15). The causal agent of AY, a non-culturable mycoplasma-like organism (MLO), is transmitted mainly by the aster leafhopper, Macrosteles fascifrons (Stal).

Bantarri and Moore in 1966 (1) first demonstrated the susceptibility of the family Gramineae to AY MLO by transmitting the MLO to and from barley, Hordeum vulgare L., cv. `Vantage'. Twenty-nine varieties of barley were shown to be susceptible to AY MLO (2). Oats, Avena sativa L., were first reported to be susceptible to AY in California in 1962 (10). In Finland in 1966, oats were found susceptible to the MLO from an "aster yellows type" disease (14). Seven varieties of oats were susceptible to a non-celery infecting or Eastern strain of AY MLO (4,7,18,19).

A celery-infecting or Western strain of AY was transmitted by another leafhopper, Endria inimica (Say), to wheat, Triticum turgidum Desf., cv. `Ramsey' (3).

Seven cultivars of wheat T. aestivum L. cv.`Thatcher', `Selkirk', `Cascade', and `Lemhi' and T. turgidum cv. `Stewart', `Ramsey', and `Pelissier' were susceptible to a celery-infecting AY MLO strain (5). Other grass hosts of AY were Bromus arvensis L., Phalaris canariensis L., and Lolium multiforum Lam. With the exception of one report in Finland (14) all other natural AY MLO infections in cereals were reported from Canada.

Hobbs et al. (12) and Kiratiya-angul et al.(13) reported MLO in diseased peanut plants. Wheat and peanuts are predominant crops of OK. The knowledge of the presence of AY in the state (9,17), the abundance of the aster leafhopper in OK, and the reports of yield losses in wheat due to AY in Canada (5), led us to investigate the susceptibility of wheat and peanut cultivars grown in OK to OK AY MLO. This paper reports the reactions of wheat and peanut cultivars grown in OK to an AY MLO isolate collected from OK.

Materials and Methods

The seeds of wheat and peanut were kindly provided by the following researchers: wheat, T. aestivum L. cvs. Chisholm, Payne, TAM 101, Triumph 64 and Vona (R. M. Hunger, Oklahoma State University, Stillwater); spring

wheat T. turgidum cvs. Stewart, Ramsey and Thatcher, and winter wheat T. aestivum cvs. Lemhi and Selkirk, (Steve Haber, Agriculture Canada, Winnipeg, Manitoba, Canada); peanut, Arachis hypogea L. cvs. Florunner, Okrun, Pronto, Tamnut, and Toalson (H. A. Melouk, Oklahoma State University, Stillwater, OK). Aster, Callistephis chinensis L., celery, Apium graveolans L., and periwinkle, Catharanthus roseus L. seed was obtained from Old's Seed Co, Madison, WI.

An Oklahoma AY isolate (AYOC 1) was collected in 1985 from carrot trap plots at the Oklahoma State Vegetable Research Station, Bixby, OK. The isolate was maintained on aster in the greenhouse by serial transfers through the aster leafhopper, M. fascifrons.

Initial healthy leafhopper colonies were started from individual insects obtained from C. Eastman, Illinois Natural History Survey, Champaign, IL. Leafhoppers were reared on barley in the greenhouse at 22-30 C. Leafhoppers used in transmission studies were infected by caging early instar nymphs on AYOC 1-infected asters for 7 days and then on barley (an AY non-susceptible feeding host) for a 2 week incubation period. Representative leafhoppers were then caged singly on healthy asters to confirm their inoculativity. Approximately 95% of such leafhopper inoculated asters

expressed AY symptoms. Only batches of leafhoppers from which the representative leafhoppers showed infectivity on aster were used in the transmission studies. All the transmission studies were conducted at 22-30 C.

In each experiment, a total of 30 wheat seedlings (5 seedlings/pot), and 30 peanut seedlings (3 seedlings/ pot), were exposed to AY MLO-infective leafhoppers. Fifty infective leafhoppers were caged for 7 days on pots containing 7-8 day old wheat seedlings, or 21-23 day old peanut seedlings. Three controls were included in each experiment; test plants caged with healthy leafhoppers, test plants caged without leafhoppers, and aster (an indicator host) caged with infective leafhoppers. After the inoculation access period, plants were sprayed with Malathion to kill the leafhoppers and held in the greenhouse to maturity for symptom observation. Representative wheat and peanut plants were checked for the presence of MLO by Dienes' staining and electron microscopy. Wheat screening experiments were performed four times and peanut screening was performed twice. Back-inoculations from representative wheat or peanut plants were conducted. Fifty healthy leafhoppers were caged on a pot with two to three inoculated wheat and peanut plants for 7 days and then on barley for a two-week incubation

period. Leafhoppers were then caged on healthy asters for a week. The aster plants were sprayed with Malathion and were held in the greenhouse for symptom observation.

Results and Discussion

In one experiment with wheat, four test plants of cv. Payne, five of cv. Chisholm and five of cv. Stewart showed distorted and sterile heads. Control plants appeared normal. No MLO were detected in the above samples by either Dienes' staining, electron microscopy or bac-inoculation. The symptoms were attributed to spider mite infestation. None of the wheat varieties tested were found susceptible to OK AY MLO (Table 1). The aster (positive) controls showed 95.5% infection, confirming the inoculativity of the leafhoppers and the reliability of transmission procedures for aster. Due to containment requirements, we were unable to test the reaction of a Canadian AY isolate (positive control) on wheat cultivars from either Canada or Oklahoma. Thus efficacy of transmission procedures for infecting wheat could not be confirmed. Survival of the leafhoppers on wheat and peanut plants for over 3 weeks confirmed that they were feeding on these plants. Back-inoculation to aster plants from representative symptomless wheat and peanut plants and from wheat plants with distorted heads failed to

produce `yellows' symptoms in the asters confirming the absence of AY MLO in the wheat and peanut varieties tested.

Five wheat cultivars from Canada reported susceptible to AY in Canada (5) were tested for susceptibility to OK AY MLO. Inability of the OK AY MLO isolate to infect these Canadian cultivars suggests the possibility of a strain difference between Canada AY and Oklahoma AY or a difference in leafhopper biotype.

In Manitoba, Canada, AY was prevalent during the 1960s, rare in the 1970s, and again was observed in 1981. AY was recorded both in experimental plots and in farmers' fields, with some of the field plots showing 65% yield loss. Early infections caused severe damage and resulted in death of the wheat (Agricultural reports on Virus and Mycoplasma Diseases on Cereals in Manitoba, Aug 1981).

Durum wheat (T. turgidum), is a widely grown species in Canada and was highly susceptible to an Eastern AY strain but less susceptible to a Western AY strain. Hard red winter wheat (T. aestivum) a widely grown species in the Southern USA including Oklahoma, was less susceptible to both Eastern and Western AY isolates than was durum wheat, though a few plants were infected (6). Peanut was reported susceptible to an unidentified MLO in Thailand (13) and India (12) but AY MLO has not been reported in peanut. Lack of transmission of OK AY MLO to OK peanut

cultivars suggests that these cultivars are not susceptible to AY MLO.

Our experiments have indicated the presence of more than one strain of AY from Oklahoma (8). Results presented in this paper suggest that the the OK AY MLO tested (AYOC 1, a prevalent isolate in OK) does not affect selected wheat cultivars grown in OK. Incidence of AY in vegetable crops in Oklahoma varied from year to year (8). Patterns of migration of insect vector populations from the south may account for this variation and for the different AY strains identified (7). The recent reports on recurrence of AY MLO in Canadian wheat suggests possible cyclic trends (16). If such a phenomenon occurs in the US, the OK wheat and peanut cultivars should be tested periodically for susceptibility to OK AY isolates.

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Table 5. Reaction of selected wheat and peanut cultivars from Oklahoma and wheat cultivars from Canada to AY MLO (AYOC 1 isolate).

Cultivars	Total plants	Infected plants	Percent infected
Wheat from Oklahoma			
Chisholm	120	0	0.0
Payne	120	0	0.0
TAM 101	120	0	0.0
Triumph	120	0	0.0
Vona	120	0	0.0
Wheat from Canada			
Lemhi	120	0	0.0
Ramsey	120	0	0.0
Selkirk	120	0	0.0
Stewart	120	0	0.0
Thatcher	120	0	0.0
Peanuts from Oklahoma			
Florunner	60	0	0.0
Okrun	60	0	0.0
Pronto	60	0	0.0
Tamnut	60	0	0.0
Toalson	60	0	0.0
Controls[a]			
Wheat[b]	75	0	0.0
Peanut[b]	50	0	0.0
Aster[c]	125	120	96.4

[a] For each species tested, controls included 5-8 plants of each cultivar listed.
[b] All wheat and peanut plants were caged with non-inoculative leafhoppers.
[c] Aster control plants were caged with inoculative leafhoppers.

APPENDICES

APPENDIX A

DIENES' STAINING OF ASTER YELLOWS MYCOPLASMA-LIKE ORGANISM (MLO) IN INFECTED PLANT TISSUE.

Introduction

Mycoplasma-like organisms (MLO) are known to be associated with more than 50 diseases of plant species. These pathogens are non-culturable prokaryotes that lack a cell wall and are bound by a single unit membrane. Electron microscopy is the most convincing method available for visualization of MLO in the phloem tissue of the infected plant, but is time-consuming and expensive. Dienes' stain is used in light microscopy and is specific for all animal mycoplasmas (1) and plant MLO (2). It was first used to detect plant spiroplasmas and MLOs (3) by Deely et al. in 1979 and has been used as a primary detection method in MLO-affected plants since then.

Materials and Methods

Dienes' Stain. The procedure for Dienes' staining of infected plants was described by Deely (4). The stain (2.5g methylene blue, 1.25g azure II, 10.0g maltose, and 0.25g sodium carbonate in 100 ml distilled water) (2) is filtered through Whatman No. 1 filter paper and diluted to

0.2% before using.

Plant Material

Greenhouse-grown periwinkle (Catharanthus roseus L.) infected with AY from AY infected carrot, lettuce, and daisy fleabane from Oklahoma; periwinkles infected with California AY (severe strain, dwarf strain and etiolated strain supplied by Bruce Kirkpatrick, University of California at Riverside, CA); periwinkle and aster infected with Canadian AY (supplied by Lloyd Chiykowski, Plant Research Institute, Canada Department of Agriculture, Ottawa, Canada, and Steve Haber, Agriculture Canada Winipeg, Manitoba, Canada); periwinkle naturally infected with an unknown MLO at the Oklahoma State Student Union and garden in Stillwater (Bob Hunger), and periwinkle infected with Spiroplasma citri saglio et al. (Jacqueline Fletcher, Oklahoma State University, Stillwater, OK) were used as test materials. Carrot, lettuce, daisy fleabane and mares' tail plants naturally infected with MLO (from trap plots at Bixby, Lane and Stillwater, OK) and asters with OK AY MLO and Canadian AY MLO were tested with Dienes' stain. Carrots infected with Sclerotium rolfsii Sacc., (Ken Conway, Oklahoma State university), peanut plants infected with Sclerotinia sp. (Hassan Melouk, Oklahoma State University), and cabbage infected with Xanthomonas sp. (from a farmer's field at Bixby) were also tested with Dienes' stain.

Sections of healthy and infected stems, petioles, leaves, midribs, flower stalks, and roots were cut by hand into water on a microscope slide with a single-edged razor blade, and covered with a cover slip. The water was withdrawn by placing blotting paper on one side of the cover slip while drops of Dienes' stain were introduced from the opposite side. The stain was allowed to stand for a few seconds to 2 min and then replaced with water. The sections were immediately examined in the bright-field microscope.

Transverse sections were preferred to longitudinal section because it was easier to locate phloem tissue in the plant material tested.

Results

The phloem of the healthy sections remained unstained (Fig. 8). However, in phloem tissue of MLO-infected stem, petiole, midrib and root several groups of cells were stained blue (Fig. 9). Xylem was colored turquoise blue and cortex was stained light blue. All the MLO-infected plants showed a positive reaction with Dienes' staining, whereas the plants infected with either fungi or bacteria gave negative results (Table 6).

Discussion

Dienes' stain, specific for wall-less prokaryotes, was first developed to detect animal mycoplasma colonies on

agar (3). Mycoplasmas are detected by the staining of typical "fried-egg" colonies. Bacteria decolorize the stain (3).

The presence of phloem-restricted, non-culturable MLO were detected by Dienes' staining in the phloem tissue of infected plants but not healthy plants. The presence of MLO in electron micrographs of the tissue that gave positive results with Dienes' stain confirmed the presence of MLO and supported the idea that the color reaction was specific to MLO. Dienes' staining is rapid, requires less expensive equipment and reagents than electron microscopy, and is a good preliminary diagnostic method to detect MLO in plant tissue.

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Table 6. Dienes' staining of cross sections of stems, leaves and petioles of plants infected with mycoplasma-like organism (MLO) and other pathogens.

Pathogen	Host	No. of samples	Reaction in phloem
MLO (AYOC 1)	aster	200	+[a]
	periwinkle	50	+
	carrot	100	+
MLO (AYOC 2)	aster	30	+
	periwinkle	20	+
	carrot	20	+
MLO (AYOC 4)	aster	20	+
	periwinkle	10	+
	carrot	20	+
MLO (AYOF)	aster	20	+
	periwinkle	10	+
	daisy fleabane	20	+
MLO (AYOL)	aster	30	+
	periwinkle	10	+
	lettuce	50	+
MLO (Canada)	aster	50	+
	periwinkle	20	+
MLO (California)	periwinkle	30	+
MLO (suspected)	periwinkle	30	+
MLO (suspected)	mares' tail	10	+
Spiroplasma citri	periwinkle	10	+
Sclerotinia sp.	peanut	10	-[b]
Sclerotium rolfsii	carrot	10	-
Xanthomonas sp.	cabbage	10	-

[a] Positive staining reaction observed in the phloem of all plants tested.

[b] No staining reaction observed in the phloem of all plants

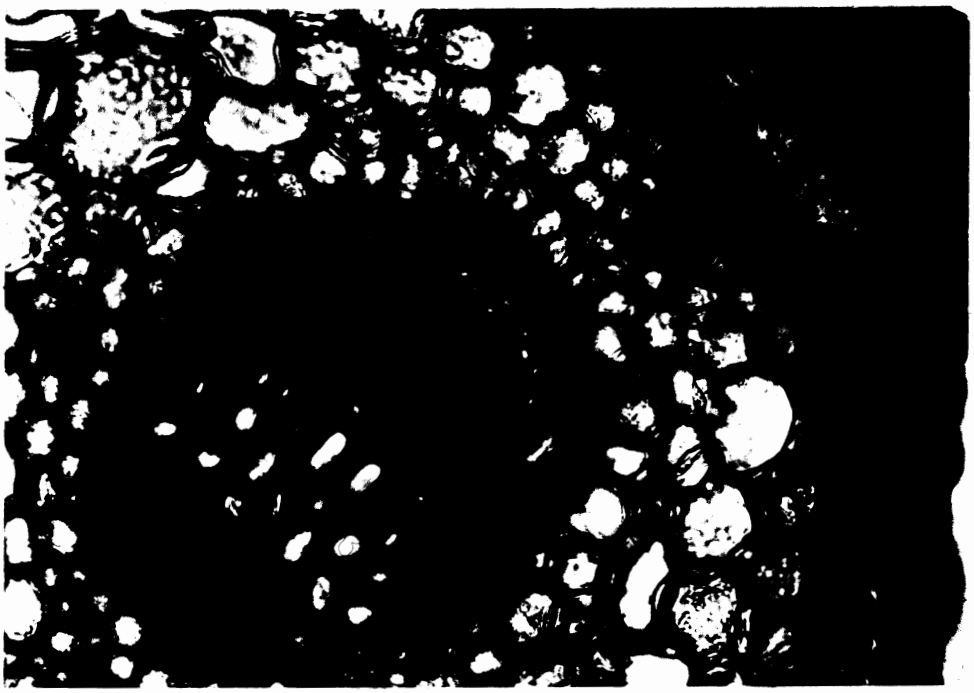

Fig. 8. Dienes' staining of a transverse section of a healthy aster petiole under bright field optics (200 X). No staining is observed in the phloem (P) region.

APPENDIX B

DETECTION OF ASTER YELLOWS MYCOPHASMA-LIKE ORGANISMS BY ELECTRON MICROSCOPY.

Introduction

Aster yellows, an extensively studied plant disease of the 'yellows' disease group, was thought to be of virus etiology for 70 years. Doi et al. in 1967 (2) first demonstrated the presence of MLO in the sieve elements of plants with several yellows diseases, including aster yellows. These diseases have traditionally been differentiated by symptomatology, host range and plant-vector relationships. The presence of MLO in the phloem tissue can be detected by diamidinophenyl indole (DAPI), Dienes' stain (1), electron microscopy (3,4,9) and serological techniques (5,6).

This paper outlines the procedures of tissue preparation of AY MLO infected plants for electron microscopy, as used in the work for this dissertation.

Materials and Methods

Oklahoma AY isolates from carrot (AYOC 1, AYOC 2 and AYOC 4), lettuce (AYOL), and daisy fleabane (AYOF), were transmitted to aster, Callistephus chinensis L., and

periwinkle, Catharantus roseus L., by the aster leafhopper, Macrosteles fascifrons (Stal). All the isolates were maintained on asters through serial transmission by aster leafhoppers, and on periwinkle by periodic grafting. Samples of AY-infected plant material observed in electron microscopy included AYOC 1-infected carrot, aster and periwinkle; AYOC 2-infected periwinkles; AYOF-infected periwinkles; AYOL infected asters, lettuce and periwinkles; and wheat and peanut plants from AYOC 1-transmission studies.

The procedure for electron microscopy of AY MLO infected plants was described by Sinha (7). Petioles, midribs and young stem regions of infected plants, were cut into 1 mm^2 pieces in 6% glutaraldehyde (3,4,7) in 0.1M potassium phosphate buffer (KPO_4 buffer), pH 7.3. and immediately fixed in fresh 6% glutaraldehyde on ice for 1 hr. After rinsing with cold 0.1 M KPO_4 buffer (3 washes, 20 min each), the tissue was post-fixed in osmium tetroxide (OsO_4) for 12 hours on ice or overnight at 4 C. Following post-fixation, the tissue was rinsed with KPO_4 buffer (3 times, 20 min each). The tissue was then dehydrated in 30%, 50%, 70% and 90% acetone (20 min each). Following 90% acetone, the tissue was stained in 5% uranyl acetate in acetone overnight. The tissue was dehydrated further in 95% and 100% acetone (2 changes for 20 min each). The tissue was transferred to propylene oxide (2

changes, 15-45 min each). Then the tissue was infiltrated with Spurr's embedding medium [10 g 4-vinylcyclohexene dioxide (resin), 0.4g 2-dimethylaminoethanol (DMAE) (accelerator), 6g DER resin grade 736 (flexibilizer), 26g nonenylsucceinic anhydride (epoxy hardener)] (8) and propylene oxide (1:3 ratio) for 1 hour, 1:1 (Spurr's: propylene oxide) for 1 hour and 3:1 (Spurr's:propylene oxide) for 1 hour. Then vials were uncapped and left in a desiccator overnight. To improve infiltration, the tissue was transferred to fresh Spurr's daily for 2-3 days without letting the Spurr's solidify (8). Blocks were polymerized at 60 C for 2-3 days, and cured for at least one wk before sectioning. Staining of the thin sections was in 5% uranyl acetate for 30 min and in lead acetate for 30 min. The sections were examined in a Joel 100CX ASID transmission electron microscope.

Healthy plant materials were treated similarly and used as controls.

Results and Discussion

No MLO were seen in sections from healthy control samples (Fig. 10). However, pleomorphic, 70-800 nm diameter, unit membrane-bound MLO were observed at 7 200 X in the phloem regions of the infected plants (Table 7) (Fig. 11). At higher magnification (100 000 X), electron-dense ribosome-like granules and DNA-like materials were present. Wheat plants with distorted heads and yellow

leaf symptoms failed to show any MLO, suggesting that the symptoms were caused by agents or conditions other than AY MLO. Similar results were obtained from symptomless wheat and all peanut samples. Electron microscopy is the most convincing diagnostic method available for the MLO, but it is not specific for any one MLO. It is also possible to miss MLO if they are present in low titer. Samples from the plants that showed MLO in electron microscopy were further tested by Western blotting, using OK AY MLO antiserum. Only the AYOC 1 group and AYOL group isolates tested positively with OK AY MLO antiserum. This suggests that the other two isolates (AYOC 2, AYOF) were MLOs other than AY or that they were present at undetectable titers.

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Table 7. Electron microscopy of healthy and mycoplasma-like organisms (MLOs) infected plants.

Isolate	Host	# of samples	Reaction in the phloem
AYOC 1	aster	6	+[a]
	periwinkle	6	+
	carrot	3	+
AYOC 2	aster	6	+
	periwinkle	6	+
AYOC 4	aster	6	+
	periwinkle	6	+
AYOF	periwinkle	6	+
AYOL	aster	6	+
	periwinkle	6	+
	lettuce	6	+
Healthy	aster	6	-[b]
	periwinkle	6	-
	wheat	6	-
	peanut	6	-

[a] MLO observed in the phloem of all plants tested.

[b] No MLO observed in the phloem of all plants tested.

APPENDIX C

DETECTION OF ASTER YELLOWS MYCOPLASMA-LIKE ORGANISMS BY MEANS OF A FLUORESCENT DNA-BINDING AGENT, 4-6-DIAMIDINO-2-PHENYLINDOLE-2 HCL (DAPI).

Introduction

Histochemical testing of mycoplasma-like organisms (MLO) in infected plant specimens is useful in screening of field and greenhouse samples. A DNA-specific dye, 4-6-diamidino-2-phenylindole-2 HCl (DAPI) binds to DNA molecules and serves as a specific probe (4,5). In the present study aster yellows MLO-infected plants were tested with DAPI to detect MLO in phloem tissue.

Materials and Methods

Oklahoma AY isolates from carrot (AYOC 1, AYOC 2, and AYOC 4), lettuce (AYOL), and daisy fleabane (AYOF) were transmitted to aster, Callistephis chinensis L., and periwinkle, Catharanthus roseus L., by the aster leafhopper, Macrosteles fascifrons Stal. All the isolates were maintained on asters through serial transmission by aster leafhoppers and on periwinkles by periodic grafting. Periwinkles with Canadian aster yellows and California aster yellows were also maintained in the greeenhouse.

The procedure for DAPI staining of MLO in infected plants was described by Dale in 1988 (1). Midrib sections 2-3 mm in length were excised from infected

plants, placed on a clean glass slide, and crushed with a spatula. The readily distinguishable vascular tissue was removed and placed in Karnovsky's fixative (3)in a beveled-edge watch glass. The tissue was fixed for 20 min, rinsed with 0.1M potassium phosphate buffer pH 7.2 (5 min), then placed in DAPI (Polysciences, Inc., Warrington, PA 18976) at a concentration of 1 ug/ml in 0.1M phosphate buffer, pH 7.0, for 3-5 min. The tissue was transferred onto a glass slide in a few drops of DAPI, crushed with a spatula and covered with a coverslip. The tissue was examined with an Olympus fluorescence mircroscope. Healthy plant material was treated in a similar fashion.

Results and Discussion.

Extracellular DNA was observed in the phloem region of the infected plants but not in the healthy tissues. This indicated the presence of MLO in infected plants. The total time from the fixation to observation of the specimen under the microscope was 40 minutes. Very few reagents were used. MLO could be detected in asters by DAPI staining as early as 10 days after inoculation. In our experiments Dienes' stain (2), a MLO-specific stain, was more useful than DAPI staining, since the former procedure took only 2-5 minutes, while the latter took 25-30 min.

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APPENDIX D

COMPARISON OF MYCOPLASMA-LIKE ORGANISMS ISOLATES BASED ON SYMPTOMATOLOGY IN ASTER, CELERY AND PERIWINKLE

Table 8. Mycoplasma-like organism isolate comparison.

Isolate	Host plant	Symptoms
AYOC 1	Aster	Vein clearing in immature leaves followed by vein clearing in other developing leaves. Chlorosis of the leaves and reduced leaf size. Severe stunting in early infected asters. Flower phyllody, virescence, and proliferation. Necrosis of the axillary shoots followed by the eventual death of the plant.
	Celery	Yellowing, vein clearing of the leaves and stunting of the plant. Symptoms appeared 34 days after inoculation.
	Periwinkle	Yellowing, leaf size reduction, proliferation, phyllody and virescence. Elongation of internodes, twisting and intertwining of the stems, elongation of flower stalks, reduction in flower size and flower necrosis.
	Carrot	Yellowing, fern leaf symptoms overall stunting, etiolated and bushy appearence. leaves

Isolate	Host plant	Symptoms
		sometimes turning dark red in color. Infected carrots tasted bitter.
AYOC 2	Aster	Mild vein clearing, slight yellowing (less than with AYOC 1), elongation of petioles, proliferation, virescence and phyllody. Less stunting of early-infected plants than with AYOC1.
	Celery	Vein clearing, yellowing and stunting. Symptoms appeared 45 days after inoculation.
	Periwinkle	Slight reduction in leaf and flower size, yellowing (but less pronounced than with AYOC 1).
	Carrot	Same as AYOC 1 infected carrot.
AYOL	Aster	Same as with AYOC 1 but with etiolation and elongation of petioles in early-infected asters. The petioles assumed upright position not seen with AYOC 1. Phyllody, virescence, stunting of the plant and eventual death of the plant.
	Celery	Yellowing, vein clearing, stunting. Symptoms were observed 45 days after inoculation.
	Periwinkle	Reduced leaf size (sometimes more pronounced than with carrot isolates). Phyllody, virescence and proliferation in flowers.

Isolate	Host plant	symptoms
	Lettuce	Yellowing, leaf size reduction, Lack of head formation in early-infected lettuce. Tapered, yellow leaves, severe stunting and early death of plants.
AYOF	Aster	No vein clearing nor chlorosis on leaves, but leaves tapered,and narrow. Elongation of internodes and virescence of the floral structures. No proliferation.
	Celery	No vein clearing but moderate yellowing. Petioles in upright position. Slight stunting of the plant. Symptoms appeared 45 days after inoculation.
	Periwinkle	Less yellowing and stunting than with other MLO isolates. No virescence nor phyllody. Flowers slightly smaller than normal flowers.
	Daisy fleabane	Yellowing, chlorosis, severe stunting, no flowers observed.

APPENDIX E

TESTING OF ASTER YELLOWS DNA PROBE ON OKLAHOMA OKLAHOMA ASTER YELLOWS-INFECTED PLANTS AND INSECT VECTORS

The Oklahoma aster yellows infected aster and celery and AY MLO-carrying aster leafhoppers hybridized with the AY MLO DNA probe developed by I. M. Lee and R. E. Davis, suggesting high homology between OK AY and the AY MLO used to make the probe. These hybridization experiments were performed by I. M. Lee at Beltsville, MD and the results are presented in Fig. 12.

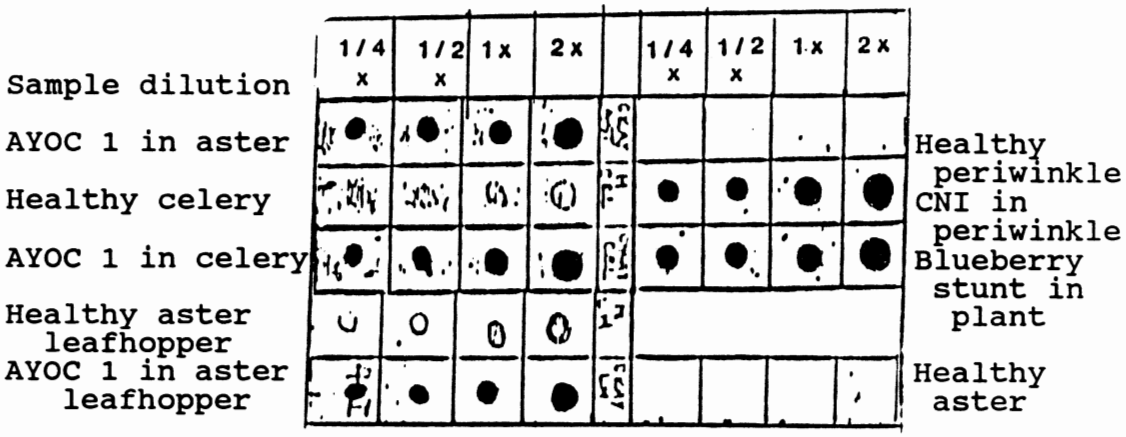

Fig. 12 Autoradiogram of Southern blot hybridization using a probe made with DNA of AY MLO against Oklahoma AY-infected aster and celery, and AY MLO-carrying aster leafhoppers, and healthy plant and insect controls. The AY MLO DNA restriction fragments hybridized with AY MLO in plant and insect preparations, but not with healthy plant and insect controls.

In another hybridization experiment, done by I. M. Lee *et al.*, among four Oklahoma AY isolates (AYOC 1, AYOC 2, AYOL, and AYOF), only AYOC 1 showed high homology to their AY MLO DNA probe. AYOL showed homology to tomato big bud MLO but not to their AY MLO. However, in our experiments, both AYOC 1 and AYOL gave positive reactions in the serological tests using OK AY MLO antiserum, suggesting that both isolates were the same or closely related. The discrepancies in results of the two tests suggest the possibility that the particular restriction fragments used for the AY probe may not be present or expressed in AYOL. Care should taken in interpreting results from these tests.

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