This is to certify that the

thesis entitled

LEAFHOPPER SAMPLING IN MICHIGAN PEACH ORCHARDS
AND SEROLOGICAL DETECTION OF A SPIROPLASMA ASSOCIATED
WITH X-DISEASE IN PLANT AND INSECT TISSUE

presented by

THOMAS MINSTER MOWRY

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Entomology

Mark E. Whalon
Major professor

Date May 18, 1982

LEAFHOPPER SAMPLING IN MICHIGAN PEACH ORCHARDS AND SEROLOGICAL DETECTION OF A SPIROPLASMA ASSOCIATED WITH X-DISEASE IN PLANT AND INSECT TISSUE

by

Thomas Minster Mowry

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Entomology

1982

ABSTRACT

LEAFHOPPER SAMPLING IN MICHIGAN PEACH ORCHARDS AND SEROLOGICAL DETECTION OF A SPIROPLASMA ASSOCIATED WITH X-DISEASE IN PLANT AND INSECT TISSUE

by

Thomas Minster Mowry

Leafhopper populations were sampled twice weekly throughout the 1980 growing season in two Michigan peach orchards. Spatial analyses of leafhoppers and X-diseased trees indicate a low level spread of the disease within the orchard possibly attributable to indigenous leafhopper populations. Serological testing of plant and insect tissue with the enzyme-linked immunosorbent assay (ELISA) produced weak, positive reactions only to peach and milkweed plant homogenates. Testing of pure spiroplasma cultures and infected celery tissue from California indicate the possible presence of Spiroplasma citri in Michigan and the need for further work in isolating the X-disease pathogen.

DEDICATION

To Dr. C. Dennis Hynes,

who must bear at least some responsibility for this madness.

ACKNOWLEDGEMENTS

I would like to thank Dr. Mark E. Whalon, my Graduate Advisor and friend, for his constant encouragement, allowing me to have a free hand and work independently, and his continual support and patience in the completion of this project. In addition, I thank my Graduate Committee, Drs. Frederick W. Stehr, Stuart H. Gage and Alan L. Jones, for their input into this research and for allowing me to pursue "what was right in my own eyes."

I would also like to thank Mrs. Mary Scripture Andrews and Mr. Robert "Scott" McCall for their excellent technical assistance in this research.

Finally, I would like to thank my wife Mary and daughters Teresa and Trista for their love and patience while I pursue yet another dream. No amount of gratitude will reflect their absolute necessity in all my endeavors.

TABLE OF CONTENTS

TABLE OF CONTENTS, continued

TABLE OF CONTENTS, continued

LIST OF TABLES

LIST OF TABLES, continued

LIST OF FIGURES

LIST OF FIGURES, continued

LIST OF FIGURES, continued

INTRODUCTION

The peach and cherry growers of the United States reap approximately $420 million annually (U.S. Bureau of the Census 1978) with about 10% of this figure going to Michigan growers (Mich. Stat. Abstr.). While no official figures are available regarding the monetary loss due to X-disease, Rosenberger (1977) and Rosenberger and Jones (1977a) showed that extensive tree losses are occurring in Michigan peach orchards, with some having more than 50% diseased trees. In some cases, entire orchards have been removed, resulting in severe losses. Cherry orchards have shown fewer diseased trees, but this may be due to the difficulty in visually identifying X-disease in cherry. In any case, X-disease is known to have severe effects on cherry, especially those trees on mahaleb rootstock (Jones and Rosenberger 1977).

The eradication of chokecherry, Prunus virginiana L., has been the major means of attempting to control the spread of X-disease in Michigan peach orchards (Jones and Rosenberger 1977). However, in the West, X-disease has been shown to spread from peach-to-peach within the orchard with the presence of X-diseased chokecherry not being necessary (Gilmer and Blodgett 1976). Rosenberger and Jones (1977a) indicate that eradication of chokecherry within 150 meters of peach orchards in Michigan had little effect on the infection rate within those orchards. These authors postulated that the X-disease pathogen may be carried into orchards from more distant chokecherry inoculum sources or transmitted from diseased to healthy peach trees within the orchard. More recently, some Michigan peach growers have questioned the eradication of

chokecherry as a means of controlling the spread of X-disease. A report by the Michigan Department of Agriculture on the Chokecherry Eradication Pilot Program in Berrien County, conducted from the fall of 1978 to the spring of 1980, showed a decline in the percent of X-diseased peach trees in the surveyed orchards from 1.54 to 1.09. This apparently small decline coincided with the removal of 93.4% of the chokecherry trees within 150 meters of the surveyed orchards and a total of 218,156 removed in the entire survey area. It is not clear if the 0.45% decrease in X-diseased peach trees is significant or if it can be attributed to the removal of chokecherry, especially since no concurrent control study was carried out.

While a number of leafhoppers have been shown to transmit the X-disease pathogen in the laboratory and greenhouse, Rosenberger and Jones (1978) have shown that there is no apparent relationship between fluctuation in the size of vector populations and transmission of the X-disease pathogen to indicator plants in the field. This calls into question the advisability of insecticide sprays (standard foliar tree applications) aimed at these leafhoppers for the control of X-disease. Those leafhoppers assumed to be the major vectors of X-disease in Michigan, e.g., Paraphlepsius irroratus (Say) and Scaphytopius acutus (Say) (Taboada et al. 1975, Rosenberger and Jones 1978), use grasses and some herbaceous weeds as primary feeding and oviposition hosts (Beirne 1956, McClure 1980a, Palmiter et al. 1960) and would not normally move into peach trees, thereby avoiding insecticide sprays directed at the trees within an orchard. Furthermore, since the fluctuation in size of these particular leafhopper populations may not be related to the transmission of the disease pathogen in the field, the possibility exists for other leafhopper species to be important in transmission in the field.

Given these considerations regarding chokecherry as an inoculum source and the laboratory-demonstrated leafhopper vectors of the X-disease pathogen, it appeared necessary to attempt to identify possible alternative plants as pathogen hosts and other leafhoppers as pathogen vectors in the field, as well as to compare the leafhopper fauna and their distributions in chemically treated and untreated peach orchards. Therefore, research was initiated to accomplish the following objectives: (1) develop the enzyme-linked immunosorbent assay (ELISA) for detection of the suspected X-disease pathogen, (2) use ELISA to screen field-collected plants and leafhoppers as possible hosts and vectors of the X-disease pathogen, (3) compare the population sizes and distributions of the various leafhopper species in chemically treated and untreated peach orchards and, (4) compare the leafhopper species distributions with the distribution of X-disease in peach orchards. It was felt that information gathered would aid in making recommendations which might check the spread of X-disease and in the design of future research for illumination of the vector-pathogen-plant associations.

LITERATURE REVIEW

X-disease

X-disease was first reported from California in 1931 where it was described as the bucksin disease of cherry (Rawlins and Horne 1931). Ten years later, Rawlins and Thomas (1941) redescribed the disease and considered it the same as X-disease. In the East, Stoddard (1934) reported observing the disease in Connecticut peach orchards in 1933. Since these early reports, X-disease has been reported in Arizona, Colorado, Idaho, Illinois, Indiana, Michigan, New Jersey, New York, North Carolina, North Dakota, Ohio, Oregon, Pennsylvania, Tennessee, Utah, Washington, Wisconsin, British Columbia, New Brunswick and Ontario (Gilmer and Blodgett 1976).

Cation (1941) first reported observing X-disease in Michigan in 1939. It was considered of minor importance until the late 1960's when its incidence began to increase in peach orchards. It is now considered the major peach disease problem in southwest Michigan (Thomas et al. 1981).

The symptoms of X-disease vary considerably over the wide geographic distribution of the disease (Gilmer and Blodgett 1976). This has resulted in the disease being described under many names, including cherry buckskin (Rawlins and Horne 1931), little cherry (Richards et al. 1948), peach leaf casting yellows (Thomas et al. 1940), peach yellow leaf roll (Schlocker and Nyland 1951), red leaf (Richards 1945), small bitter cherry (Lott 1947), western X-disease (Reeves and Hutchins 1941), western X little cherry (Richards et al. 1949), western X red leaf (Richards 1945), wilt and decline (Richards et al. 1946), and yellow-red virosis (Hildebrand and Palmiter 1938, Palmiter and Hildebrand 1943). It was Stoddard (1934, 1938) who suggested the name X-disease which has, at this time,

apparently replaced all other terminology. His reasoning for naming the disease was as follows:

> The name "X-disease" was suggested because in mathematics the character "X" stands for an unknown quantity. At the beginning of our investigations X represented the disease very aptly and even now there is sufficient mystery in some of its manifestations to warrant the name (Stoddard 1938).

At the present time, it would appear that the name coined by Stoddard is still very apt indeed.

X-disease has been reported in areas that emcompass almost the entire continental United States and southern Canada (Gilmer and Blodgett 1976). Whitcomb and Williamson (1979) have mapped the distributions of eastern and western X-disease along with the distributions of eastern and Great Plains chokecherry, Prunus virginiana L. and P. virginiana var. melanocarpa, respectively. There is a high geographical correspondence in these distributions. The role of chokecherry as the primary source of inoculum for X-disease is not, however, as simple as these distributions indicate. In the East, chokecherry appears to be of major importance in the spread of X-disease, with eradication of this wild host within 500 feet of orchards resulting in significant reduction in disease spread (Parker et al. 1933, Lukens et al. 1971). In the West, the presence of chokecherry is not necessary for major spread of X-disease (Gilmer and Blodgett 1976) where peach-to-peach (Jensen 1957), cherry-to-cherry and cherry-to-peach (Nielson and Jones 1954) transmissions have been demonstrated. Recently, the proximity of pear orchards to peach orchards where X-disease is spreading in California has indicated the possibility that pear may be a source of inoculum (Purcell et al. 1981).

The role of chokecherry in the spread of X-disease in Michigan is not as

well defined as in the East or the West. Rosenberger and Jones (1977a) could find no apparent correlation between eradication of chokecherry near peach orchards and the spread of the disease within those orchards. It is possible that there is a gradient in the importance of chokecherry as an inoculum source when viewing the spread of X-disease from east to west.

The important economic hosts of X-disease are peach, Prunus persica L. Batsch; nectarine, P. persica var. nectarina (Ait.) Maxim.; Japanese plum, P. salicina Lindl.; tart cherry, P. cerasus L.; and sweet cherry, P. avium L.. Other cultivated hosts include almond, P. dulcis (Mill.); apricot, P. armeniaca L.; mahaleb cherry, P. mahaleb L.; Korean cherry, P. ssiori F. Schmidt; western sand cherry, P. besseyi Bailey; bitter cherry, P. emarginata (Hook.) Walp.; hollyleaf cherry; P. ilicifolia (Nutt.) Walp.; Manchu cherry, P. tomentosa Thunb.; and wildgoose plum, P. munsoniana Wight and Hedr. (Gilmer and Blodgett 1976).

The important wild hosts of X-disease are common, or eastern, chokecherry, P. virginiana L.; and western chokecherry, P. virginiana var. demissa (Gilmer and Blodgett 1976). Other wild hosts that harbor the disease without apparent symptoms include flowering cherry, P. japonica Thunb.; pin cherry, P. pensylvanica L.; American plum, P. americana Marsh.; damson plum, P. insititia L.; and European plum, P. domestica L. (Gilmer et al. 1954, Gilmer and Blodgett 1976).

Kunkel (1944) was the first to demonstrate herbaceous hosts of the X-disease pathogen. By means of dodder, Cuscuta campestris Yuncker, he transmitted the pathogen to carrot, Daucus carota L.; parsley, Petroselinum crispam (Mill.) Num.; periwinkle, Vinca rosea L.; and tomato, Fragaria X ananassa Duch. Jensen (1955) infected celery, Apium graveolens L., by

leafhopper transmission of the X-disease pathogen. Other herbaceous plants shown to be hosts by experimental transmission include chrysanthemum, Chrysanthemum carinatum L.; China aster, Callistephus chinensis Nees; radish, Raphanus sativus L.; cauliflower, Brassica oleracea var. botrytis L.; turnip, Brassica rapa L.; filaree, Erodium moschatum L'Her.; strawberry, Fragaria vesca L.; and coriander, Coriandrum sativum L. (Jensen 1971). Of the herbaceous hosts of the X-disease pathogen, celery has proven to be the most widely used in experimental studies involving the disease (Jensen 1955, 1956, 1957a, 1957b, 1969, Purcell 1979, Whitcomb et al. 1966a). Milkweed, Asclepias syriaca L., has been shown to be a naturally infected host of the X-disease pathogen (Gilmer 1960).

The symptoms of X-disease in peach vary somewhat from east to west. In the East, foliar symptoms include large, chlorotic, watersoaked spots that appear after approximately six weeks of growth in the spring. These spots later turn red and separate from the leaf, resulting in a tattered appearance, and the leaves curl under, longitudinally. Eventually, the leaves drop, leaving diseased branches with a rosetted tuft of young leaves at their ends. Fruits from diseased branches usually abort and drop early. If they persist, they are more pointed than normal, contain non-viable seeds and have a bitter flavor (Gilmer and Blodgett 1976, Jones and Rosenberger 1977, Rosenberger 1977).

In the West, the symptoms are similar to those in the East except that the leaf tatters before any red or yellow chlorosis occurs. Later in the season, newly developed watersoaked spots become necrotic and may not separate from the leaf. In both localities, trees survive from two to four years, but rarely more than three years following infection (Gilmer and Blodgett 1976).

Foliar symptoms in commercial cherry are not as apparent as in peach. A general, mild chlorosis followed by defoliation has been observed, but has not definitely been attributed to X-disease. Disease symptoms are usually first seen in the fruit, which are small, pointed and pale red to greenish white in color. Trees on mazzard rootstock generally survive for several years while those on maheleb rootstock frequently decline rapidly in midsummer and die in the year of infection (Gilmer and Blodgett 1976, Jones and Rosenberger 1977).

X-disease infected chokecherry produces near normal growth for six to eight weeks in the spring, after which they turn bright orange or red. Some defoliation occurs and the fruit, if any is produced, are pointed, remain pale red and fail to mature. Infected chokecherry will survive three to four years with an increasing number of branches dying during this time. Toward the end, any leaves produced are usually smaller than normal and no fruit is evident (Gilmer and Blodgett 1976, Jones and Rosenberger 1977, Rosenberger 1977).

The etiology of X-disease was considered viral until the 1970's (Hildebrand and Palmiter 1938, Gilmer 1960, Gilmer et al. 1954, Jensen 1955, 1969, Kunkel 1944, Nyland 1955, Whitcomb et al. 1966a, 1966b, 1967, 1968). Holmes (1941) went so far as to name the supposed virus Marmar lacerans even though no viral characterization had been done. The work of Doi et al. (1967) and Ishiie et al. (1967) in the discovery of mycoplasmas associated with several plant diseases generated much activity around diseases of unknown etiology, especially those of the yellows type (Whitcomb 1980). Although Whitcomb et al. (1968b) suspected a mycoplasma etiology, it wasn't until 1970 that mycoplasma-like organisms (MLO's) were observed with the electron microscope in association with insect and plant hosts of X-disease (Huang and Nyland 1970, Nasu et al. 1970). Jones et

al. (1974) showed this same association with X-diseased peach trees in Michigan.

At this time, there is some controversy surrounding the prokaryotic etiology of X-disease (Whitcomb 1981). Some reseachers have isolated spiroplasmas from infected plant tissue (Kloepper and Garrott 1980, Thomson et al. 1978). However, all electron microscopy of X-disease infected plant and insect tissue have revealed the presence of MLO's only, even from tissue from which spiroplasmas were isolated (Granett and Gilmer 1971, Huang and Nyland 1970, Jones et al. 1970). The possibility exists for a dual infection of a cultivatible spiroplasma and a non-cultivatible MLO in diseased tissue (Whitcomb 1981), but if this is the case, the pathogenic role of each organism is not yet understood. Nasu et al. (1974a, 1974b) isolated an MLO from infected celery and leafhopper tissue with no spiroplasmas being cultured or observed by electron microscopy. These are the only reports of subsequent pathogenicity of a microorganism isolated from X-disease tissue. The evidence seems to point toward MLO etiology with spiroplasmas being isolated from dual infection situations or contamination. However, the repeated association of spiroplasmas with X-disease through pathogen isolation attempts has rendered the etiological outcome of this controversy as yet undetermined.

Leafhopper Vectors of X-disease

The first insect shown capable of transmitting the X-disease pathogen was the geminate leafhopper, Colladonus geminatus (Van D.) (Wolfe et al. 1950). It was able to transmit the pathogen from diseased peach, cherry and chokecherry to healthy peach in both greenhouse and field experiments (Kaloostian 1951a, 1951b, Wolfe et al. 1951). Since these first reports, C. geminatus has been used

frequently in leafhopper-plant-pathogen interaction studies (Jensen 1953a, 1956, 1969, Jensen and Thomas 1954, Jensen et al. 1952, Nielson and Jones 1954, Wolfe and Anthon 1953, Wolfe et al. 1951). Fourteen additional leafhopper species have also been shown capable of transmitting the X-disease pathogen, including Acinopterus angulatus Lawson (Purcell 1979); Colladonus clitellarius (Say) (Gilmer 1954, Gilmer et al. 1966); C. montanus (Van D.) (Jensen 1957b, 1969, Whitcomb et al. 1966a, Wolfe 1955a); Euscelidius variegatus (Kirsch.) (Jensen 1969); Fieberiella florii (Stal) (Anthon and Wolfe 1951, Gilmer et al. 1966, Jensen 1957a, Wolfe 1955b); Gyponana lamina DeL. (Gilmer et al. 1966); Keonolla confluens (Uhl.) (Anthon and Wolfe 1951); Orientus ishidae (Mat.) (Rosenberger and Jones 1978); Osbornellus borealis DeL. & M. (Jensen 1957a); Paraphlepsius irroratus (Say) (Gilmer et al. 1966, Rosenberger and Jones 1978); Norvellina seminuda (Say) (Gilmer et al. 1966); Scaphoideus spp. (probably diutius DeL. & M., melanotus Osb. and/or carinatus Osb.) (Rosenberger and Jones 1978); Scaphytopius acutus (Say) (Gilmer et al. 1966, Rosenberger and Jones 1978, Wolfe and Anthon 1953); and S. nitridus (DeL.) (Purcell 1979). Of these fifteen known vector species, Keonolla confluens (Uhl.) belongs to the subfamily Tettigellinae, Gyponana lamina DeL. to the subfamily Gyponinae, and the remaining thirteen to the subfamily Deltocephalinae (Beirne 1956, Oman 1951).

Particular leafhopper species appear to be of regional importance in the transmission of the X-disease pathogen. Colladonus geminatus (Van D.) is considered the most important vector in the West (Gilmer and Blodgett 1976, Kaloostian 1951b, Wolfe et al. 1950), Scaphytopius acutus (Say) in the East (Gilmer and Blodgett 1976, Gilmer et al. 1966, Palmiter et al. 1960) and Paraphlepsius irroratus (Say) in Michigan (Rosenberger and Jones 1978, Taboada

et al. 1975). These determinations are based primarily upon the transmission efficiency of the leafhopper and its ability to survive on Prunus hosts. For example, while Colladonus montanus is numerically dominant to C. geminatus in some peach-growing areas of California, it survives poorly on Prunus hosts, making transmission of the X-disease pathogen to these plants rather difficult (Jensen 1953b, 1957b). P. irroratus does not survive well on Prunus hosts, but demonstrated a higher transmission efficiency than did S. acutus in experiments in Michigan (Rosenberger and Jones 1978). The seven leafhopper species that have been shown to vector the X-disease pathogen and are known to occur in Michigan are pictured in Figure 1.

Investigations into leafhopper-pathogen interactions are of limited value at the present time. The work of Whitcomb et al. (1966a, 1967, 1968a, 1968b) has been the most definitive to date and showed histopathological effects thought to have been due to the X-disease pathogen in the hemolymph and alimentary tract as well as salivary, neural, adipose, circulatory and connective tissues. These studies were approached from the virological point of view and some of the findings normally related to viral activity, e.g., crystal formation in the alimentary tract, may not be related to the presence of MLO's. Lee and Jensen (1963) also found these crystals in infected leafhoppers and one hypothesis they ventured to explain their presence was the possible modification of plant constituents in those plants that were diseased and upon which the insects fed. This is partially supported by their finding that the crystals tended to disappear when the leafhoppers were returned to healthy plants to feed. The crystals, then, were possibly a result of feeding on diseased plants rather than due to the pathogenic effects of the X-disease pathogen. In light of the possible

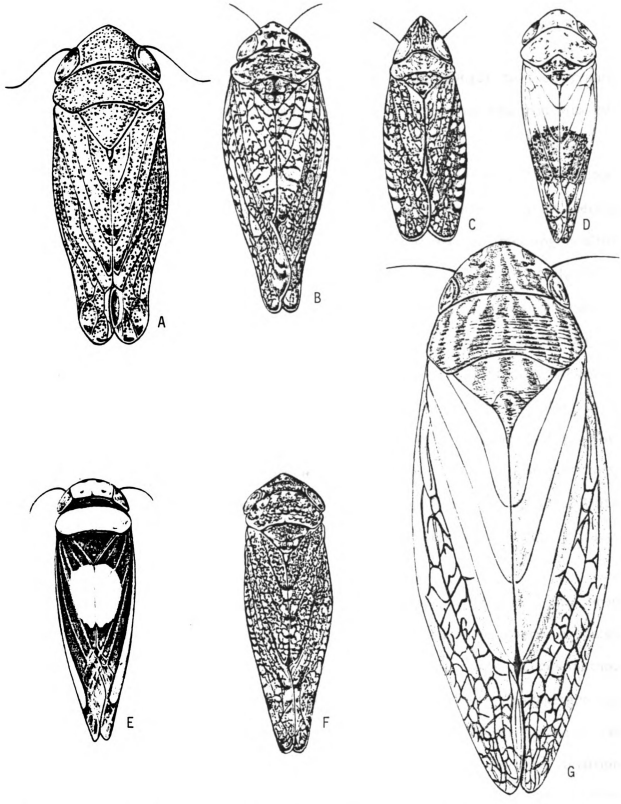


Figure 1.--Leafhoppers which are known to vector the X-disease pathogen and found in Michigan. (A) Fieberiella florii (Stal), (B) Orientus ishidae (Mat.), (C) Scaphytopius acutus (Say), (D) Norvellina seminuda (Say), (E) Colladonus clitellarius (Say), (F) Paraphlepsius irroratus (Say), (G) Gyponana lamina DeL.

mycoplasma etiology of X-disease, Whitcomb et al. (1968b), to their credit, called for a re-examination of their findings and for further research into this area.

The X-disease pathogen has been shown to be lethal to the vector, Colladonus montanus (Van D.). This has been demonstrated both by feeding leafhoppers on infected plants (Jensen 1958, 1959) and by injecting healthy leafhoppers with infectious extracts from diseased insects (Jensen et al. 1967). In addition, Jensen (1971) showed that the fecundity of C. montanus was reduced when infected with the X-disease pathogen either by injection or feeding on diseased plants. Also using C. montanus, Nasu et al. (1970) observed pleomorphic MLO's in the cytoplasm of the brain and salivary gland and one MLO form showed some similarity to spiroplasma plant pathogens. It appears, then, that the X-disease pathogen is both circulative and propagative in its insect vectors, with an incubation period ranging from 20 to 50 days (Gilmer et al. 1966, Wolfe and Anthon 1953) and as short as 11 days when injected with infectious extracts (Whitcomb et al. 1966a).

The control of X-disease, at least in the East and Midwest, has been primarily through eradication of chokecherry near peach and cherry orchards (Lukens et al. 1971, Jones and Rosenberger 1977). The results of this method have been questionable, if not disappointing (Lacy et al. 1979, Rosenberger and Jones 1977a, 1978). The injection of trees with tetracycline antibiotics has shown promise in restoring diseased trees to a temporary, productive condition (Nyland 1971, Rosenberger and Jones 1977b), but this is treatment after the fact and in Connecticut it has not been particularly effective (Lacy et al. 1979). Control of the leafhopper vectors has been considered an essential, but not much

practiced, means of slowing the spread of X-disease (Lacy et al. 1979, Rosenberger and Jones 1977a).

The role of leafhopper host plants in orchard ground cover has been shown to be an important factor in invasion of the orchard by X-disease pathogen vectors (McClure 1980a, 1980b). Manipulation of this ground cover to eliminate suitable host plants for leafhopper vectors is seen as a possible means of controlling the spread of X-disease by reducing within-orchard vector populations (Lacy et al. 1979, McClure 1980a, 1980b, Rosenberger and Jones 1977a, 1978). Purcell and Elkinton (1980), however, found that orchard ground cover in California cherry orchards had little effect on trap catches of leafhopper vectors in the tree canopy. This may prove to be a significant finding in other areas where leafhopper vectors are readily able to complete their life cycle on Prunus hosts, e.g., Scaphytopius acutus on peach (McClure 1980a, Palmiter et al. 1960).

Chemical control of the leafhopper vectors of X-disease has not been successful (Lacy et al. 1979, Rosenberger and Jones 1977a). This may be due to the low residual activity of presently used organophosphate insecticides which would allow for reinvasion of the orchard by leafhoppers within a relatively short time after spraying. The fact that the vectors may gain protection in the perennial ground cover of the orchard may also influence poor chemical control (Rosenberger and Jones 1977a). The late season transmission of the X-disease pathogen demonstrated by Rosenberger and Jones (1978) may be an important factor because this transmission occurs after the seasonal insecticide spraying has stopped in most orchards.

At present it appears that the control of X-disease in any definitive sense

may only be accomplished by an integrated approach involving chokecherry eradication, rogueing of diseased trees, orchard cultural practices and chemical insecticides. An integrated approach will have to be regionally modified in order to account for the varying importance of the factors involved.

Enzyme-linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) originated in the medical sciences in the early 1970's (Engvall and Perlmann 1971, 1972, Engvall et al. 1971, Van Weemen and Schuurs 1971). These early techniques employed polystyrene tubes coated with 1 ml of antibody solution which is quite a large amount of this reagent, especially when larger numbers of samples must be processed. Voller et al. (1974) described a microplate method of ELISA that allowed for more efficient, rapid and economical assays to be carried out which is now the standard method used in ELISA procedures. There are several types of assays employed in ELISA depending on the purpose of the assay. These include the competitive, double antibody sandwich, modified double antibody sandwich, and inhibition methods for detection and measurement of antigen, the indirect method for detection and measurement of antibodies, and the solid phase anti-IgM method for the detection and measurement of immunoglobulin-M antibodies (Voller et al. 1979).

The first application of ELISA in agriculture was the double antibody sandwich method for the detection of arabis mosaic and plum pox viruses (Voller et al. 1976). This was followed by more detailed studies involving the same method (double sandwich) of ELISA and the same viruses (Clark et al. 1976a, 1976b). While these first works detailed the double antibody sandwich method of

ELISA for plant viruses, the definitive work, and the one upon which almost all subsequent ELISA procedures in phytopathology and entomology have been based, was that of Clark and Adams (1976, 1977), who spelled out the technique and investigated various experimental procedures and their effect on the results of the assay. Since this work, ELISA has been used extensively in phytopathology and occasionally in entomology for the detection and quantification of viruses, bacteria, fungi and spiroplasmas (Mowry et al. 1981).

Spiroplasma citri was the first mycoplasma plant pathogen to be detected by ELISA. It was detected in plant tissues (Bove et al. 1979a, 1979b, Clark et al. 1978, Saillard 1978) and in leafhopper vectors (Bove et al. 1979a). This was followed by the detection of the corn stunt spiroplasma in both plant and insect tissue (Raju and Nyland 1981). To date, these are the only plant pathogenic mycoplasmas for which ELISA has been developed.

In addition to the entomological applications of ELISA with the above mentioned spiroplasmas, the double antibody sandwich method has been used to detect potato leafroll virus (Clarke et al. 1980) and cucumber mosaic virus (Gera et al. 1978) in viruliferous aphids, small iridescent viruses in Galleria mellonella L. larvae (Kelly et al. 1978a) and nuclear polyhedrosis virus in Heliothis armigera (Hub.) larvae (Kelly et al. 1978b). An indirect sandwich ELISA was used to detect baculovirus in both larvae and adults of the rhinoceros beetle, Oryctes rhinoceros L. (Longworth and Carey 1980). ELISA has also been used in the quantification of predation by the southern green stink bug, Nezara viridula L. (Ragsdale 1980). We find, then, a broad range of applications of ELISA in both phytopathology and entomology.

METHODS AND MATERIALS

Enzyme-linked Immunosorbent Assay (ELISA)

Antiserum Generation

A spiroplasma in pure culture, isolated from X-diseased celery tissue infected by leafhopper transmission from X-diseased chokecherry and supplied by Dr. Alan L. Jones, Department of Botany and Plant Pathology, Michigan State University, was used as antigen. It was the 5th clone in the 4th passage and designated as Ex-CL5-P4. In order to produce 10 ml of antigen suitable for injection, 1000 ml of spiroplasma culture was prepared. One ml of Ex-CL5-P4 was aseptically inoculated into each of 10, 100 ml portions of a modification of the C-G3 culture medium of Liao and Chen (1977) (Appendix A). This culture was incubated at 32°C until the phenol red indictor had turned yellow, approximately 2-4 days, indicating probable log phase growth of the spiroplasmas.

The entire spiroplasma culture was harvested by placing 40 ml of culture into each of 25 sterile, 50 ml polypropylene centrifuge tubes and centrifuging at 14,350 x g (10,600 rpm) for 20 minutes in a Sorvall SS-1 Superspeed centrifuge (Sorvall, Inc., Newtown, Connecticut). The supernatant was discarded and the pellet was gently washed four times with 0.5 ml phosphate buffered saline (PBS) (Appendix A), pH 7.4. Care was taken not to dislodge or resuspend the pellet during the washing procedure. After washing, 0.5 ml PBS was used to resuspend the pellets in two tubes by adding the buffer to the first tube, resuspending the pellet, then transferring this to the second tube and resuspending its pellet. This suspension, containing the pellets from two tubes, was added to a sterile, glass

injection vial. This was done for the first 24 tubes, with the pellet of the 25th tube being resuspended in 0.5 ml PBS and added directly to the injection vial, producing a volume of 6.5 ml antigen-PBS suspension. Groups of five tubes were then rinsed with 0.5 ml PBS/group, with each rinse being added to the injection vial, producing a volume of approximately 9 ml. Finally, 1 ml PBS was used to rinse all 25 tubes and this was added to the injection vial for a final volume of 10 ml. The antigen-PBS suspension was then frozen and thawed 15 times by alternating between ethanol + dry ice and warm water in order to thoroughly disrupt the spiroplasma cells. Subsequent antigen preparations were produced in the same manner, except that the spiroplasma culture was grown-up by inoculating 10 ml of Ex-CL5 into 500 ml of medium.

Two adult New Zealand White rabbits were used to produce antiserum. Prior to any injections, both rabbits were bled for non-specific serum. Four intravenous injections of antigen alone, in increasing doses, were administered into the marginal vein of the left ear. The ear was carefully shaved in the injection area to allow for easy access to the vein and facilitate future bleeding. The area around the central artery and the marginal vein, but not the shaved area, was bathed with xylene to dilate the blood vessels. Three intramuscular injections of antigen + Freund's incomplete adjuvant Grand Island Biological Co. (GIBCO), Grand Island, New York followed by three more intramuscular injections of antigen + Freund's complete adjuvant (GIBCO), were administered into alternating hip muscles, with one being administered inside the right thigh. Incomplete adjuvant contains a mineral oil and an emulsifier that allows "slow release" of the antigen, prolonging the immune response. In addition, complete adjuvant contains heat-killed bacteria (Mycobacterium sp.) which stimulates the

immune system to increased antibody production (Freund and McDermott 1942). The antigen-adjuvant emulsion was prepared by placing equal amounts of each into a sterile, 25 ml crucible and drawing this mixture into and ejecting it out of a 5 ml plastic, disposable syringe (Becton, Dickinson and Co., Rutherford, New Jersey) until it was almost too thick to dispense. The injection and bleeding schedules are detailed in Tables 1 and 2.

Both rabbits were ear-bled by making an angular incision into the marginal ear vein with a sterile razor blade. Before each bleeding, the ear was reshaved to prevent hair from obstructing blood flow and causing coagulation. Xylene was again used to dilate blood vessels and enhance blood flow. The incision was made deep enough for good blood flow, as shallow incisions resulted in rapid coagulation necessitating further incisions which unnecessarily traumatized the rabbit. Approximately 50 to 100 ml of blood was taken at each bleeding, before coagulation stopped blood flow, that produced 14 to 30 ml of serum (Tables 1 and 2). Large sterile test tubes were held beneath the bleeding ear to collect blood. After blood flow had stopped, the ear was disinfected with alcohol and the rabbit recaged. The collected blood was left to stand at room temperature for one hour, allowing the red blood cells to separate from the serum and form a large clot. The clot was gently dislodged from the tube wall and the tubes were refrigerated overnight at 6^{o}C. The serum was then decanted off and centrifuged at 3200 x g (5000 rpm) for 10 minutes to remove the remaining red blood cells and other large impurities. It was then filter sterilized by drawing it into a 50 ml plastic, disposable syringe, attaching a 25 mm Swinney-type filter holder containing a 0.22 µm Millipore filter (both from Millipore Filter Corp., Bedford, Massachusetts) previously autoclaved at 120^{o}C for 20 minutes and forcing the

Table 1.--Rabbit No. 109 injection and bleeding schedule, 1979-1980.

Date	Antigen(ml)	Adjuvant (ml) Incomp	Comp	Total/Type	Bled	Serum(ml)
09/24	-	-	-	-	NS[a]	-
09/28	0.2	-	-	0.2/IV	-	-
10/05	0.4	-	-	0.4/IV	-	-
10/12	1.0	-	-	1.0/IV	-	-
10/19	2.0	-	-	2.0/IV	-	-
10/26	1.0	1.0	-	2.0/IM	-	-
10/31	0.8	0.8	-	1.6/IM	-	-
11/05	2.0	2.0	-	4.0/IM	-	-
11/13	-	-	-	-	S[b]	22
11/19	-	-	-	-	S[b]	23
12/28	1.5	-	1.5	3.0/IM	-	-
12/31	1.5	-	1.5	3.0/IM	-	-
01/02	2.5	-	2.5	5.0/IM	-	-
01/12	-	-	-	-	S[b]	18
01/19	-	-	-	-	S[b]	14
01/31	-	-	-	-	S[b]	21

[a]Non-specific serum bleeding.

[b]Specific serum bleeding.

Table 2.--Rabbit No. 110 injection and bleeding schedule, 1979-1980.

Date	Antigen(ml)	Adjuvant (ml) Incomp	Comp	Total/Type	Bled	Serum(ml)
09/24	-	-	-	-	NS[a]	-
09/28	0.2	-	-	0.2/IV	-	-
10/05	0.4	-	-	0.4/IV	-	-
10/12	1.0	-	-	1.0/IV	-	-
10/19	2.0	-	-	2.0/IV	-	-
10/26	1.0	1.0	-	2.0/IM	-	-
10/31	1.3	1.3	-	2.6/IM	-	-
11/05	1.5	1.5	-	3.0/IM	-	-
11/13	-	-	-	-	S[b]	25
11/19	-	-	-	-	S[b]	27
12/28	1.3	-	1.3	2.6/IM	-	-
12/31	1.5	-	1.5	3.0/IM	-	-
01/02	2.5	-	2.5	5.0/IM	-	-
01/12	-	-	-	-	S[b]	20
01/19	-	-	-	-	S[b]	25
01/31	-	-	-	-	S[b]	30

[a]Non-specific serum bleeding.

[b]Specific serum bleeding.

serum through the filter into sterile injection vials which were stored frozen at -20°C for future use as antiserum to the spiroplasma antigen.

The antiserum was titered using the metabolic inhibition test described by Williamson et al. (1979). The test was performed in microtiter plates (Dynatech Laboratories, Alexandria, Virginia) that contained 96 wells in eight rows labelled A to H, and 12 columns, numbered 1 to 12. A spiroplasma culture, for use as antigen in the test, was prepared by inoculating 1 ml of Ex-CL5-P10 into each of two, 100 ml portions of the M1A medium described by Jones et al. (1977) (Appendix A). The test was performed when the culture reached log phase growth, indicated by a color change in the medium from red to yellow. The M1A medium was used to make all antiserum and antigen dilutions.

A 1:81 dilution of antiserum and the following dilutions of antigen were prepared: undiluted, 1:2, 1:4, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. One hundred µl of M1A medium was added to all wells in columns 1 through 9, 11 and 12. One hundred fifty µl was added to the wells in column 10 and these were used as medium control wells. To all the wells in column 1, rows A through H, 50 µl of the 1:81 antiserum dilution was added, producing a 1:243 antiserum dilution in the first well of each row. Threefold dilutions of antiserum were made by serially transferring 50 µl across each row through column 9, but not columns 10, 11 and 12. The 50 µl to be transferred out of the wells in column 9 was discarded, leaving 100 µl in all wells of the plate except those in column 10. A 50 µl amount of each antigen dilution, one dilution for each row, was then added to wells 1 through 9, 11 and 12. Columns 11 and 12 were, therefore, antiserum-free and served as antigen control wells. At this point, a fresh vial of guinea pig complement (GIBCO) was rehydrated with PBS and an 8% solution was prepared

by mixing 0.8 ml complement and 9.2 ml M1A medium. This complement solution was filter sterilized through a 0.45 μm filter as described earlier and 50 μl was added to all wells in the microtiter plate. When mycoplasmas are grown in media that contain sera previously heat inactivated, as does modified C-3G and M1A, the growth inhibitory activity of their specific antisera is markedly reduced. This is apparently related to a heat-labile component of complement, as the addition of guinea pig complement enhances the growth inhibitory activity of the antisera (Taylor-Robinson et al. 1966). The microtiter plate, with all wells now containing 200 μl of various reagents, was covered with a styrene lid (Dynatech) to maintain sterility and incubated at 30°C. The plates were read for color changes daily for four days and the metabolic inhibition titer was expressed as the reciprocal of the highest antiserum dilution to prevent a color change at the highest antigen dilution that produced a color change in the control wells. A schematic representation of the microtiter plate showing dilution and control locations is presented in Figure 2. The dilutions in Figure 2 are slightly different than those of Williamson et al. (1979) as they apparently made a mathematical error in computing their final dilutions (Table 3, p. 347).

Antiserum Purification

These methods of antiserum purification, enzyme conjugation and ELISA protocol follow that of Clark and Adams (1977). All glassware intended to come in contact with the antiserum and purified γ-globulin from this point on was first siliconized by filling their interiors with siliconizing solution (Appendix A), letting them stand for 10 minutes, pouring off the solution and thoroughly air drying them before use. Glass wool was submerged in a beaker containing

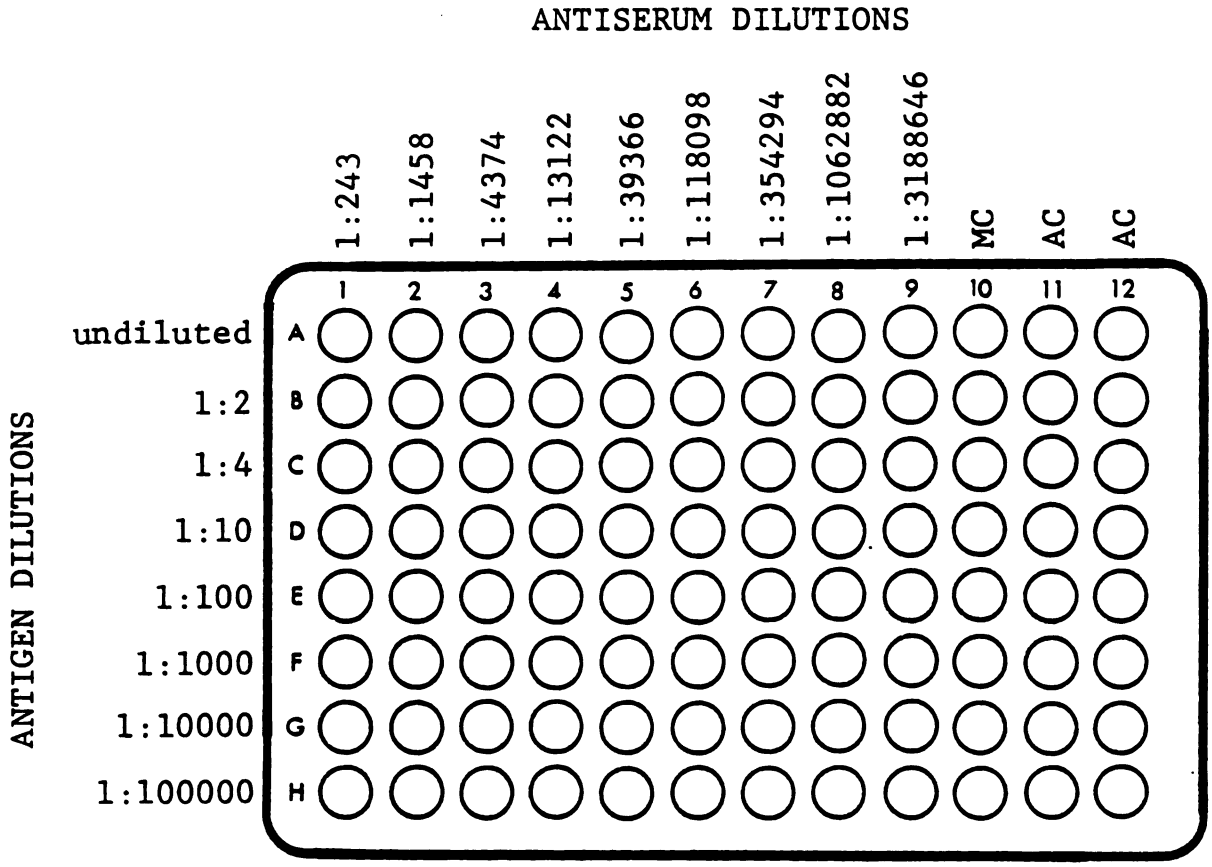


Figure 2.--Microtiter plate showing antiserum and antigen dilution locations for the metabolic inhibition test. (MC = medium control; AC = antigen control).

siliconizing solution. This treatment prevents glassware from adsorbing protein.

To 0.5 ml of antiserum in a 15 ml test tube, 4.5 ml of distilled water was added. To this, 5 ml of saturated ammonium sulfate solution (Appendix A) was added slowly, with gentle shaking, and the tube was allowed to stand for one hour. The precipitate was collected by centrifugation at 3200 x g (5000 rpm) for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 ml 0.5X PBS. Using a Pasteur pipette, the suspension was transferred to 1 cm diameter dialysis tubing, securely tied at both ends with string, and dialized against 500 ml 0.5X PBS, twice for four hours and once overnight, at 6°C.

Following dialysis, the antiserum was further purified by column chromatography using DE 22 (diethylaminoethyl) cellulose (Whatman Ltd., Maidstone, Kent, England). The DE 22 was first equilibrated by mixing dry cellulose into 10X PBS at 1 g cellulose/25 ml buffer. This slurry was then poured into a 9 cm Buchner funnel attached to a filtration flask and equipped with Whatman No. 1 filter paper (Whatman Ltd.). The slurry was washed, using vacuum filtration, with 10X PBS until the pH of the filtrate was equal to that of the original buffer (7.4). The slurry, vacuum dried to a moist cake, was transferred to a clean funnel and the washing procedure was repeated using 5X PBS and again, using 1X PBS, and finally, using 0.5X PBS. The minimum amounts of buffer necessary for these washing steps were: 4 liters-10X PBS, 3 liters-5X PBS, 4 liters-1X PBS and 3 liters-0.5X PBS. The conductivity of the filtrate and the original buffer should also be the same, but the equipment to measure conductivity was not available. However, enough extra washings were performed at each buffer concentration to be reasonably sure that this condition was met.

After the final washing, the excess PBS was vacuumed off in preparation

for the removal of fines. These are very small particles in the slurry that inhibit the efficiency of the cellulose in the column by altering its flow characteristics. The DE 22 was resuspended in 0.5X PBS at 1 g wet cellulose/6 ml buffer. This was mixed well and poured into a graduated cylinder and timing was started. The height, h, of the mixture in the cylinder was noted and it was allowed to settle for t time, where $t = nh$, with $n = 1.8$. The value of n depends upon the degree of fines removal required and ranges from 1.3 and 2.4 for DE 22 cellulose. A value of 1.3 means that almost all fines are removed, while 2.4 means only the finest particles are removed. A value of 1.8 is suitable for the elution of γ-globulin. After t time, the height of the wet settled volume (WSV), i.e., the volume of the settled DE 22, was quickly noted and the overlying buffer was drawn off to a height in the cylinder of 1.2 x WSV. Then 0.5X PBS was added to a height of 1.5 x WSV and the DE 22 was now ready for pouring into the column.

The chromatographic column was made by cutting off the top of a 10 ml disposable glass pipette (American Scientific Products, McGaw Park, Illinois). A small piece of glass wool was pushed down the pipette to the tip to prevent the DE 22 from escaping. A 6 cm piece of 2 mm ID silicon tubing was forced over the end of the pipette and a Mohr pinchcock clamp (American Scientific Products) was installed on this tubing. The column was then clamped vertically to a ringstand, a small amount of 0.5X PBS was placed in the column and the air bubbles were worked out of the glass wool. The equilibrated DE 22 was poured gently down the side of the column, being careful to avoid air bubbles, to a height of approximately 6-8 cm. This was allowed to settle for 0.5 hour. The clamp was then opened, allowing buffer to flow through, further settling the column. The final height of the DE 22 cellulose was 3-5 cm.

Just prior to the insertion of the antiserum, the overlying buffer was allowed to drain through the column until the top of the column was exposed, but moist. The previously prepared antiserum, as much as 3-4 ml, was carefully layered over the top of the DE 22. The clamp on the silicon tubing was opened and 2 ml fractions were collected from the start of the chromatography. Once the antiserum had completely flowed into the DE 22, 0.5X PBS was allowed to flow, by gravity, through silicon tubing onto the top of the DE 22 column, from a beaker clamped on the ringstand above the chromatographic column. A Hoffman clamp was placed on the tubing and the flow was adjusted to keep 2-4 mm of buffer overlaying the DE 22. The assembled column chromatography apparatus is pictured in Figure 3.

At least six, 2 ml fractions were collected from every column chromatography run. Each fraction was read with ultraviolet light at 280 nm on a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, California). All fractions that read 1.4 O.D. and above were combined and the O.D. of this combination was adjusted to 1.4 by adding the appropriate amount of a fraction that read less than 1.4. This reading corresponded to an approximate γ-globulin concentration of 1 mg/ml (Clark and Adams 1977). Figure 4 shows a typical absorbance plot for successive fractions in this method of column chromatography. The purified antiserum was divided in half and stored at 6^{o}C for later use as coating γ-globulin in ELISA and enzyme conjugation. Typically, 1 ml of raw antiserum produced approximately 2 ml of purified γ-globulin.

Enzyme Conjugation

One ml of enzyme, alkaline phosphatase, Type VII, 5 mg protein/ml (Sigma Chemical Co., St. Louis, Missouri), was centrifuged at 3200 x g (5000 rpm) for 10

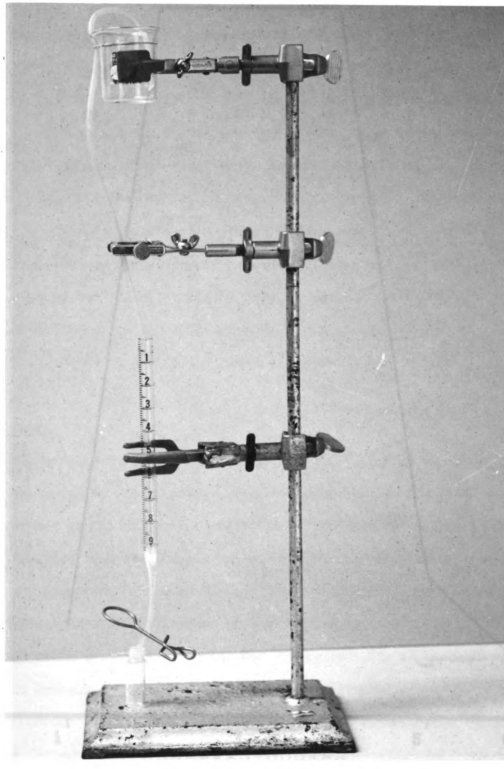


Figure 3.--Column chromatography apparatus for the purification of antiserum.

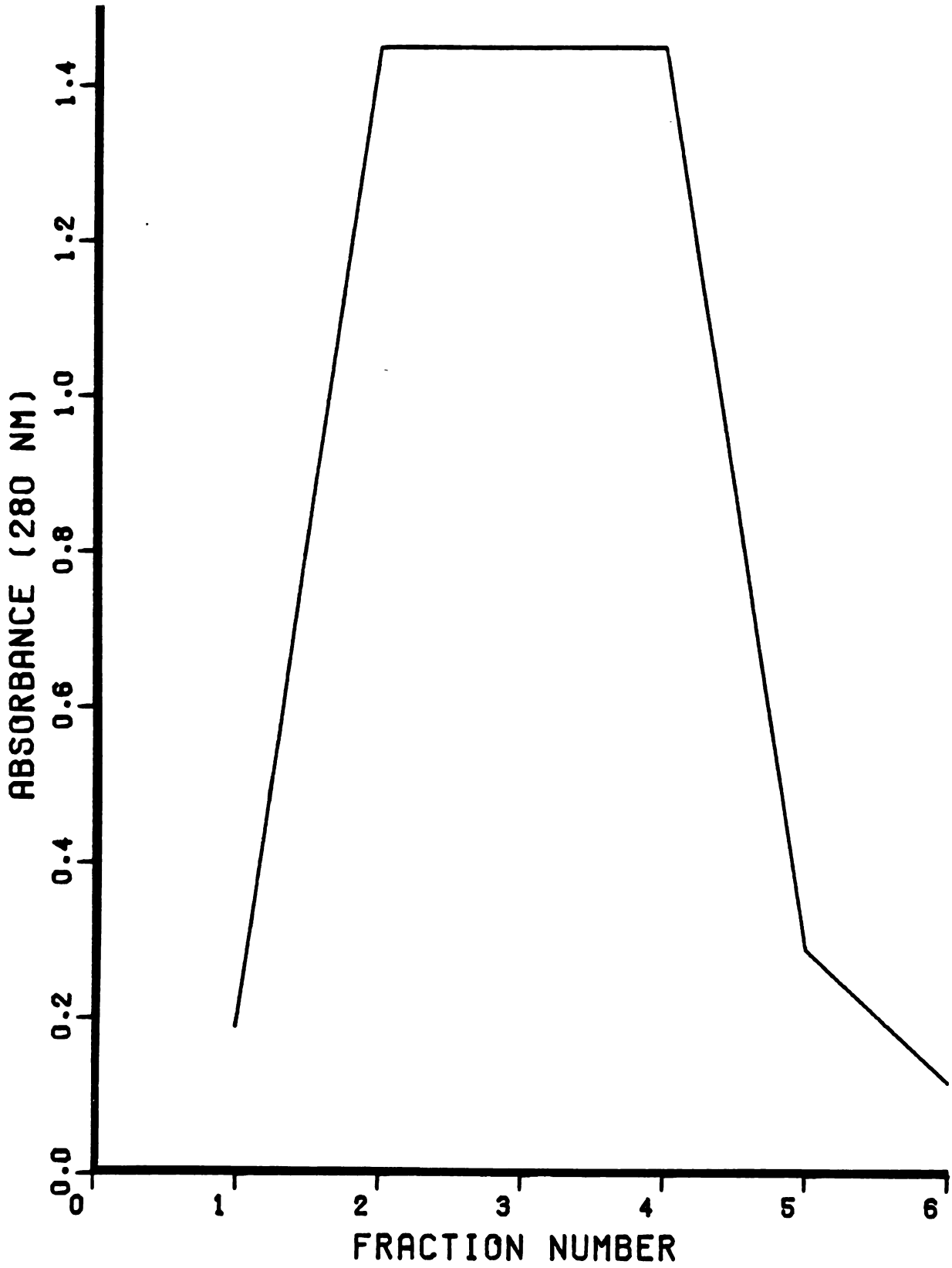


Figure 4.--Typical absorbance plot for the γ-globulin fractions eluted through DE-22 cellulose.

minutes. The supernatant was discarded and the pellet was dissolved in 2 ml of purified γ-globulin which was added directly to the centrifuge tube. This mixture was dialyzed three times, as described in the antiserum purification procedure. Following dialysis, the mixture was transferred to a test tube and 1 µl 25% glutaraldehyde, Grade I (Sigma), was added per 2 ml of mixture (Avrameas 1969). This was left to stand at room temperature for 4 hours at which point a very faint, sandy brown color developed in the tube. The mixture was again dialyzed three times, as above. It was then transferred to a screw top vial of appropriate size as the preparation from all dialysis tubes were combined at this point and 5 mg bovine serum albumin (Sigma) per ml was added. The enzyme-labelled γ-globulin was then stored at 4° for future use.

ELISA Protocol

All ELISA tests were performed in MicroELISA™ plates (Dynatech) which are the same design as the microtiter plates described earlier but made of a plastic especially suited for protein adsorption. The procedure described here, and used throughout this research, is the double antibody sandwich method of ELISA. From information gathered through system evaluation (described later), it was determined that 1:500 dilutions of both coating γ-globulin and enzyme-labelled γ-globulin, made using coating and conjugate buffers (Appendix A), respectively, were adequate for antigen detection at all levels.

Two hundred µl coating γ-globulin was added to all wells in the MicroELISA™ plate using a Dynadrop™ SR-1 semi-automatic single reagent dispenser (Dynatech) (Figure 5). This instrument dispenses reagent into eight wells simultaneously and a plate can be filled in 10-15 seconds. The plate was then sealed by stretching Handiwrap™ over its surface and incubated at 37°C in

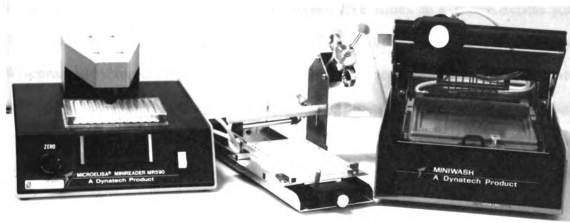


Figure 5.--Equipment used in the performance of the enzyme-linked immunosorbent assay (ELISA).

a Lab-Line Imperial II incubator (Lab-Line Instruments, Inc., Melrose Park, Illinois) for 4 hours. Following incubation, the plate was washed three times with PBS-Tween (Appendix A) using a Miniwash^{TM} washer/aspirator (Dynatech) (Figure 5) that alternately fills and aspirates the plate wells, eight at a time, as the operator manually advances the plate. Three washings may be accomplished in 15 minutes, letting the plates stand for 3 minutes after each filling. After the final aspiration, the plate was firmly shaken five times to remove excess PBS-Tween and the surfaces dried with Kimwipes^{TM} (Kimberly-Clark Corp., Neenah, Wisconsin). After drying, 200 μl of the previously prepared samples to be tested were pipetted into the appropriate wells using an automatic, adjustable volume Finnpippette^{TM} (Arthur H. Thomas Co., Philadelphia, Pennsylvania). The plate was resealed with Handiwrap^{TM} and incubated for 16 hours at 6^{o}C. The plate was again washed three times, dried, as above, and 200 μl enzyme-labelled γ-globulin was added to all wells using the Dynadrop^{TM} SR-1 dispenser. The plate was resealed and incubated for 4 hours at 37^{o}C. The plate was again washed three times, dried and 250 μl enzyme substrate, p-nitrophenyl phosphate (Sigma) in substrate buffer (Appendix A), was added to all wells. The enzyme substrate was prepared immediately prior to use. The plate was allowed to stand at room temperature for 1 hour at which time 50 μl 3M NaOH was added to each well to stop the enzymatic reaction. The results were assessed visually or the absorbance of each well was read spectrophometrically on a MicroELISA^{TM} MR590 Minireader^{TM} (Dynatech) (Figure 5). This instrument measures absorbance at 405 nm (A_{405}) directly through the plate well, eliminating the need to transfer the contents to cuvettes for reading in other types of spectrophotometers. All 96 wells could be read and recorded in less than 5 minutes by one person.

System Evaluation

For system evaluation and all subsequent ELISA tests, 200 ml of M1A medium was prepared in 500 ml portions. By the method described previously, 1000 ml of spiroplasma culture was prepared and this culture, along with the uninoculated medium, was divided into 1 ml aliquots and frozen at -20°C for future use as antigen and media controls. A test was considered ELISA positive if the absorbance of the sample was at least twice that of the medium controls (Voller et al. 1979). Modified C-3G medium was also included in initial system evaluation.

ELISA was performed as described in the protocol. This test involved four MicroELISA™ plates, two for the antigen and two for the media. Coating γ-globulin was diluted 1:100, 1:500, 1:1000 and 1:10000 in coating buffer. Antigen (=sample in protocol) was diluted 1:10 in conjugate buffer and seven, serial, twofold dilutions were prepared from this. Both modified C-3G and M1A media were diluted 1:10, 1:100 and 1:1000. The enzyme-labelled γ-globulin was diluted 1:100 in conjugate buffer and five, serial, twofold dilutions were prepared from this. Diagrams of the MicroELISA™ plates showing location and distribution of the various reagents are presented in Figures 6 and 7. The test was visually assessed as the Minireader™ was not yet available.

Following the above test, a simplified evaluation was performed. The coating γ-globulin and the enzyme-labelled γ-globulin were diluted 1:100, 1:500 and 1:1000 in coating and conjugate buffers, respectively. Antigen and M1A medium were both diluted 1:10 and 1:100 in conjugate buffer. The diagram of this test is presented in Figure 8. The test was performed according to the protocol, replicated on a second plate and visually assessed at the conclusion.

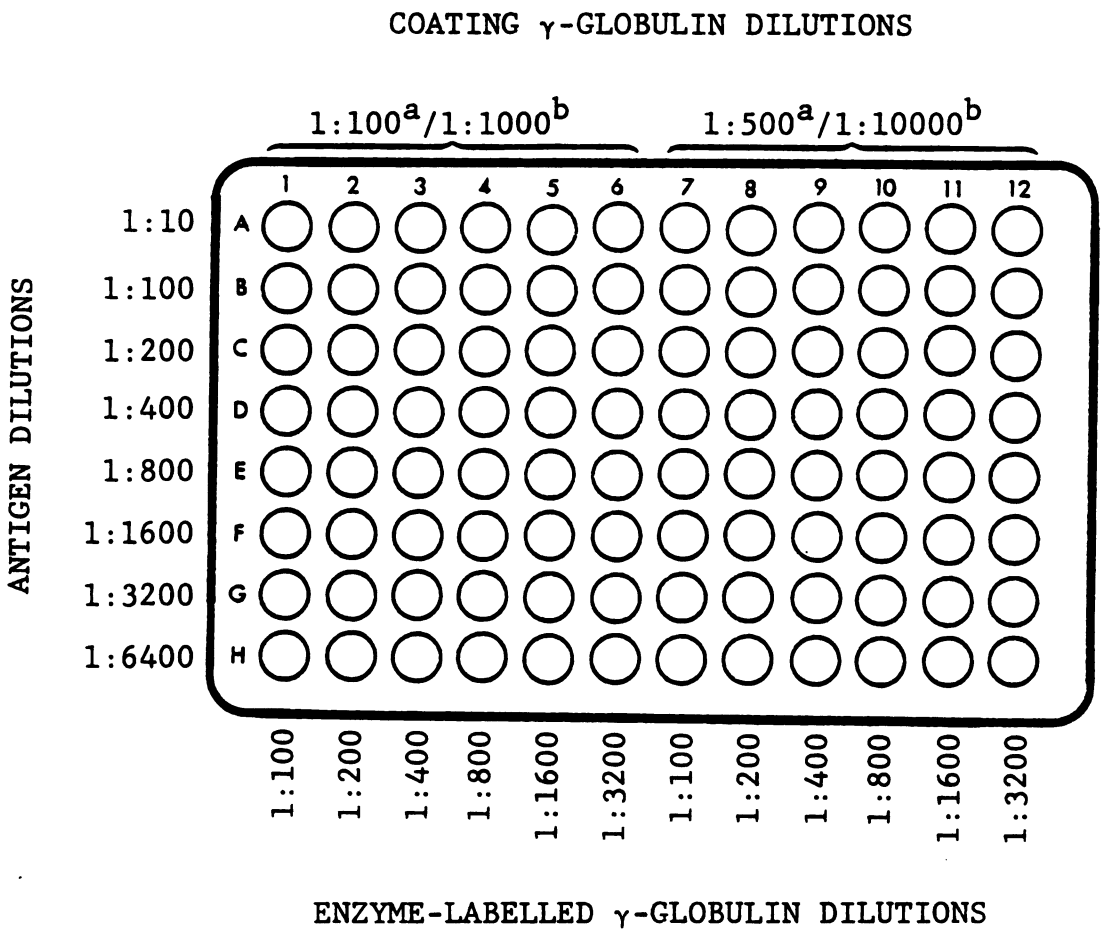


Figure 6.--MicroELISA™ plate showing coating γ-globulin, enzyme-labelled γ-globulin and antigen dilution locations for the ELISA system evaluation test. ([a]plate 1; [b]plate 2).

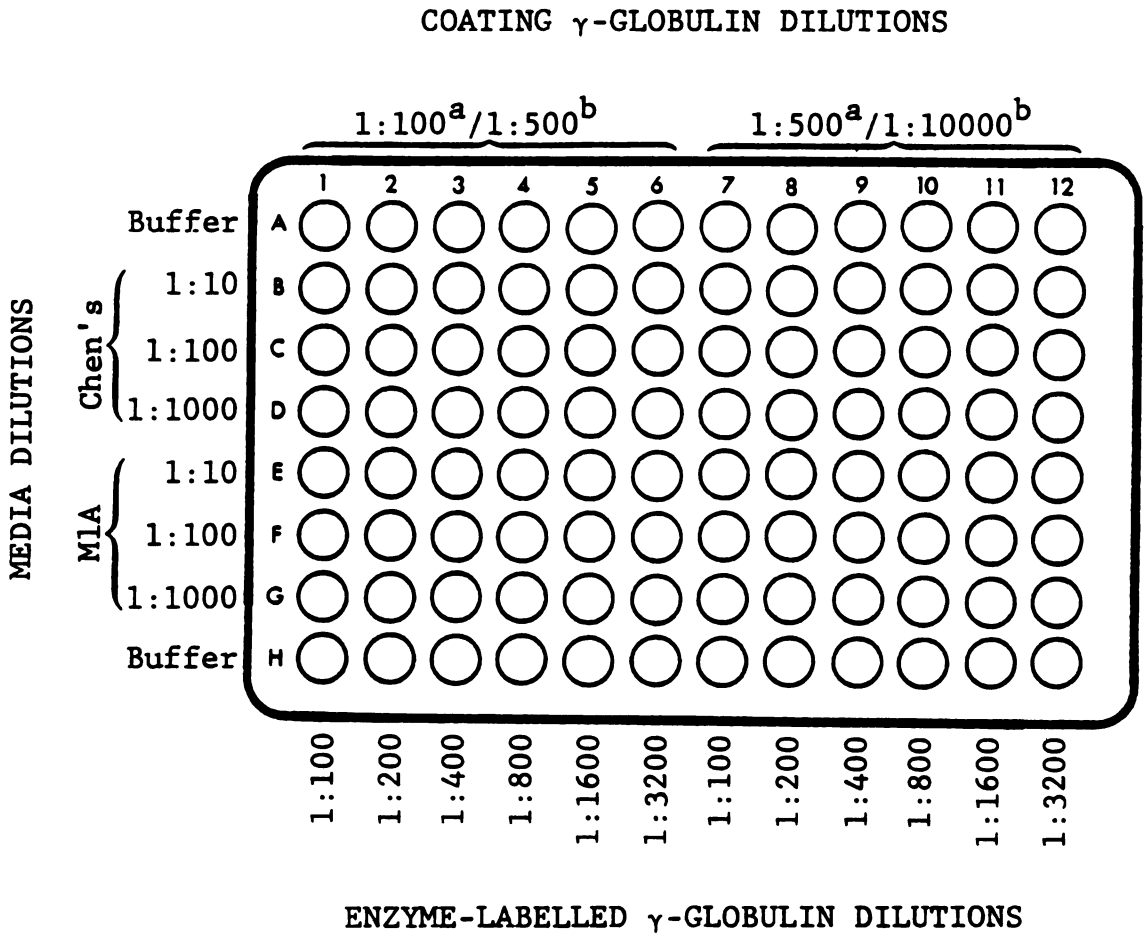


Figure 7.--MicroELISA^{TM} plate showing coating γ-globulin, enzyme-labelled γ-globulin and media dilution locations for the ELISA system evaluation test. (^{a}plate 1; ^{b}plate 2).

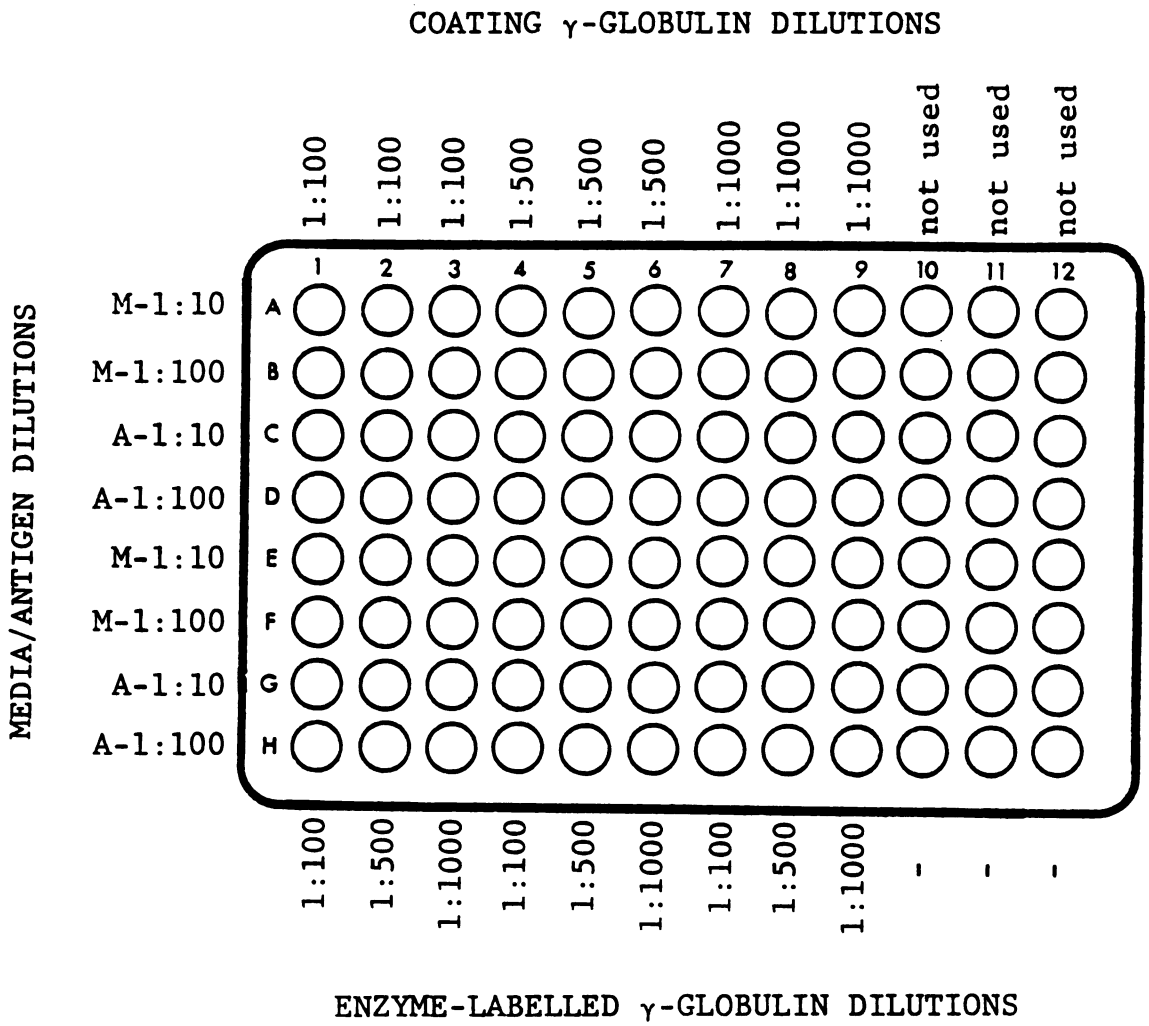


Figure 8.--MicroELISA^{TM} plate showing coating γ-globulin, enzyme-labelled γ-globulin, antigen and medium dilution locations for the simplified ELISA system evaluation test. All media wells contain M1A medium.

Culture Quantification and ELISA Sensitivity

The sensitivity of this ELISA technique was assessed by the number of spiroplasmas it could detect. This necessitated the enumeration of the spiroplasmas in the control culture. This quantification was accomplished using a method described by Liao and Chen (1977). Three 1 ml aliquots of the frozen culture were thawed and mixed together in a 16 x 125 mm glass culture tube. This was also done with three 1 ml aliquots of M1A medium. Both tubes were vortex mixed for 3 minutes on a Vortex-Genie mixer (Scientific Industries, Inc., Springfield, Massachusetts) to break up any clumps of spiroplasmas in the culture. The culture was then diluted 1:10 with M1A medium and 3 µl was placed on a meticulously clean microscope slide using a 5 µl microsyringe (Hamilton Co., Reno, Nevada). This was covered with a No. 1, 18 x 18 mm, cover glass so that the culture was spread evenly, and completely, under the glass and no air bubbles were trapped. The slide was viewed under dark field oil immersion at a magnification of 1500X with a Wild M20 compound microscope equipped with a dark field immersion condenser (Wild Heerbrugg, Ltd., Heerbrugg, Switzerland). Ten randomly selected fields were located and the spiroplasma cells in each field were counted and recorded. The microscope was focused up and down to insure cells in all planes were counted. This procedure was performed on three slide preparations and the average number of cells in 30 fields was computed. The diameter of the microscope field was measured with a slide micrometer (Wild) and the area computed. The number of cells per ml was computed according to the following formula:

$$\text{cells/ml} = Y/a \times A \times B \times C \times D$$

where, Y = average number of cells per 30 fields, a = area of the microscope field = 0.02 mm^2, A = area of cover grass = 324 mm^2/slide, B = conversion factor = 1 slide/3 µl, C = conversion factor = 1000 µl/ml and D = dilution factor = 10. If the same enumeration procedure is used consistently, this equation simplifies to:

$$\text{cells/ml} = (5.4 \times 10^7) \times Y$$

and any number of fields can be counted to arrive at a value for Y.

The same portion of culture from which the sample was drawn for spiroplasma enumeration was tested with ELISA. Four antigen dilutions were prepared, 1:10, 1:20, 1:40, and 1:80, in conjugate buffer. Tenfold and hundredfold dilutions were made from each of these four, resulting in twelve antigen dilutions. One ml of each dilution was transferred into each of 12, 12 x 75 mm glass culture tubes. Each of these 1 ml aliquots was sonicated for 30 seconds at 20% power using a Blackstone SS-2 Ultrasonic generator equipped with a model BP-2 probe and 0.125 inch (3.1 mm) diameter probe tip (Blackstone Ultrasonics, Inc., Sheffield, Pennsylvania). Dilutions of unsonicated and sonicated medium were prepared in the same manner. The diagram of this test is presented in Figure 9. The test was performed according to the protocol, replicated on a second plate and the A_{405} of all wells was read with the Minireader^{TM}. From the number of cells per ml computed above, the cells per ml of each antigen dilution was computed. These concentrations were plotted against their corresponding absorbances to establish a standard curve for the quantification of spiroplasmas in test samples.

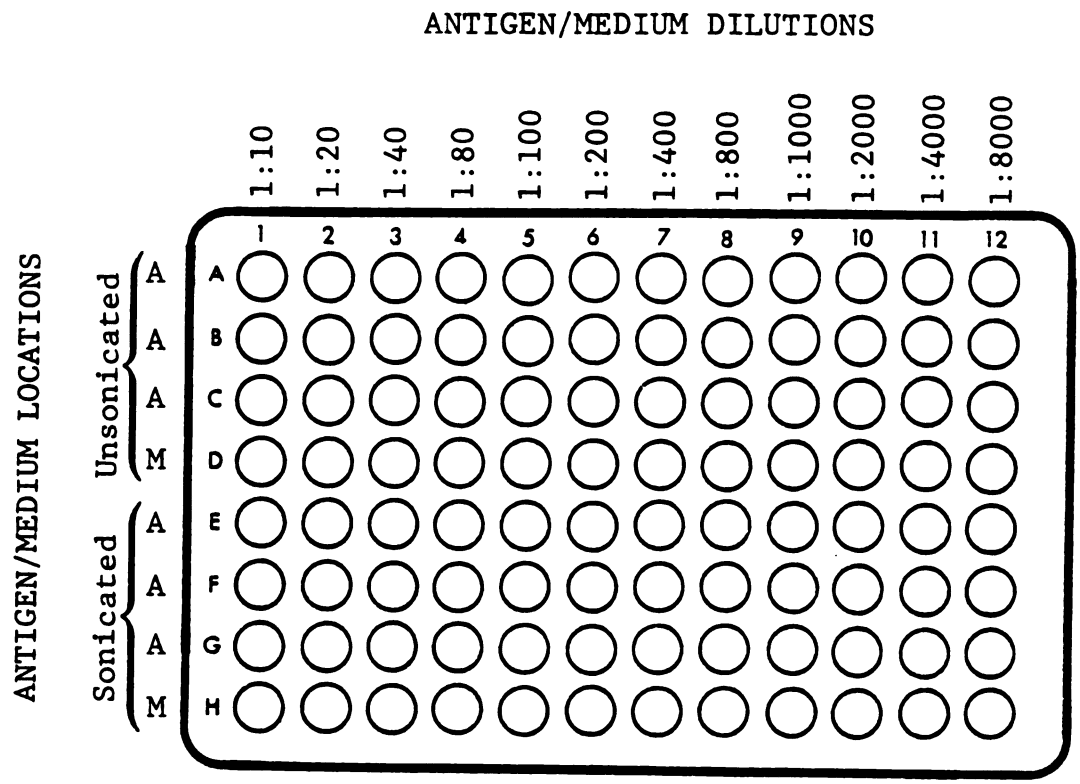


Figure 9.--MicroELISA^{TM} plate showing unsonicated and sonicated antigen and medium dilution locations for the ELISA sensitivity test. All media wells contain M1A medium.

ELISA Specificity

In order to assess the specificity of this ELISA technique, 10 spiroplasma isolates, isolated in association with several plant diseases and supplied by Dr. Alan L. Jones, were tested. These included AY-I3-P3 and AY-I6M-P3, two isolates associated with aster yellows, E-P49 and G-3N, two isolates of the corn stunt spiroplasma, MOROC, ISRAEL and C-189, three strains of Spiroplasma citri, PY-1B-P14, an isolate associated with the peach yellow leaf roll strain of X-disease and Ex-CL5, the isolate used to establish this ELISA. All isolates were vortexed for 3 minutes and diluted 1:10, 1:100, 1:1000 and 1:10000, as were medium and antigen controls. One ml of each dilution was prepared and sonicated as described above. The diagram of this test is presented in Figure 10. The test was performed according to the protocol, replicated on a second plate and the A_{405} was recorded.

Leafhopper Sampling and Testing with ELISA

Description of Sample Sites

Two sites were selected for leafhopper sampling. The first was a commercial peach orchard operated for a U-pick market (Location: T4N R1E Sec 4 NE 1/4). It was maintained on a regular pesticide spray schedule until harvest. There were about 1165 peach trees of several varieties, including Red Haven and Harbrite, and 96 apricot trees in rows running north and south arranged in five blocks separated by tractor paths (Figure 11). The trees were planted on 12 ft. (3.7 m) centers in rows spaced 20 ft. (6.1 m) apart. The orchard was bordered on the north and east by an alfalfa/grass hay field, on the west by a

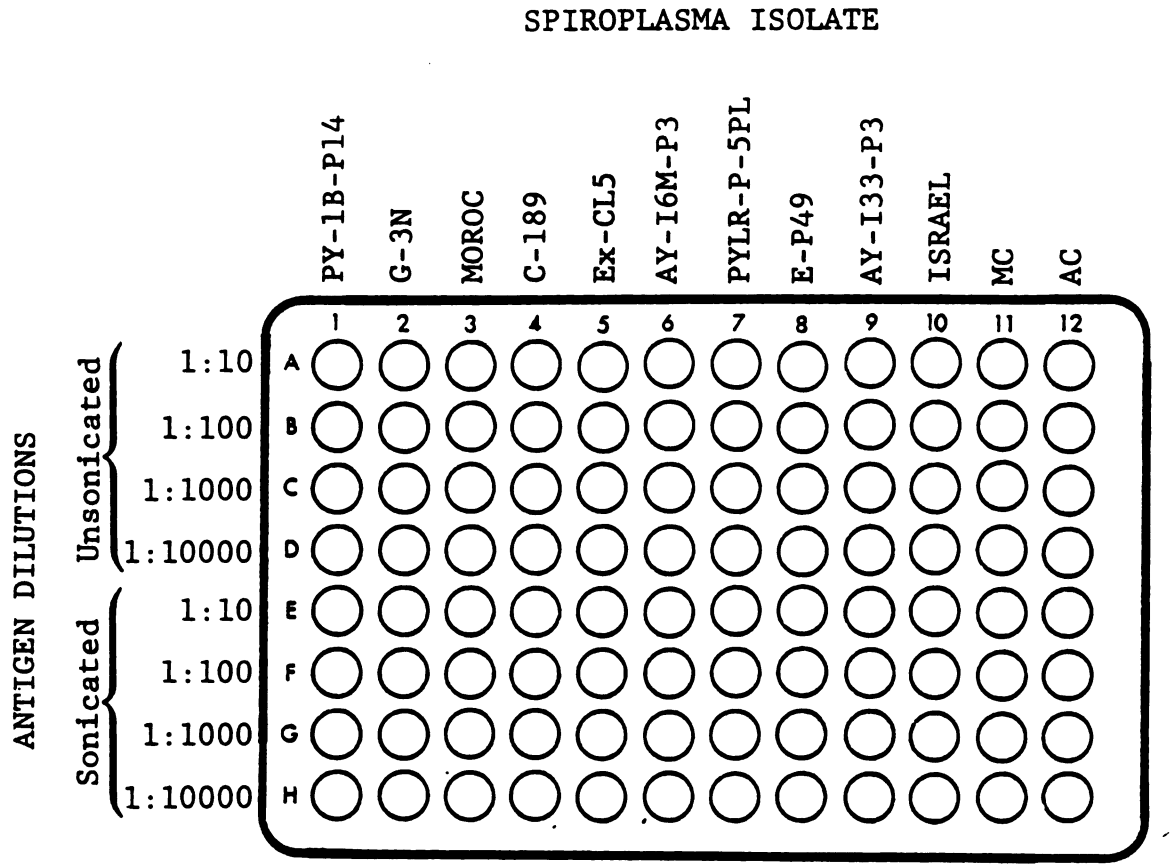


Figure 10.--MicroELISA^{TM} plate showing spiroplasma isolate dilution locations for the ELISA specificity test. (MC = medium control; AC = antigen control).

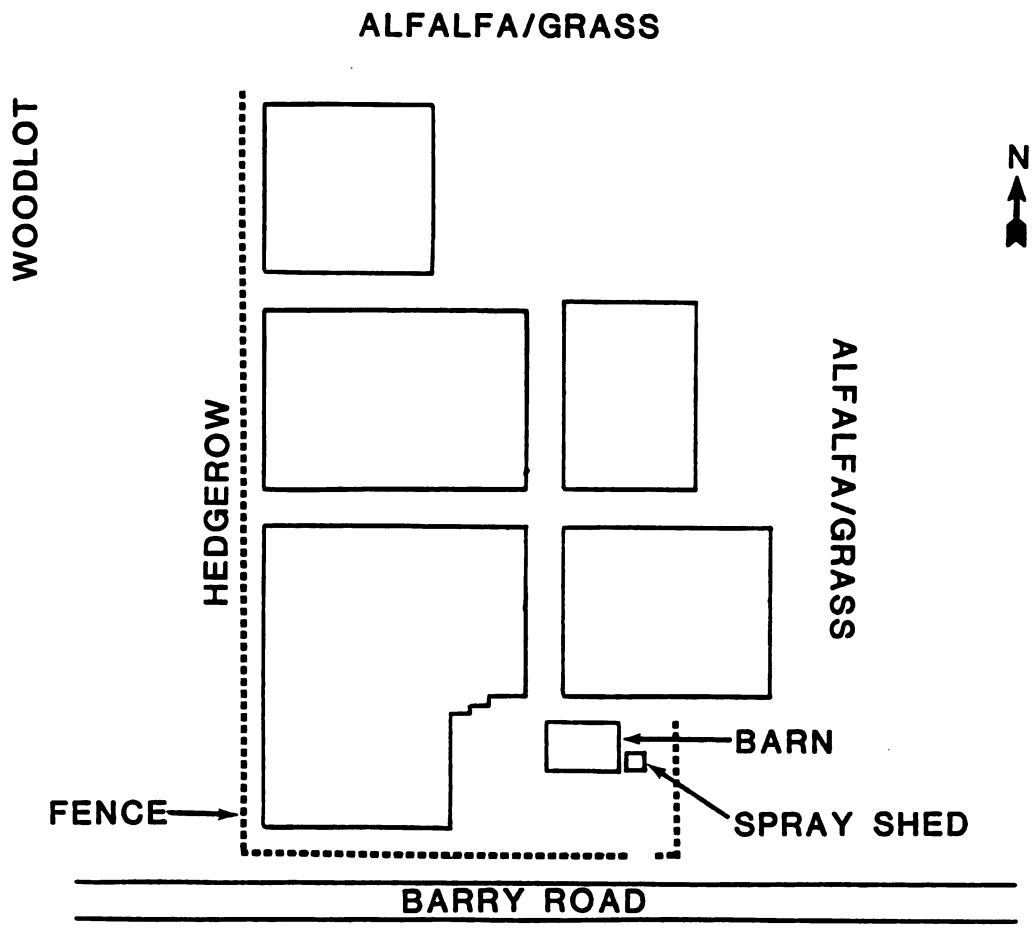


Figure 11.--Commercial orchard used for leafhopper sampling.

fence row and woodlot and on the south by a two-lane asphalt road. The ground cover consisted primarily of orchard grass (Dactylis glomerata L.) and red clover (Trifolium pratense L.) with many herbaceous weeds scattered throughout the orchard. The trees were marked for X-disease symptoms in 1978, 1979, 1980 and 1981 with approximately 4.1%, 15.9%, 22.9% and 25.0%, respectively, showing symptoms.

The second site was a three-row peach block on the campus of Michigan State University (Location: T4N R1W Sec 31 SE 1/4). Originally, there were 123 trees running north and south that were planted for varietal research. The trees were planted on 6 ft. (1.8 m) centers in rows spaced 12 ft. (3.7 m) apart (Figure 12). At the time of this research, 45 trees remained, with the rest being rogued out for various reasons, many due to X-disease. Five trees, 11.1% of the those remaining, showed symptoms of X-disease. For the duration of this research, no pesticides were applied to this block, which had probably not received much, if any, attention for several years previous. The site was bordered on the north by a two-lane asphalt road, on the south by several vegetable research plots, on the east by a row of pine trees adjacent to a forest research facility, and on the west by several rows of both peaches and cherries running east and west. The ground cover was primarily orchard and other grasses with very little red clover. There were a number of other herbaceous weeds with a great deal of milkweed (Asclepias syriaca L.). The block was left unmowed for the entire season.

Tree Sampling

In an attempt to obtain absolute estimates of leafhopper populations in the trees, a modification of the device described by Dempster (1961) was

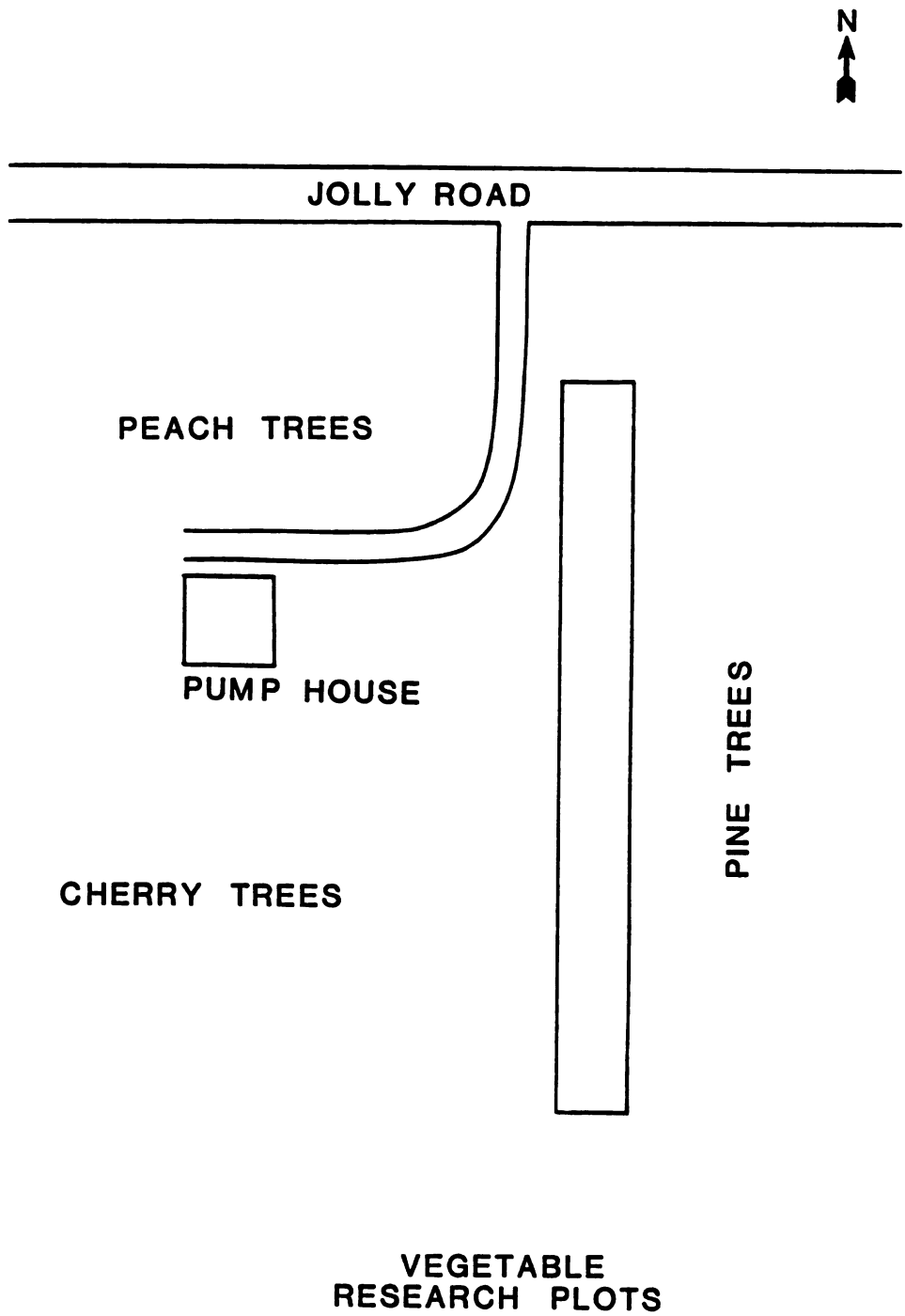


Figure 12.--Michigan State University peach block used for leafhopper sampling.

constructed (Figure 13). Screen-door handles were attached to the bottom of two stainless steel steam tray pans measuring 29 x 23 x 10 cm. A 5 cm wide square flange of 1/4 inch (0.64 cm) plywood was bolted around the open edges of the pan. To this flange was glued 5 x 5 cm foam rubber strips to act as gaskets. These two halves of the trap were clamped together using two elastic bicycle tie-downs which were stretched around each side and hooked to the handles on the bottom of each pan.

The trap was used by carefully approaching the tree limb to be sampled and quickly enclosing it with the two halves and clamping them together with the tie-downs. The foam rubber gaskets allowed limbs up to 5 cm in diameter to be sampled. A rubber tube attached to a small E cyclinder of carbon dioxide was inserted into the trap between the gaskets and gas was pumped in for 15-30 seconds. After 1 minute, the trap was opened and the limb was vigorously shaken while holding one-half of the trap underneath to catch all insects on the limb. The number of leaves caught inside the trap were counted and any leafhoppers were placed in 10 x 15 cm plastic bags. The bags were sealed, labelled and returned to the lab where they were frozen for future leafhopper identification and counting.

A computer program was written that randomly generated sample locations (Appendix B). The trees in each of the five blocks at the commercial site and the MSU block were numbered and divided into eight octants by three imaginary perpendicular planes. The program generated four random sample locations for each of the six blocks that consisted of two numbers, one corresponding to a tree in the specific block and the other to an octant within that tree. Both sites were sampled at 3-4 day intervals from June 9 to August 28, 1980.

Figure 13.--Tree sampling device showing carbon dioxide cylinder and its connection to the box.

Ground Sampling

An attempt was also made to obtain absolute leafhopper population estimates in the ground cover of both sample sites. To do this, a modification of the procedures described by Heikinheimo and Raatikainen (1962) and Johnson et al. (1958) was employed. A cone 34 cm high with upper and lower diameters of 19 and 40 cm, respectively, was constructed from 1 mm thick, semi-transparent fiberglass (Figure 14). A 5 cm vertical flange of fiberglass was attached to the upper edge of the cone to accommodate a vacuum hose. Two 15 cm holes were cut, opposite one another, into the side of the cone. These were covered with two layers of 1.5 cm thick black rubber with one slit cut in each piece, perpendicular to one another, to allow hand entry into the interior of the cone. All fastening of the fiberglass was done with pop rivets. The edges of the cone were covered with duct tape to prevent fraying of the fiberglass. The bottom of the cone encompassed an area of 0.125 m^2.

The trap was used by inserting a nylon net into the upper hole in the cone, carefully approaching the sample area and quickly setting the cone into the ground cover before any insects could escape. An assistant then approached the cone with a gasoline powered D-vac^{TM} (D-vac, Ltd., Riverside, California) equipped with a 20 cm ID suction hose. The hose was placed over the upper hole in the cone and the vacuum motor started. By inserting a hand through the holes in the side of the cone, all the plant material trapped was uprooted and vacuumed into the sample. The bare ground was raked over with the fingers to insure that all insects trapped were taken up into the sample. With the motor still running, the hose was removed from the cone, the insect net and sample remaining inside the hose. The net was carefully removed, held closed with the

Figure 14.--Ground sampling device showing D-vac™ attached and method of use in the ground cover.

hand and the motor shut off. The sample was transferred to a 19 x 45 cm plastic bag, previously marked with date and location, by inverting the insect net into the bag and thoroughly shaking out its contents. The bag was sealed, returned to the lab and frozen for future leafhopper extraction.

Leafhoppers were extracted from the sample by sifting the sample through three layers of screen. Three square frames were constructed of 1 x 4 inch (2.54 x 10.16 cm) pine. One side of the first frame was covered with 0.5 inch (1.27 cm) mesh welded wire screen, the second with 0.25 inch (0.64 cm) welded wire screen and the third with 0.0625 inch (0.16 cm) aluminum window screen. The frames were stacked, largest mesh on top, and the sample placed in the top frame. It was sifted through the first two frames, which removed large plant material and other debris. The contents of the third frame, and all material falling through it, was thoroughly sorted for leafhoppers. All leafhoppers found in the sample were placed in 12 x 75 mm glass culture tubes containing PBS, appropriately marked, sealed with corks and frozen for future identification and testing with ELISA. All leafhoppers were identified to species using the descriptions and keys of Beirne (1956) and Oman (1951). Voucher specimens of all identified species have been deposited in the Michigan State University Entomological Museum (Appendix F).

The locations for ground cover sampling were derived in a manner similar to that for tree sampling. A computer program was written to generate random sample locations based on the number of grids in the five commercial blocks and one MSU block (Appendix C). A grid was delineated by the tree spacing at the two sites. It was measured as the distance from one tree to the next in the same row by one half the distance from the tree to the corresponding tree in the

adjacent row. In the commercial orchard, where the tree spacing was 12 x 20 ft. (3.7 x 6.1 m), the grid size was 12 x 10 (3.7 x 3.0 m) while in the MSU block, where tree spacing was 6 x 12 ft. (1.8 x 3.7 m), the grid size was 6 x 6 (1.8 x 1.8 m). The grids in each of the six blocks were numbered and the computer program generated four random sample locations for each block based on these numbers. Each grid was sampled at its middle. Both sites were sampled every 3-4 days from June 9 to October 10, 1980.

Preparation of Insect Tissue for ELISA

Initially, leafhoppers were tested individually. One leafhopper was placed in a 12 x 75 mm glass culture tube containing 1 ml conjugate buffer. The insect was ground up using a 6 mm diameter glass rod rounded at the end in a Bunsen burner and roughened with coarse, garnet sandpaper. The insect-buffer solution was sonicated at 25% power with the Blackstone sonicator described previously. This solution was pipetted, in 200 µl aliquots, into two wells in each of two MicroELISA^{TM} plates resulting in four replicates of each insect. The test was performed according to protocol and the A_{405} recorded.

Leafhoppers were also tested in groups of 10 of the same species. The insects were placed in a 30 ml Potter-Elvehjem tissue grinder containing 10 ml conjugate buffer and equipped with a teflon pestle. The pestle was clamped into a Model 3 Variable Speed stirring motor (Eastern Industries, New Haven, Connecticut) and the sample was homogenized at full speed until no visible insect pieces remained. The sample was then filtered through four layers of cheese cloth into a 15 ml polycarbonate centrifuge tube and centrifuged at 23,000 x g (13,500 rpm) in a Sorvall SS-1 centrifuge and the pellet resuspended in

1 ml conjugate buffer. This solution was sonicated and distributed into MicroELISA^{TM} plates and the test completed as described for individual leafhopper tests.

Plant Testing with ELISA

Plant Collection

Herbaceous plants were collected from peach and cherry orchards, as well as from near diseased chokecherry, in central and southwestern Michigan (Table 3). These plants were uprooted, placed in 19 x 45 cm plastic bags, appropriately labelled, and returned to the lab where they were frozen for future testing. Cultivated carrot, Daucus carota L., and cultivated onion, Alium cepa L., that exhibited symptoms of aster yellows were supplied by other researchers at Michigan State University. Dr. Alexander H. Purcell, University of California, Berkeley, was kind enough to supply celery, Apium graveolens L., tissue for testing with this ELISA. Twelve samples were sent coded, wrapped in moist paper toweling and sealed in plastic bags. Ten of the samples were infected with seven pathogens, including two infected with the Berkeley strain of peach yellow leaf roll (PYLR) X-disease, one with a mild strain of PYLR, one with another mild strain of PYLR, one with a mild X-disease strain in young leaves, one with the same strain in old leaves, two possibly infected with Spiroplasma citri, one with aster yellows and one with corn stunt. The remaining two samples were healthy controls.

Woody plant samples were collected by clipping stems, leaves and fruit from the tree with pruning shears (Table 4) which were handled the same as the herbaceous samples. The pruning shears were rinsed in alcohol between samples.

Table 3.--Herbaceous plants tested with ELISA and their collection locations. MSU = Michigan State University sample site; COMM = commercial sample site; EL-CC = X-diseased chokecherry site in East Lansing, Michigan; SWM = southwest Michigan.

Plant	Collection Location[a,b]			
	MSU	COMM	EL-CC	SWM
Cudweed *Gnaphalium obtusifolium* L.	-	-	1	-
Curled Dock *Rumex crispus* L.	-	1	-	-
Dandelion *Taraxacum officinale* L.	-	1	-	-
Dogbane *Apocynum cannabinum* L.	-	-	1	-
Field Goldenrod *Solidago nemoralis* Ait.	-	1	-	-
Horse Nettle *Solanum carolinense* L.	-	-	-	1
Lamb's Quarter *Chenopodium album* L.	-	1	-	2
Milkweed *Asclepias syriaca* L.	1	1	-	4
Motherwort *Leonurus cardiaca* L.	-	1	-	-
Pennsylvania Smartweed *Polygonum pensylvanicum* L.	-	-	1	-
Quackgrass *Agropyron repens* (L.) Beauv.	-	1	-	-
Ragweed *Ambrosia artemisiifolia* L.	-	-	-	2
Red Clover *Trifolium pratense* L.	-	1	-	-
Rough Pigweed *Amaranthus retroflexus* L.	1	-	-	-

Table 3.--(cont'd.).

Plant	Collection Location[a,b] MSU	COMM	EL-CC	SWM
Roundleaved Mallow *Malva neglecta* L.	1	-	-	-
Tumbleweed *Amaranthus albus* L.	1	-	-	-
White Clover *Melilotus alba* Desr.	-	1	-	-
Wild Carrot *Daucus carota* L.	-	-	1	-
Wild Grape *Vitis* sp.	-	1	1	-
Wild Strawberry *Fragaria virginiana* Duch.	1	1	-	-

[a]Number indicates the number of samples collected.

[b]MSU location: T4N R1W Sec 31 SE ¼; COMM location: T4N R1E Sec 4 NE ¼; EL-CC location: T4N R2W Sec 24 SW ¼; SWM locations: T3S R15W Sec 20 NW ¼ and T3S R16W Sec 25 SW ¼.

Table 4.--Woody plants tested with ELISA and their collection locations. MSU = Michigan State University sample site; COMM = commercial sample site; EL-CC = X-diseased chokecherry site in East Lansing, Michigan; SWM = southwest Michigan.

Plant	Collection Location[a,b]			
	MSU	COMM	EL-CC	SWM
Tart Cherry *Prunus cerasus* L.	-	-	-	15
Peach *Prunus persica* L. Batsch	1	8	-	5
Black Cherry *Prunus serotina* Ehrh.	-	1	-	-
Chokecherry *Prunus virginiana* L.	-	1	1	-
Apricot *Prunus armeniaca* L.	-	2	-	-
Apple *Malus sylvestris* Mill.	-	-	-	1
Running Juneberry *Amelanchier stolonifera* Wieg.	-	-	1	-
Red-Panicle Dogwood *Cornus racemosa* Lam.	-	-	1	-

[a]Number indicates the number of samples collected.

[b]MSU location: T4N R1W Sec 31 SE ¼; COMM location: T4N R1E Sec 4 NE ¼; EL-CC location: T4N R2W Sec 24 SW ¼; SWM locations: T3S R15W Sec 20 NW ¼ and T3S R16W Sec 25 SW ¼.

In addition to several samples taken randomly in peach trees, two X-diseased peach trees were sampled for no, mild and severe symptom tissue in an effort to locate the X-disease pathogen. For this reason also, another sample involved two diseased branches from separate peach trees. These branches were cut into 10 cm sections. Each section was sequentially labelled from base to tip and placed in 10 x 15 cm plastic bags for transport to the lab. On one occasion, root samples from X-diseased peach trees were taken just after they had been rogued out.

Preparation of Plant Tissue for ELISA

Initially, 1 g of plant tissue, stems, leaves or fruit, was ground up in 5 ml conjugate buffer using a No. 2 mortar and pestle. The homogenate was filtered through eight layers of cheesecloth and tested without further treatment. This method proved unsatisfactory due to non-specific reactions in the ELISA test, probably caused by large particulate matter in the wells. The method was modified by grinding 1 g of tissue in 10 ml of buffer, filtering through the cheesecloth and centrifuging the filtrate at 1000 x g (2500 rpm) for 3 minutes. This clarified extract gave very reproducible results. The method finally arrived at, however, was that of Raju and Nyland (1981). One g of tissue, usually midveins and petioles, was homogenized in 15 ml conjugate buffer using a Waring Commercial Blender, Model 5010G, equipped with an MC-1, 37 ml, stainless steel minicontainer (Waring Products Div., Dynamics Corp. of America, New Hartford, Connecticut) at high speed for 1 minute. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3200 x g (5000 rpm) for 5 minutes. The supernatant was poured into a polycarbonate centrifuge tube and centrifuged

at 18,400 x g (12,000 rpm) for 20 minutes at 6°C. The supernatant was now discarded, the pellet resuspended in 1 ml conjugate buffer and the solution sonicated for 15 seconds. The sample was now ready for testing. All plant sample tests were performed according to the protocol and the A_{405} recorded.

In order to assess the possible effects of plant constituents on the performance of this ELISA, several woody and herbaceous plant preparations were inoculated with antigen in pure culture. The sample preparation itself was used to make a 1:10 dilution of antigen. This was pipetted into the MicroELISA™ plate adjacent to its uninoculated counterpart. The test was, again, performed according to protocol and the A_{405} recorded.

RESULTS AND DISCUSSION

Enzyme-linked Immunosorbent Assay

Antiserum Generation

The results of the metabolic inhibition test are presented in Table 5. The non-specific serum from rabbit No. 109 failed to inhibit spiroplasma growth at all antigen dilutions while that from rabbit No. 110 produced weak inhibition. There were indications however, that growth would have been manifested had the test been allowed to proceed for a longer time. These results indicate that the inhibition of spiroplasma growth by specific antiserum may be attributed almost totally to specific antibody activity.

The metabolic inhibition titers of the specific antisera from both rabbits, particularly those for sera obtained on 1-12-80 and 1-19-80, compare very favorably with those obtained by Williamson et al. (1979) for spiroplasma-specific antisera. These antisera, then, are quite adequate for purification and use in ELISA. The specific antiserum from rabbit No. 110 obtained on 1-12-80 was used for all ELISA testing in this research.

System Evaluation

The visual results of the initial system evaluation are presented in Figures 15 and 16. At the 1:10 antigen dilution, strong reactions were obtained with coating γ-globulin dilutions up to 1:1000 and enzyme-labelled γ-globulin dilutions up to 1:800. As can be seen, the other antigen dilutions resulted in less observable reactions as all reagent dilutions increased. The 1:10000 coating γ-globulin dilution produced only weak reactions at all enzyme-labelled γ-globulin dilutions and was, therefore, immediately disregarded as a possible operating

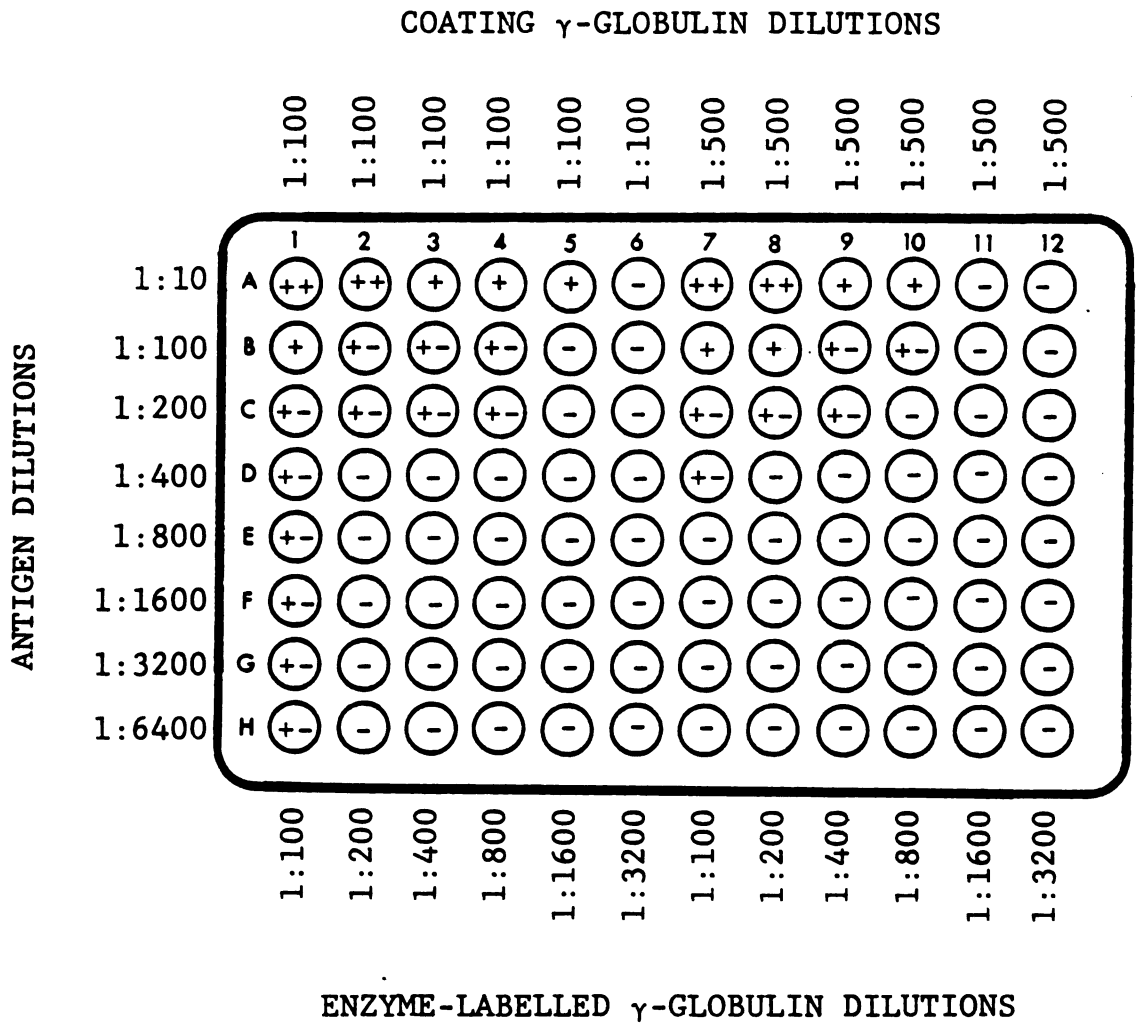
Table 5.--Metabolic inhibition titers for the antisera obtained from the two rabbits used in antiserum generation.

Date	Type	Metabolic Inhibition Titers No. 109	No. 110
9-24-79	NS[a]	0	4,374
11-13-79	S[b]	118,098	118,098
11-19-79	S[b]	354,294	354,294
1-12-80	S[b]	354,294	354,294
1-19-80	S[b]	354,294	354,294
1-31-80	S[b]	NT[c]	NT[c]

[a]Non-specific serum.

[b]Specific serum.

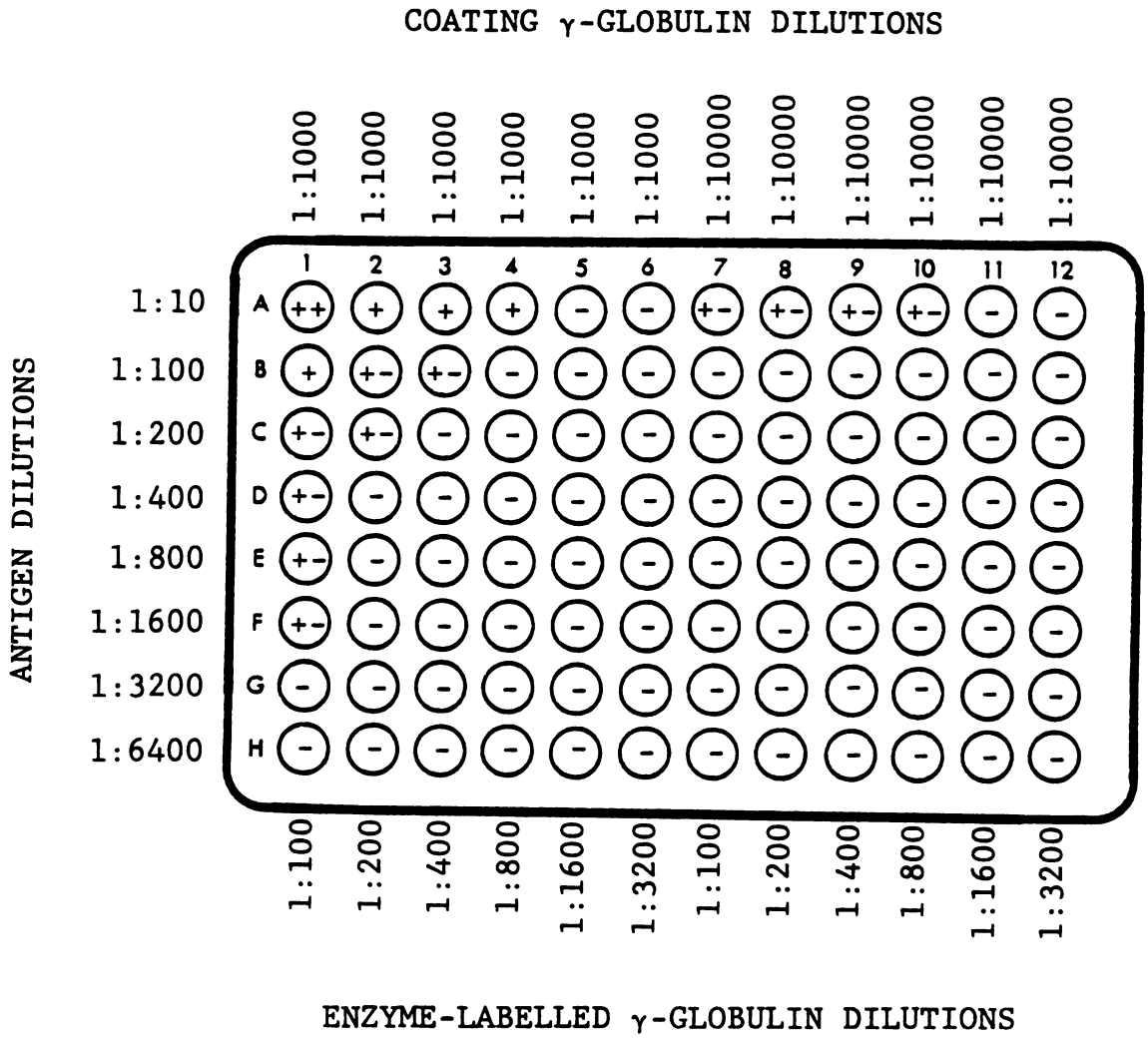
[c]Not tested.



Visual key:

++ very strong reaction
+ strong reaction
+- weak reaction
- no reaction

Figure 15.--Visual results of the ELISA system evaluation test with antigen for plate 1.



Visual key:

++ very strong reaction
+ strong reaction
+- weak reaction
- no reaction

Figure 16.--Visual results of the ELISA system evaluation test with antigen for plate 2.

dilution. This same reasoning eliminated all enzyme labelled γ-globulin dilutions of 1:1600 and above. No observable reactions were obtained from the buffer and media controls.

The simplified system evaluation test showed a more clear delineation of the detection abilities of the various reagent dilutions. There is a distinct decrease in the observable reaction beyond a 1:500 dilution of both coating and enzyme-labelled γ-globulins at the 1:10 antigen dilution. Again, there was no observable reactions in the medium controls.

Based on these two tests, 1:500 dilutions of coating and enzyme-labelled γ-globulins were chosen for all future ELISA testing. In all probability, dilutions of 1:1000, or, possibly, somewhere between 1:500 and 1:1000, would have been adequate. Because ELISA is not dependent upon optimal antigen and antibody concentrations, as are other serological tests, e.g., the ring precipitin test, the less diluted reagents were chosen in order to enhance the chances of antigen detection at low levels.

The spectrophotometer can detect enzymatic reactions far beyond the visual detection level. Had this test been read spectrophometrically, or if a rough estimate of antigen titers in test material was known, the higher dilutions may have been chosen. The development of purified γ-globulin is a time consuming, and sometimes costly, endeavor. Therefore, the highest dilutions of coating and enzyme-labelled γ-globulins that allow for reasonable antigen detection are to be preferred for conservation of these reagents, especially if large numbers of samples are to be tested.

These tests, and those subsequent to it, also reveal that there are no heterologous reactions with the spiroplasma culture media. The method used for

preparing the antigen for injection is adequate in terms of eliminating media contaminants. This is important when evaluating other spiroplasma strains in culture with a given ELISA technique.

Culture Quantification and ELISA Sensitivity

The counting of spiroplasma cells using dark field microscopy resulted in an average of 16.8 cells/field (Table 6). This resulted in a culture concentration of 9.07×10^8 cells/ml. This is in favorable agreement with the results of others using this technique (Liao and Chen 1977). However, it is felt that this may be an overestimate of the spiroplasma cell concentration. The cells were highly motile, moving through all planes of the microscope field, as well as laterally. If anything, cells were counted more than once, rather than any cells being missed. This means that this ELISA is somewhat more sensitive than these results show, but, much care was taken in counting the cells and the concentration estimate is as accurate as can be expected using this technique.

Based on the spiroplasma concentration of 9.07×10^8 cells/ml, each antigen dilution in the sensitivity test was assigned its appropriate concentration. These dilutions, concentrations and A_{405} values for both sonicated and unsonciated antigen are presented in Table 7. At higher spiroplasma cell concentrations, the first two for unsoncated and the first three for sonicated antigen, no absorbance differences are observed due to a maximum amount of enzyme present in the plate wells. A 1:500 dilution of coating γ-globulin results in a set amount of antibody protein, probably at a maximum, being adsorbed onto the surfaces of the plate wells. This means a set amount of antigen may be bound which in turn limits the amount of enzyme-labelled γ-globulin that attaches to bound antigen. The enzyme present in the plate wells, then, will

Table 6.--Number of spiroplasma cells counted using dark field microscopy at a magnification of 1500X.

Field	Slide Number		
	1	2	3
1	24	21	10
2	15	21	13
3	21	15	19
4	18	22	17
5	21	13	15
6	13	15	20
7	23	17	16
8	13	14	19
9	17	10	15
10	18	15	14
Totals:	183	163	158
Means:	18.3	16.3	15.8

Overall Mean: 16.8

Table 7.--Antigen dilutions, cell concentrations and absorbance values for the ELISA sensitivity test.

Antigen Dilution	Cells/ml	Absorbance (405 nm)[a]	
		Unsonicated	Sonicated
1:10	90700000	1.61	1.63
1:20	45350000	1.61	1.62
1:40	22675000	1.54	1.62
1:80	11337500	1.12	1.56
1:100	9070000	1.02	1.56
1:200	4535000	0.56	1.31
1:400	2267500	0.31	0.70
1:800	1133750	0.16	0.43
1:1000	907000	0.15	0.41
1:2000	453500	0.08	0.20
1:4000	226750	0.05	0.10
1:8000	113375	0.04	0.07
Medium Control[b]	-	0.03	0.04

[a]Mean of readings from six wells on two plates.

[b]Mean of readings from 25 wells on two plates.

hydrolyze only so much substrate before it is saturated. Therefore, based on this test, cell concentrations higher than 45,350,000 cells/ml for unsonicated and 22,675,000 cells/ml for sonicated antigen will not be reflected in the A_{405} readings.

It is clear that sonication greatly enhances the sensitivity of ELISA. Below concentrations of 9,070,000 cells/ml, the A_{405} readings are at least doubled for the sonicated antigen, except for the lowest concentration. Using the criterion of a positive result being an A_{405} reading twice that of the controls, i.e., 0.06 and 0.08 for unsonicated and sonicated antigen, respectively, and extrapolating from Table 7, this ELISA detects approximately 302,000 cells/ml of unsonicated and 151,000 cells/ml of sonicated antigen. This confirms that sonication doubles the sensitivity of this ELISA.

The data in Table 7 are plotted in Figure 17 producing enzyme saturation-type curves. From this relationship, standard curves were constructed by plotting the common logarithm of the spiroplasma cell concentration against a modification of the LOGIT transformation of the A_{405} value. The LOGIT transformation is one of several transformations that will convert sigmoid curves to a more linear form (Ashton 1972) and is expressed by the relationship

$$\text{LOGIT } P = \ln [P/(1-P)]$$

where $P = A_{405}$. The A_{405} values were first multiplied by 0.6 to bring them all into the domain (0,1) and from these the LOGIT transformation was applied. In order to gain increased linearity from the resulting relationship, the common logarithm of (LOGIT P) + 5 was used. The total transformation is expressed as

$$y = \log_{10}[\text{LOGIT}(0.6A_{405}) + 5].$$

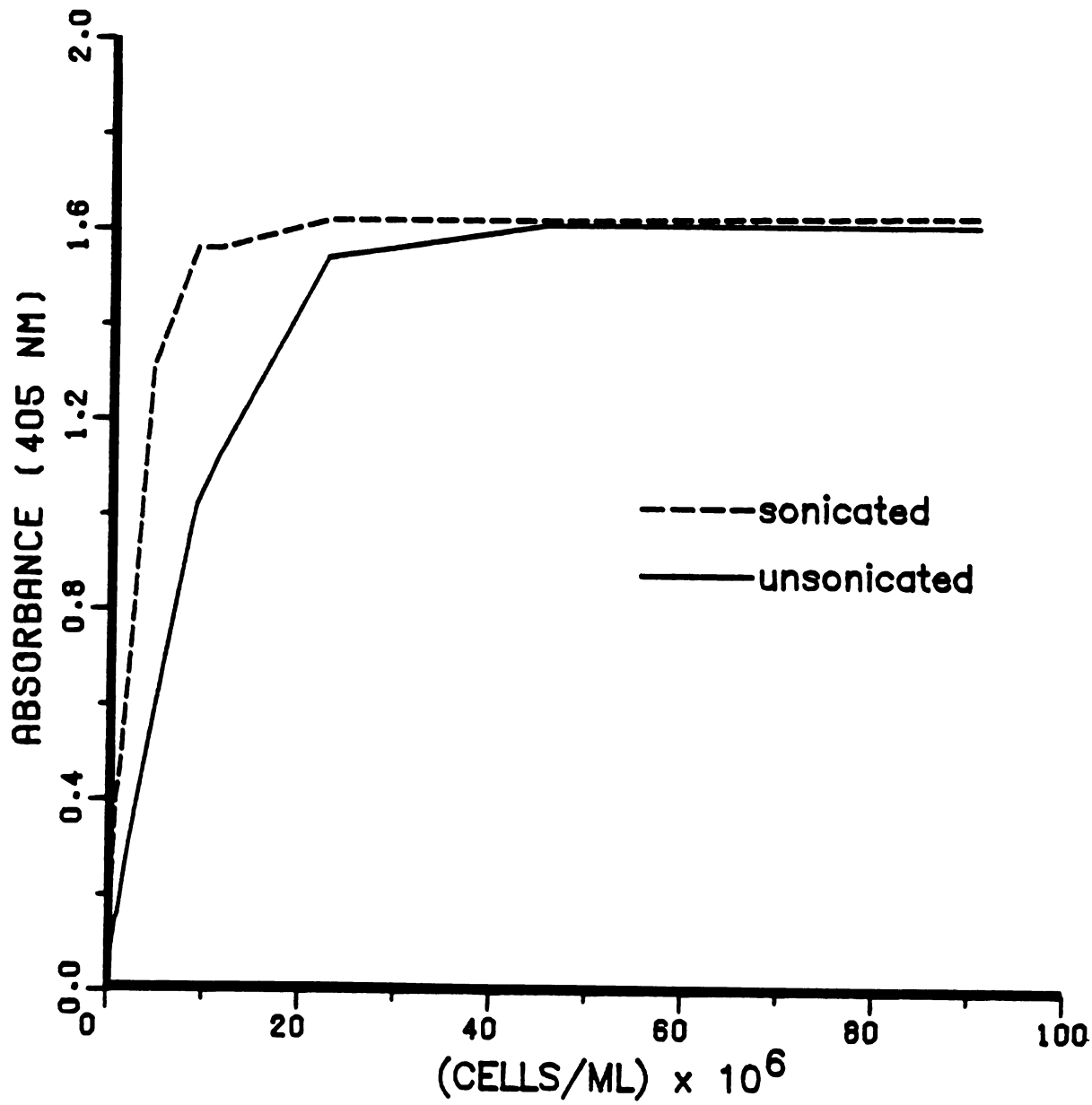


Figure 17.--Absorbance at 405 nm of the various spiroplasma cell concentrations for the ELISA sensitivity test.

The addition of 5 to the LOGIT values is necessary to eliminate negative values so that the common logarithm could be applied.

These transformed data, along with their corresponding regression lines, are plotted in Figure 18. These plots indicate that absorbance is remarkably accurate in predicting spiroplasma cell concentrations in test samples ($y = -1.405 + .304x$, $r = .991$ for unsonicated and $y = -0.932 + .250x$, $r = .970$ for sonicated antigen). Even more accuracy can be obtained if the data points that fall in the area of enzyme saturation, as discussed above, are eliminated. These would be the first point and the first two points for unsonicated and sonicated antigen, respectively. These data are plotted in Figure 19 and show a slight increase in accuracy of prediction ($y = -1.530 + .325x$, $r = .998$ for unsonicated and $y = -1.232 + .301x$, $r = .994$ for sonicated antigen). In order to use the above regression equations for the estimation of spiroplasma cell concentrations from known A_{405} readings, they must be expressed in the form

$$\log_{10}[\text{LOGIT}(0.6A_{405}) + 5] = a + b\,[\log_{10}(x)]$$

where a and b are the regression parameters and x is the spiroplasma cell concentration. Table 8 shows the expected cell concentrations derived from the relationships of Figure 19. For both unsonicated and sonicated antigen, the most accurate cell concentration estimates may be had from A_{405} readings in the range 0.4 to 0.6. Therefore, antigen titer estimates from ELISA results should be made from absorbance readings in this range. In addition, the greater % errors observed for sonicated antigen indicate that some accuracy is sacrificed in order to obtain greater sensitivity. This is most probably due to the inability of sonication to fragment spiroplasma cells equally. This, however, is not a

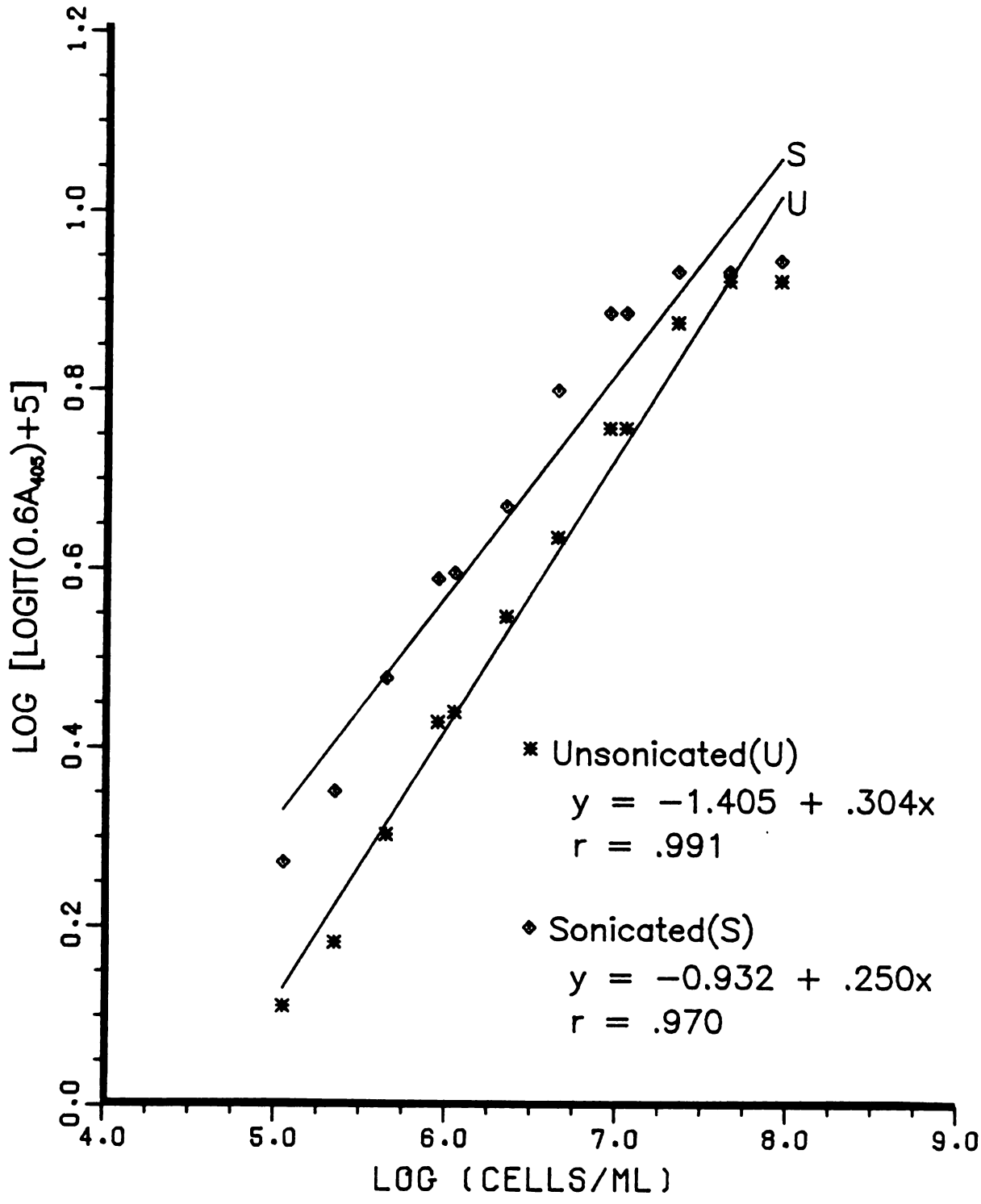


Figure 18.--Standard curves for estimating spiroplasma cell concentrations from known absorbance values.

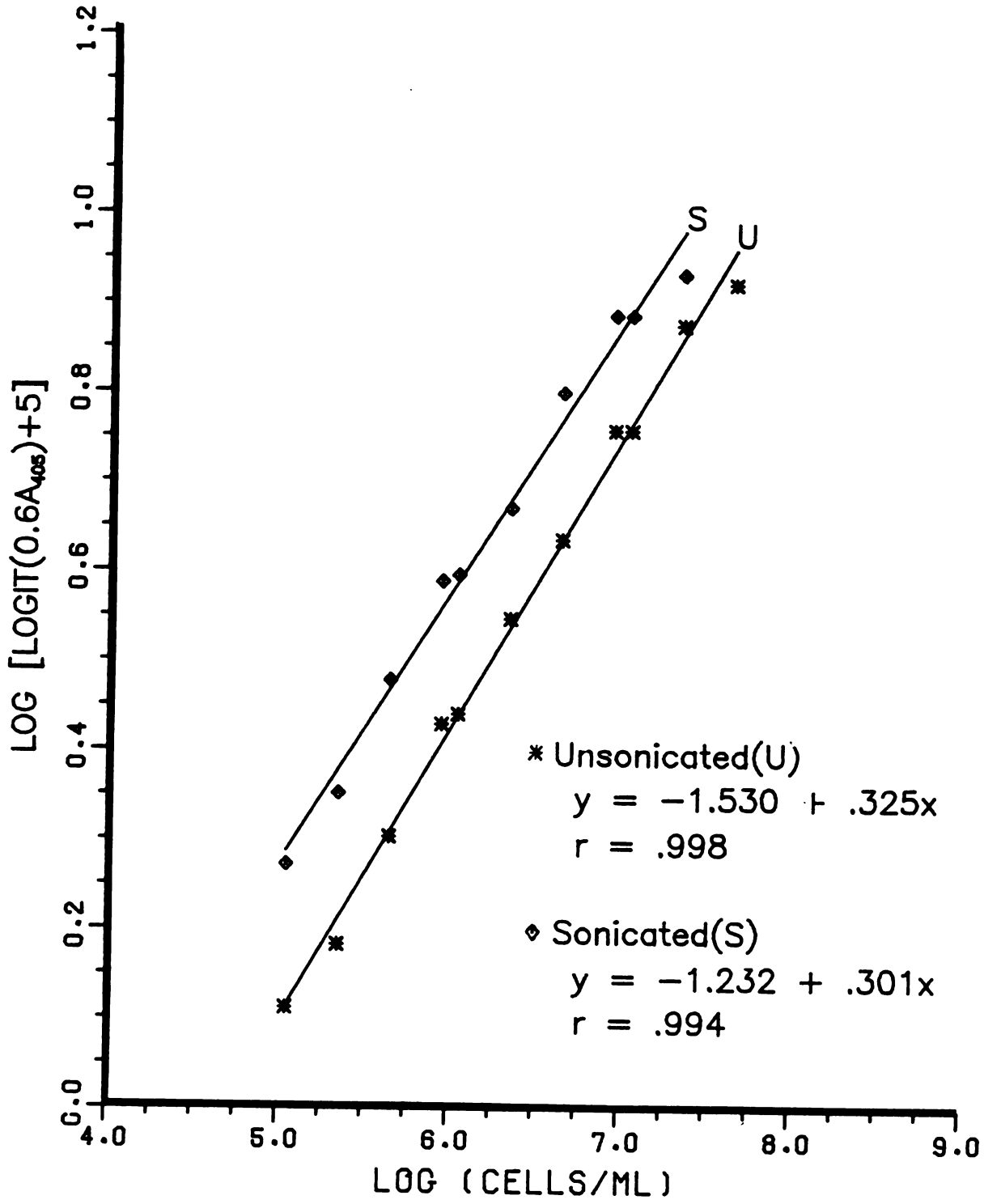


Figure 19.--Modified standard curves for estimating spiroplasma cell concentrations from known absorbance values.

Table 8.--Prediction accuracies derived from the modified standard curves for estimating spiroplasma cell concentrations.

Observed Cells/ml	Unsonicated			Sonicated		
	A_{405}	Expected Cells/ml[a]	%Error	A_{405}	Expected Cells/ml[b]	%Error
90700000	1.61	---[c]	---[c]	1.63	---[c]	---[c]
45350000	1.61	34925210	22.0	1.62	---[c]	---[c]
22675000	1.54	25108940	10.7	1.62	15444550	31.9
11337500	1.12	10901790	3.8	1.56	10837670	4.4
9070000	1.02	9439112	4.1	1.56	10837670	19.5
4535000	0.56	4599013	1.4	1.31	5608727	23.7
2267500	0.31	2459227	8.5	0.70	2084003	8.1
1133750	0.16	1156469	2.0	0.43	1182327	4.3
907000	0.15	1067088	17.7	0.41	1120088	23.5
453500	0.08	438889	3.2	0.20	480596	6.0
226750	0.05	186648	17.8	0.10	182284	19.4
113375	0.04	112906	0.4	0.07	99616	12.1

[a]Found using: $\log_{10}[\text{LOGIT}(0.6A_{405}) + 5] = -1.530 + .325(\log_{10}x)$.

[b]Found using: $\log_{10}[\text{LOGIT}(0.6A_{405}) + 5] = -1.232 + .301(\log_{10}x)$.

[c]Points not used due to enzyme saturation at these antigen concentrations.

significant problem in insect vector-plant-pathogen applications for ELISA, where detection, not quantification, of pathogens is of primary importance.

ELISA Specificity

The A_{405} values for the sonicated antigen dilutions of the 10 spiroplasma isolates tested with ELISA are presented in Table 9. This ELISA does not distinguish between the isolate used to establish the technique, Ex-CL5, and the isolates of Spiroplasma citri (MOROC, ISRAEL, C-189), suspected western X-disease (PYLR-P-5PL), suspected peach yellows (PY-1B-P14) or suspected aster yellows (AY-I6M-P3, AY-I3-P3). At the 1:10 dilution, all isolates, except G-3N and E-P49, are at, or very near, the saturation level. The differences in A_{405} readings at higher dilutions are explained by the varying dilution titers. For example, the dilution titer of the MOROC isolate of S. citri is 1000 times higher than that of the ISRAEL isolate and this is reflected in the A_{405} readings. As discussed earlier, there is some controversy surrounding the spiroplasma isolates associated with X-disease and aster yellows, with some researchers holding that these isolates are S. citri because they cannot be serologically distinguished. These data support this view and help explain the negative results obtained with this ELISA in tests of field-collected tissue (see insect and plant tissue ELISA results). These data also indicate the possible presence of S. citri in Michigan because Ex-CL5 was isolated from tissue collected in Michigan. This could be an important area of follow-up research, especially in light of the newly established etiological role of S. citri in the brittle root disease of horseradish from Illinois (Raju et al. 1981, Fletcher et al. 1981). There is an indication of a possible role of S. citri in the etiology of X-disease. However, it is unknown at this time whether this possible role is pathogenic in nature or simply a non-virulent, occasional dual infection.

Table 9.--Absorbance at 405 nm for four dilutions of ten spiroplasma isolates in pure culture for the ELISA specificity test.

Isolate	Dilution Titer[a]	Absorbance (405 nm)[b,c]			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
PY-1B-P14	10^{8}	1.64	0.76	0.16	0.08
G-3N	10^{6}	0.08	0.06	0.07	0.06
MOROC	10^{10}	1.63	1.60	0.48	0.18
C-189	10^{8}	1.63	1.17	0.36	0.09
Ex-CL5	10^{9}	1.63	1.58	0.66	0.14
AY-I6M-P3	10^{8}	1.62	1.60	0.68	0.26
PYLR-P-5PL	10^{9}	1.65	1.59	0.50	0.22
E-P49	10^{7}	0.07	0.06	0.06	0.08
AY-I3-P3	10^{8}	1.63	1.61	0.62	0.20
ISRAEL	10^{7}	1.59	0.39	0.10	0.07
Medium Control	-	0.05	0.05	0.05	0.05
Antigen Control	-	1.66	1.58	0.47	0.12

[a]Reciprocol of the highest dilution to produce a color change in a dilution series growth test; supplied by Dr. Alan L. Jones.

[b]Mean of two wells from two plates.

[c]All values are for sonicated preparations.

This ELISA did not produce reactions to either of the corn stunt spiroplasma (CSS) isolates, G-3N and E-P49. This is in direct contrast to the ELISA results shown by other researchers where CSS had rather strong reactions with antiserum to S. citri (Bove and Saillard 1979). This may be due to the Ex-CL5 isolate having more specific antigenic determinants than S. citri, i.e., Ex-CL5 may have antigenic determinants in common with S. citri but not with CSS, while S. citri has antigenic determinants in common with both Ex-CL5 and CSS. This is apparently the case indicated by the results presented here as the dilution titers of G-3N and E-P49 cannot account for the lack of a reaction.

Leafhopper Sampling and Testing with ELISA

Tree Sampling

In 418 tree samples taken at both sample sites, a total of 48 leafhoppers were captured. These included 40 Empoasca spp. and one each of Athysanus argentarius Metc., Draeculacephala antica (Walk.), Endria inimica (Say), Erythroneura sp., Gyponana sp., Latalus sayi (Fitch), Paraphlepsius irroratus (Say) and Scaphoideus amplus DeL. & Mohr. These were all males, except for the Gyponana sp. and 13 Empoasca spp. In addition, seven Philaenus spumarius L. (Cercopidae) and 10 Delphacidae were captured. At both sites, the total leaves trapped by the tree samples was 21,303 and the overall leafhopper density was 0.0022 leafhoppers/leaf. At these very low densities, it was decided to terminate the tree sampling as of August 28, 1980, as this method required much time and expense.

It is interesting to note that the known vector, P. irroratus, and the possible vector, Gyponana sp., were both captured in the commercial orchard

within rows immediately adjacent to the alfalfa field bordering the east side. These rows also contained X-diseased trees in the immediate area of the sample. Of course, with only two leafhoppers captured, no conclusions can be drawn, but this may indicate that future sampling schemes should be designed with adjacent habitat in mind.

Twenty-four leafhoppers were captured at each sample site. The densities, however, were 0.0015 and 0.0047 leafhoppers/leaf for the commercial and MSU sites, respectively. The density was more than three times greater at the MSU site, probably reflecting the lack of insecticide use at that location. The number of samples taken at the commercial and MSU sites were 342 and 76, respectively, resulting in 0.07 and 0.32 leafhopper/sample.

These data indicate that the tree sampling method used here was not especially effective for estimating leafhopper densities in the tree canopy, as they were most probably underestimated. However, these estimates cannot be rejected out-of-hand without further data. This would be difficult to obtain, as the sample size required to validate the method would be excessive. The data do, however, reflect the expected trend of lower leafhopper densities in an orchard where insecticides are regularly used as opposed to one where they are not (see Ground Sampling discussion). The future use of this method should be restricted to situations of very high leafhopper density or when very large numbers of samples may be taken.

Ground Sampling

The leafhopper species captured at both sample sites are listed, with their numbers, in Table 10. Of the more than 1100 insects trapped, only one, P. irroratus, was a known vector of the X-disease pathogen. Species that are known

Table 10.--Leafhopper species, Cercopidae, Delphacidae and their total numbers captured in ground samples at the commercial and Michigan State University sample sites.

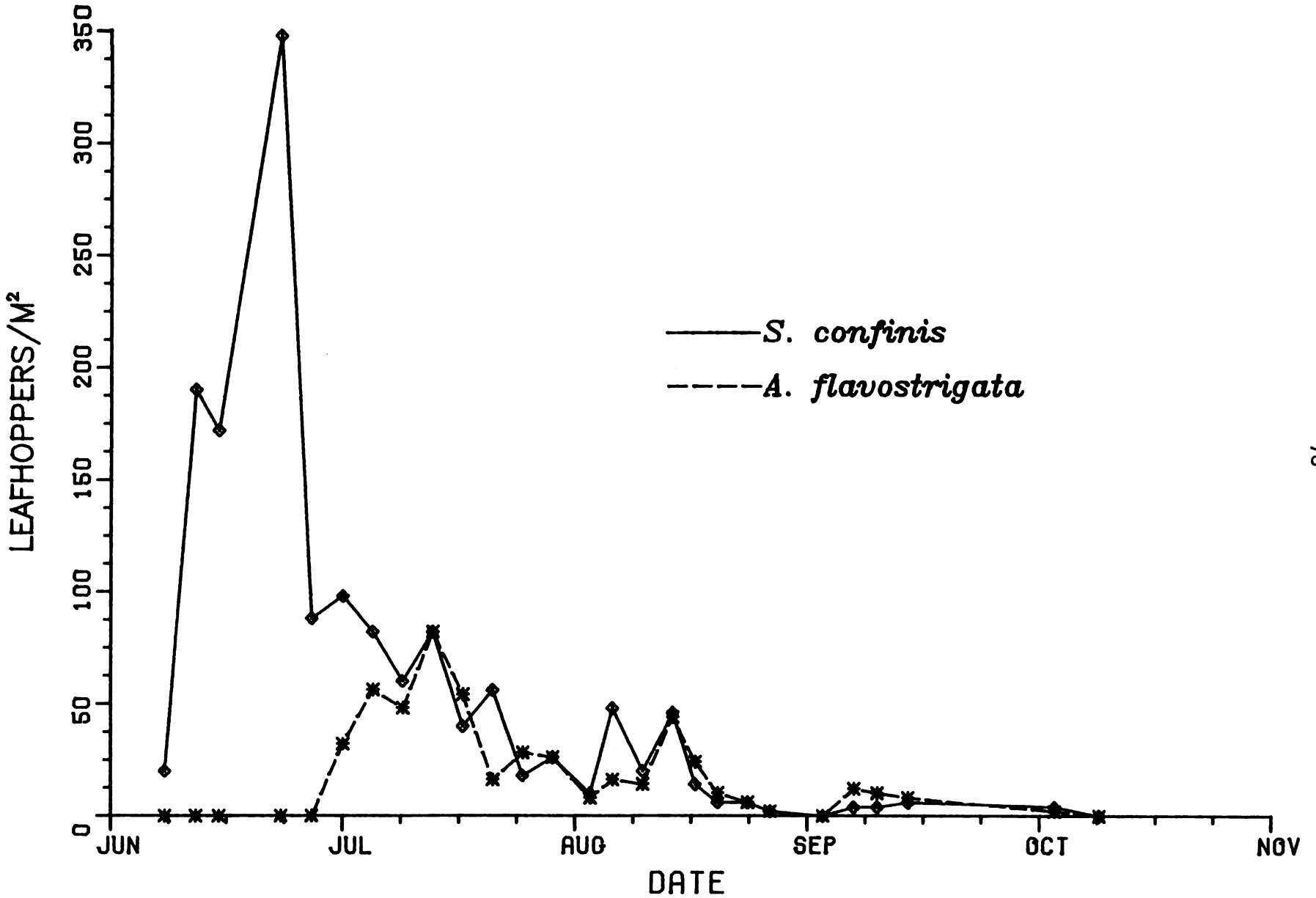
Species	Sample Site COMM	MSU	Total
Streptanus confinis (Reut.)	187	726	913
Aphrodes flavostrigata (Don.)	35	249	284
Athysanus argentarius Metc.	13	54	67
Draeculacephala antica (Walk.)	6	42	48
Psammotettix ferratus (DeL. & Dav.)	1	29	30
Doratura stylata (Boh.)	1	26	27
Aphrodes fuscofaciata (goeze)	4	18	22
Dicraneura mali (Prov.)	8	13	21
Endria inimica (Say)	10	5	15
Latalus sayi (Fitch)	1	9	10
Commellus comma (Van D.)	2	5	7
Macrosteles fascifrons (Stal)	6	0	6
Aphrodes bicincta (Schrank)	1	5	6
Parabolocratus viridis (Uhl.)	1	5	6
Amblysellus curtisii (Fitch)	0	1	1
Psammotettix lividellus	0	1	1
Graminella nigrifrons (Forbes)	1	0	1
Philaenus spumarius L. (Cercopidae)	13	8	21
Delphacidae	1021	98	1119

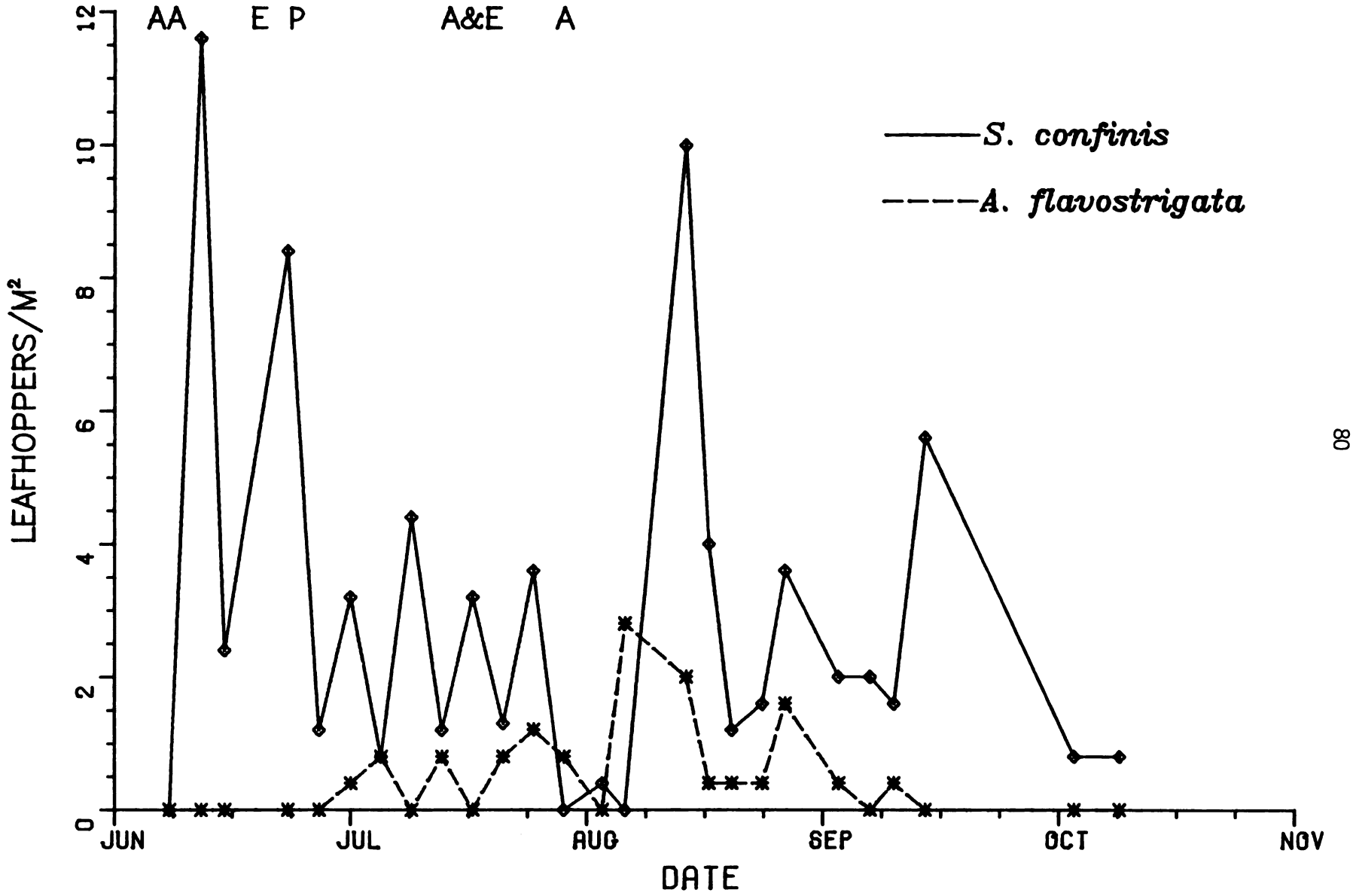
to vector the pathogens of other diseases of mycoplasma etiology include E. inimica, for aster yellows, M. fascifrons, for aster yellows and clover phyllody, A. bicincta, for stolbur disease, European aster yellows and clover phyllody, and G. nigrifrons, for corn stunt (Nielson 1968).

The species complex, as well as the relative numbers captured, at both sites are very similar. The much reduced numbers at the commercial site are undoubtedly due to insecticide applications. The seasonal population trends of the two most numerous species for both sites, S. confinis and A. flavostrigata, are plotted in Figures 20 and 21. The leafhopper density was calculated according to the following:

$$\text{leafhoppers/m}^2 = (n/a)/b$$

where n = number of leafhoppers trapped on a particular date, a = number of samples taken and b = area of the sampling device = 0.125 m^2. It is obvious that insecticides tremendously depress these leafhopper population levels relative to the unsprayed situation, even for those insects in the ground cover. While azinphosmethyl certainly reduces leafhopper numbers, apparently endosulfan, and possibly phosmet, have an even greater effect. What effect this level of leafhopper population reduction may have on the spread of X-disease within an orchard is unknown at this time. It should be noted that following the termination of insecticide applications toward the end of July, the leafhopper populations at the commercial site did tend to increase, but they never approached the densities recorded during the same period at the MSU site. This indicates that a thorough examination of the relationship between leafhopper numbers and the spread of X-disease must be made before late season insecticide applications can be recommended for leafhopper vector control. It should be


LEAFHOPPERS/M²
0
50
100
150
200
250
300
350
JUN
JUL
AUG
SEP
OCT
NOV
DATE
S. confinis
A. flavostrigata


AA
E P
A&E
A
S. confinis
A. flavostrigata
LEAFHOPPERS/M²
0
2
4
6
8
10
12
JUN
JUL
AUG
SEP
OCT
NOV
DATE

kept in mind, also, that the depression of leafhopper population levels in the ground cover follow insecticide applications to the tree canopy. It might be expected that ground cover insecticide applications would further reduce populations but, again, the necessity for this level of reduction remains to be proven.

In the commercial orchard, the number of Delphacidae captured in the ground cover was more than ten times that for the MSU site. This is probably due to the insecticide applications which may have induced an increase in delphacid populations by eliminating natural enemies.

Due to the fact that almost no vector species were captured in this research, the spatial distribution of leafhoppers within the orchard was investigated using S. confinis. This was done because S. confinis was (1) the most numerous leafhopper trapped, (2) it is closely related, taxonomically, to some of the more important known vectors, being in the subfamily Deltocephalinae and the tribe Deltocephalini which also contains the genera Paraphlepsius, Colladonus, Norvellina, Fieberiella and Scaphoideus, and, (3) it has been observed on yellow sticky board traps, along with P. irroratus, at a height of approximately 1.8 m in cherry trees (T. Mowry, personal observation). In addition, spatial analysis was performed only for the commercial site because the MSU site was too small to reflect any spatial distribution differences and historical data for the distribution of X-disease were only available for the commercial site.

Table 11 records the frequencies of the leafhopper counts for all sample units taken on each of the 29 sampling days. In attempting to fit the leafhopper count frequencies to known, discreet frequency distributions on a preliminary basis with the χ^2 test for goodness-of-fit (Elliott 1977), 11 samples fit the negative binomial distribution (NBD), indicating aggregation, 12 samples fit the

Table 11.--Frequencies of Streptanus confinis for all sampling days at the commercial sample site in 1980.

Date	n	Leafhopper Counts 0	1	2	3	4	5	6	7	8	9	10	Total
06/09	20	20	-	-	-	-	-	-	-	-	-	-	0
06/13	20	14	1	-	1	2	-	-	-	1	1	-	29
06/16	20	18	-	1	-	1	-	-	-	-	-	-	6
06/20						SAMPLE SPOILED							
06/24	20	12	4	2	-	1	-	-	-	-	1	-	21
06/28	20	18	1	1	-	-	-	-	-	-	-	-	3
07/02	20	15	3	1	1	-	-	-	-	-	-	-	8
07/06	12	10	2	-	-	-	-	-	-	-	-	-	2
07/10	20	17	-	-	1	2	-	-	-	-	-	-	11
07/14	20	17	3	-	-	-	-	-	-	-	-	-	3
07/18	20	17	-	2	-	1	-	-	-	-	-	-	8
07/22	16	13	3	-	-	-	-	-	-	-	-	-	3
07/26	20	15	4	-	-	-	1	-	-	-	-	-	9
07/30	20	20	-	-	-	-	-	-	-	-	-	-	0
08/04	20	19	1	-	-	-	-	-	-	-	-	-	1
08/07	20	20	-	-	-	-	-	-	-	-	-	-	0
08/11						RAINED OUT							
08/15	20	12	1	1	4	-	2	-	-	-	-	-	25
08/18	20	16	2	-	1	-	1	-	-	-	-	-	10
08/21	20	17	3	-	-	-	-	-	-	-	-	-	3
08/25	20	17	2	1	-	-	-	-	-	-	-	-	4
08/28	20	17	1	-	-	2	-	-	-	-	-	-	9
09/01						RAINED OUT							
09/04	20	17	2	-	1	-	-	-	-	-	-	-	5
09/08	20	15	5	-	-	-	-	-	-	-	-	-	5
09/11	20	17	2	1	-	-	-	-	-	-	-	-	4
09/15	20	16	2	1	-	-	-	-	-	-	-	1	14
10/04	20	18	2	-	-	-	-	-	-	-	-	-	2
10/10	20	18	2	-	-	-	-	-	-	-	-	-	2
Totals	508	425	46	11	9	9	4	0	0	1	2	1	187

Poisson distribution, indicating randomness, and the rest could not be analyzed due to no counts being recorded. It is believed that the random distribution results were due to low leafhopper counts rather than the actual spatial pattern of the insects. Agreement is made with Pielou (1977) that it would be unreasonable to postulate that the spatial pattern was random. Therefore, the samples were lumped on approximately a weekly basis to produce 10 combined samples that allowed more reasonable spatial analysis. These are reflected in Table 12.

For the purpose of comparing the spatial pattern of the leafhoppers with that of the X-diseased trees in the commercial orchard, it was necessary to know if it could be safely assumed that the leafhopper spatial pattern was aggregated throughout the entire season. Each of the 10 combined samples was analyzed for goodness-of-fit to the NBD using the Kolmogorov-Smirnov test (Sokal and Rohlf 1969). For this test, the mean (x) and variance (s^2) of each sample were calculated along with the parameter k of the NBD using the procedures described by Elliott (1977). Initial estimates of k were calculated using the moment estimation method according to the following equation:

$$k = {}_x{}^2/(s^2-x).$$

More accurate estimates were calculated using the iterative maximum-likelihood equation

$$n \cdot \ln(1 + x/k) = \Sigma [A_x/(k+x)]$$

where n = sample size, x = frequency class (leafhopper count), A_x = total number of counts exceeding x and i = total number of frequency classes. The results of these analyses are presented in Table 13.

Table 12.--Frequencies of Streptanus confinis for combined sampling days at the commercial sample site.

Date	n	Leafhopper Counts 0	1	2	3	4	5	6	7	8	9	10	Total
06/09-06/16	60	52	1	1	1	3	-	-	-	1	1	-	35
06/24-06/28	40	30	5	3	-	1	-	-	-	-	1	-	24
07/02-07/10	52	42	5	1	2	2	-	-	-	-	-	-	21
07/14-07/18	40	34	3	2	-	1	-	-	-	-	-	-	11
07/22-07/30	56	48	7	-	-	-	1	-	-	-	-	-	12
08/04-08/15	60	51	2	1	4	-	2	-	-	-	-	-	26
08/18-08/21	40	33	5	-	1	-	1	-	-	-	-	-	13
08/25-08/28	40	34	3	1	-	2	-	-	-	-	-	-	13
09/04-09/11	60	52	6	1	-	-	-	-	-	-	-	1	18
Totals	508	425	46	11	9	9	4	0	0	1	2	1	187

Table 13.--Fit of the negative binomial distribution to the observed combined sample data from the commercial sample site.

Date	n	$\bar{x}$	s^2	k	df	$D^{a,b}$
06/09-06/16	60	0.58	3.16	0.063	10	0.036
06/24-06/28	40	0.60	2.55	0.212	10	0.027
07/02-07/10	52	0.40	0.99	0.190	5	0.028
07/14-07/18	40	0.28	0.61	0.172	5	0.013
07/22-07/30	56	0.21	0.54	0.222	6	0.028
08/04-08/15	60	0.43	1.37	0.100	6	0.045
08/18-08/21	40	0.33	0.89	0.192	6	0.023
08/25-08/28	40	0.33	0.89	0.132	5	0.027
09/04-09/11	60	0.23	0.32	0.700	4	0.008
09/15-10/10	60	0.30	1.77	0.101	11	0.031

[a]Kolmogorov-Smirnov test statistic.

[b]No values were significant at any α. All samples fit the NBD.

All of the combined samples fit the NBD (Table 13) which is an indication of aggregation throughout the season. However, the application of a common k (k_c), if possible, is a better indication that a particular level of aggregation is a fairly constant characteristic of a population (Elliott 1977). It is permissible to derive a k_c for a series of samples if there is no relationship between x and k (expressed at 1/k). Plotting these parameters, there was no apparent relationship, indicating that a common factor of aggregation exists (Figure 22). Based on this information, four k_c's were calculated. The first of these, k_{c1}, was a moment estimate of k_c described by Elliott (1977) based on the statistics x' and y' where

$$x' = x^2 - s^2/n \quad \text{and} \quad y' = s^2 - x$$

which were calculated for each sample. The estimate of k_c is

$$k_{c1} = \Sigma y'/\Sigma x'$$

over all samples. The second estimate, k_{c2}, was a weighted estimate described in detail by Bliss and Owen (1958). A FORTRAN computer program to generate k_{c1} and k_{c2} is listed in Appendix D. The third estimate, k_{c3}, was the arithmetic name of the k's for each of the 10 combined samples. Finally, the fourth estimate, k_{c4}, was a maximum-likelihood estimate using the total seasonal data as one large sample, i.e., the "TOTALS" line in Table 12.

These four estimates of k_c were used, in turn, to refit each of the observed combined samples to the NBD (Table 14). These indicate that the spatial pattern of S. confinis in the commercial orchard was aggregated throughout the seasonal sampling period. It is, therefore, possible to compare the total seasonal spatial

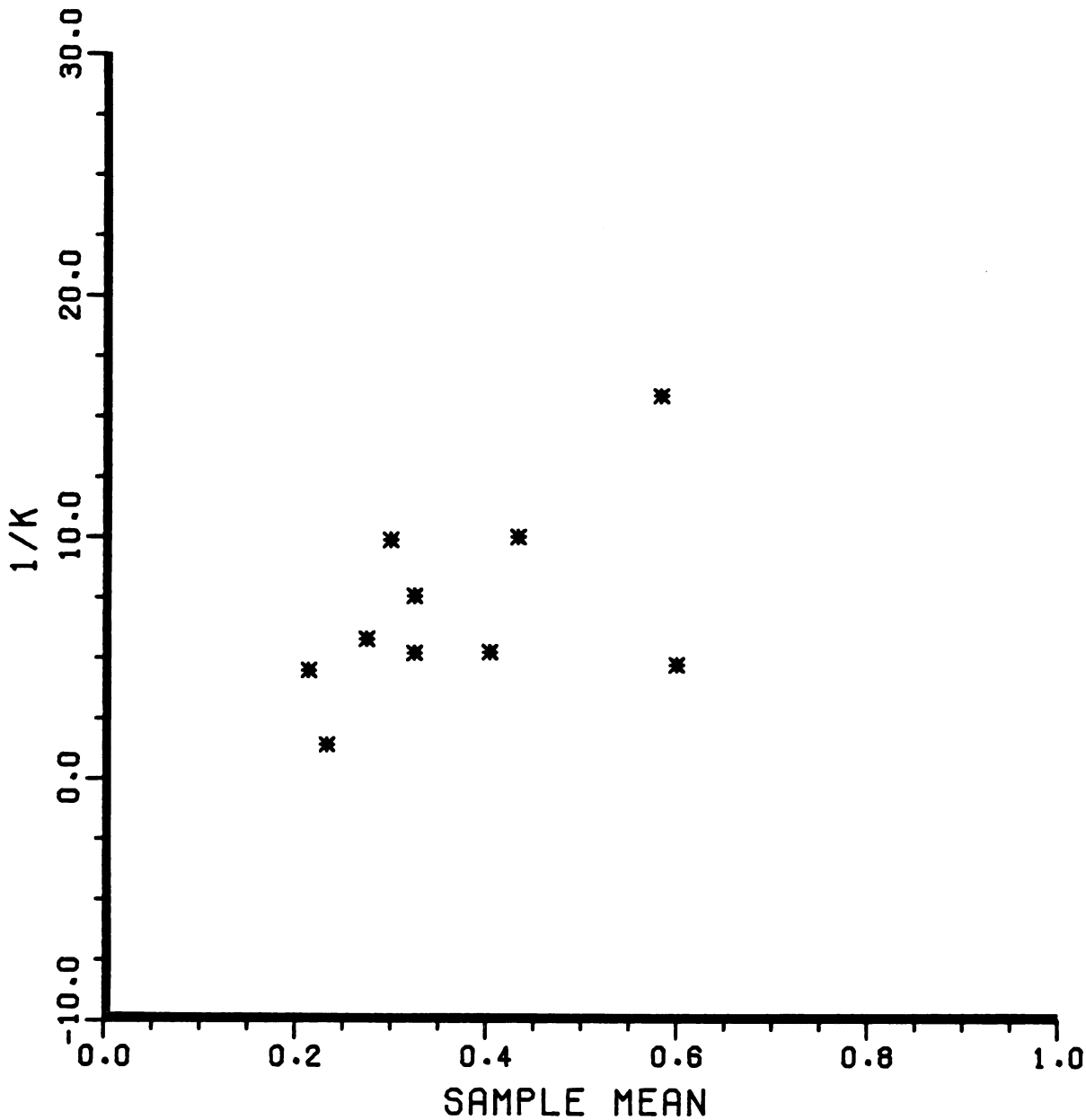


Figure 22.--Negative binomial parameter k as a function of the sample mean for each combined sample from the commercial sample site.

Table 14.--Fit of the negative binomial distribution to the observed combined sample data using four estimates of common k.

Date	n	$\bar{x}$	s^2	$D^{a,b,c}$				df
				k_{c1}	k_{c2}	k_{c3}	k_{c4}	
06/09-06/16	60	0.58	3.16	0.068	0.064	0.112	0.069	10
06/24-06/28	40	0.60	2.55	0.046	0.050	0.027	0.045	10
07/02-07/10	52	0.40	0.99	0.026	0.026	0.030	0.026	5
07/14-07/18	40	0.28	0.61	0.014	0.014	0.014	0.014	5
07/22-07/30	56	0.21	0.54	0.030	0.030	0.028	0.030	6
08/04-08/15	60	0.43	1.37	0.045	0.045	0.061	0.045	6
08/18-08/21	40	0.33	0.89	0.023	0.025	0.023	0.022	6
08/25-08/28	40	0.33	0.89	0.027	0.026	0.033	0.027	5
09/04-09/11	60	0.23	0.32	0.057	0.059	0.037	0.056	4
09/15-10/10	60	0.30	1.77	0.033	0.033	0.038	0.033	11

[a]Kolmogorov-Smirnov test statistic.

[b]No values were significant at any α. All samples fit the NBD.

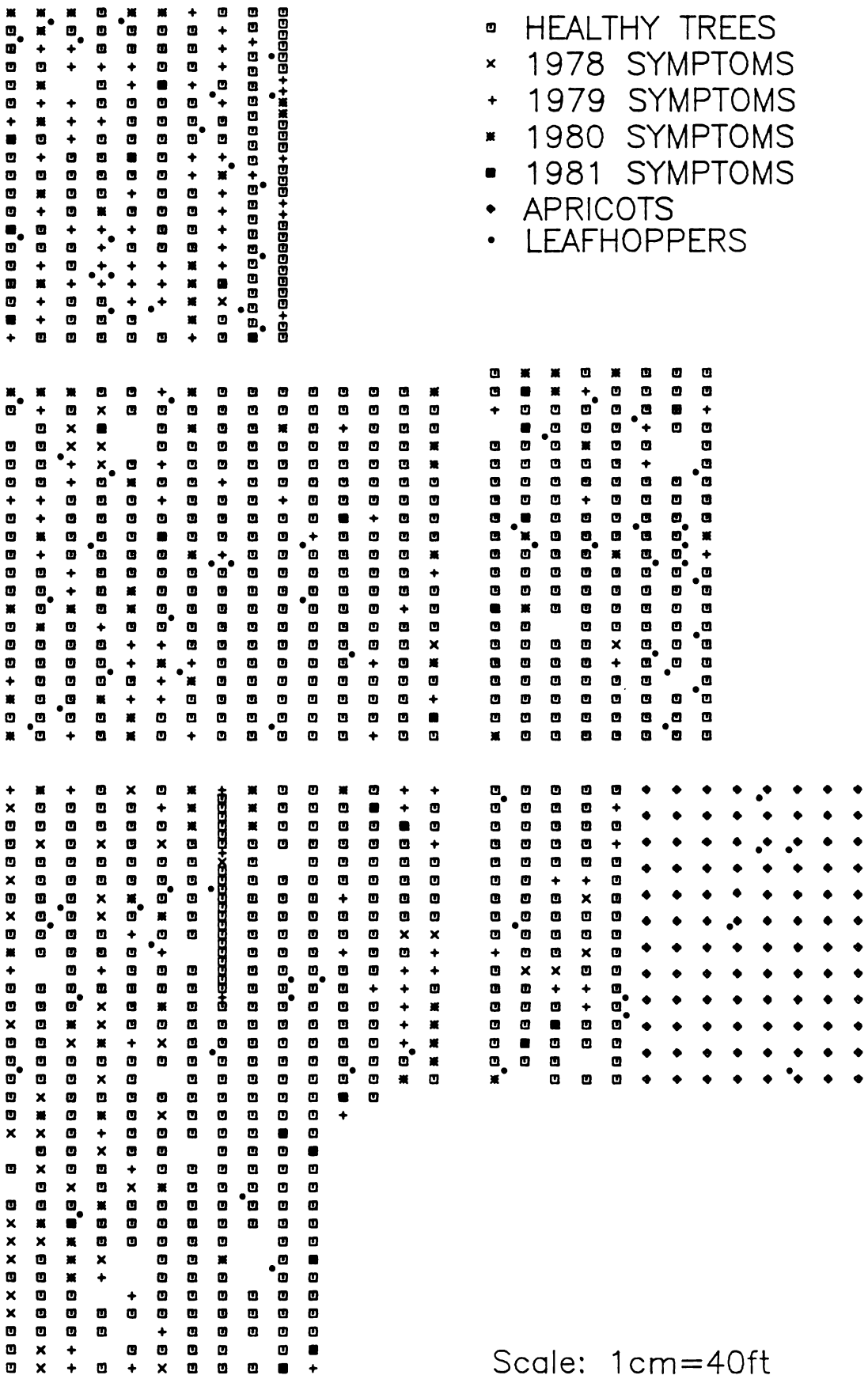
[c]$k_{c1} = 0.135$; $k_{c2} = 0.128$; $k_{c3} = 0.214$; $k_{c4} = 0.136$.

pattern for the leafhopper with that of the X-diseased trees in the orchard. In addition, this seasonal pattern of aggregation strongly indicates that the population of S. confinis was resident to the commercial orchard. However, the possibility exists that immigrating leafhoppers would seek out those orchards best suited for their survival. In either case, the fact that they are aggregated is important in terms of their spatial relationship to X-diseased trees which, if coincidental, would allow for control measures to be applied with, at least, some level of confidence. To further address this subject, the spatial pattern of the X-diseased trees in the commercial orchard was analyzed.

Figure 23 is a to-scale map of the commercial orchard showing the location of X-diseased trees and the year in which they were first observed to have symptoms. Fifty random locations were located on the map using the random sample program for ground sampling (Appendix C). Seven quadrats were constructed, each twice the area of the quadrat immediately preceding it, with the first representing 576 ft^2 (51.8 m^2). Each quadrat was placed on the 50 random locations so that the location was at their exact centers and the number of X-diseased trees falling with the quadrat was recorded. From these data, Morisita's index of dispersion, I_δ, was calculated according to the following equation:

$$I_\delta = n[\Sigma(x^2) - \Sigma x] / [(\Sigma x)^2 - \Sigma x]$$

where n = number of sample units = 50 and x = number of X-diseased trees in a sample unit (Morisita 1959). The spatial pattern is judged as random if $I_\delta = 1$, aggregated if $I_\delta > 1$ and regular if $I_\delta < 1$. Departures from randomness are tested against the x^2 distribution by calculating the test statistic as


HEALTHY TREES
1978 SYMPTOMS
1979 SYMPTOMS
1980 SYMPTOMS
1981 SYMPTOMS
APRICOTS
LEAFHOPPERS
Scale: 1cm=40ft

$$X^2 = I_\delta(\Sigma x-1) + n - \Sigma x$$

with n-1 degrees of freedom (Elliott 1977). Table 15 lists the important statistics obtained in using this method.

The largest value of I_δ was obtained for the quadrat size 16q (9216 ft^2; 829.4 m^2). This can also be seen by plotting quadrat size against I_δ (Figure 24). A plot of this shape indicates an aggregated spatial pattern with uniform intra-clump distribution (Elliott 1977). This is precisely what would be expected in an orchard where the trees are planted in a uniform manner and the spatial pattern of X-disease is aggregated. An estimate of the size of the clumps may be obtained by plotting the ratio

$$(I_\delta \text{ for quadrat } q)/(I_\delta \text{ for quadrat } 2q)$$

against quadrat size 2q and repeating the process for each successive pair of quadrats. In this graph, peaks will occur where quadrat size is approximately equal to clump size. Figure 25 reveals that the smallest approximate size occurs at quadrat size 8q (4608 ft^2; 414.7 m^2). Another peak apparently occurs at some point beyond 64q indicating a larger clump may exist made up of the smaller clumps. This phenomenon may reflect the "artificial clumps" formed by the division of the orchard by tractor paths or the actual peak may occur at a point where the quadrat size is approximately equal to the size of the orchard which, obviously, forms one large clump of X-diseased trees. In any case, the larger clump size is of little statistical or biological interest at this point. This analysis shows that the population of X-diseased trees in the commercial orchard has an aggregated spatial pattern consisting of clumps with a mean size of approximately 4600 ft^2 (414 m^2).

Table 15.--Morisita's index of dispersion and associated statistics obtained by quadrat sampling of the commercial sample site.

Quadrat Size[a]	$\bar{x}$	s^2	Σx	Σx^2	$(\Sigma x)^2$	I_δ	X^{2b}
q	0.94	0.94	47	91	2209	1.018	49.83
2q	0.98	1.18	49	107	2401	1.233	60.18
4q	1.52	2.30	76	230	5776	1.351	75.33
8q	3.96	8.84	198	1226	39204	1.318	111.65
16q	6.06	19.10	303	2791	91809	1.359	157.42
32q	13.6	66.24	680	12560	462400	1.286	243.19
64q	26.2	149.56	1310	41800	1716100	1.181	285.93

[a] $q = 576\ ft^2\ (51.8\ m^2)$.

[b] $\chi^2_{(.05)} = 66.34$; df = 49.

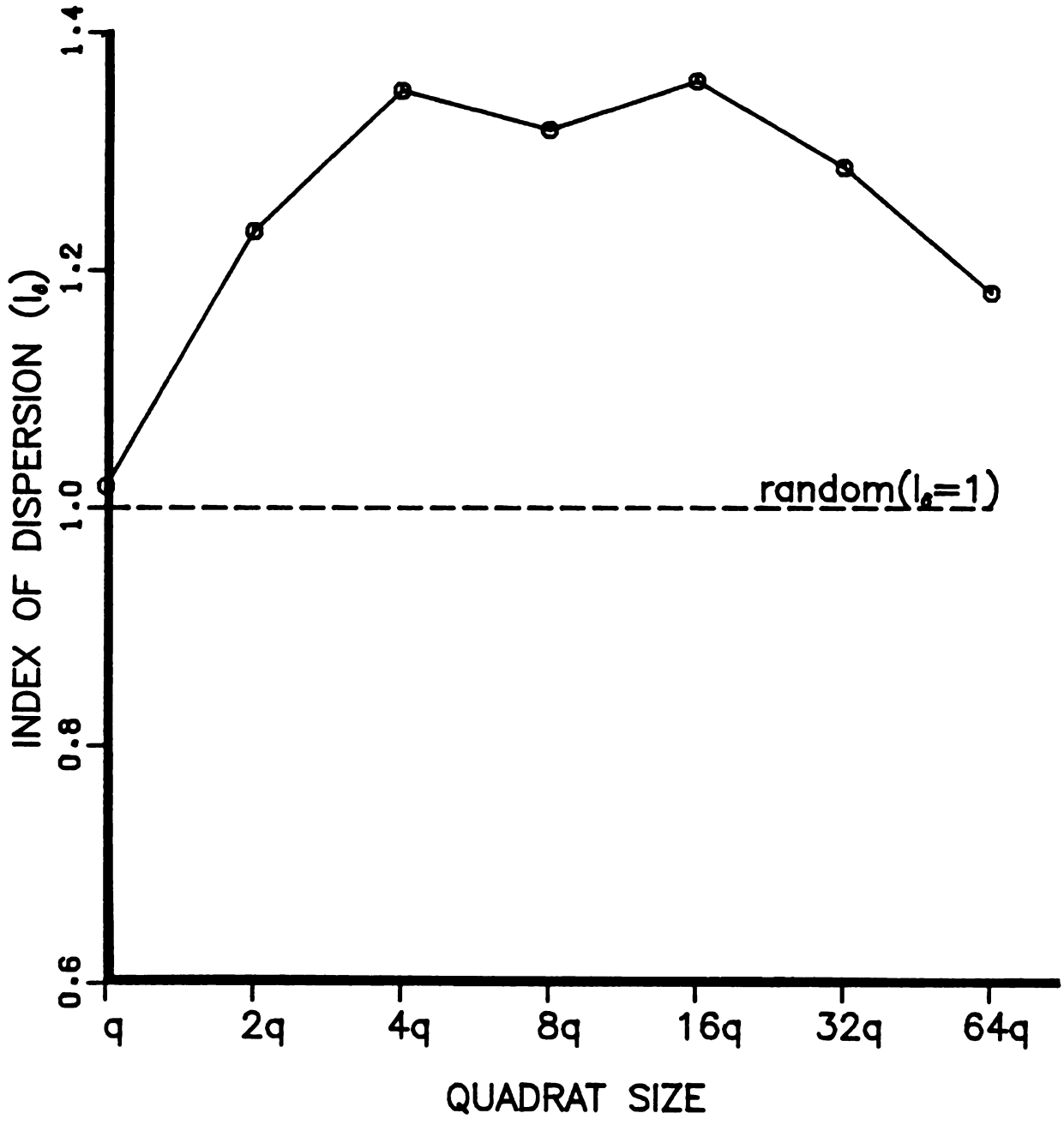


Figure 24.--Morisita's index of dispersion as a function of quadrat size for the commercial sample site.

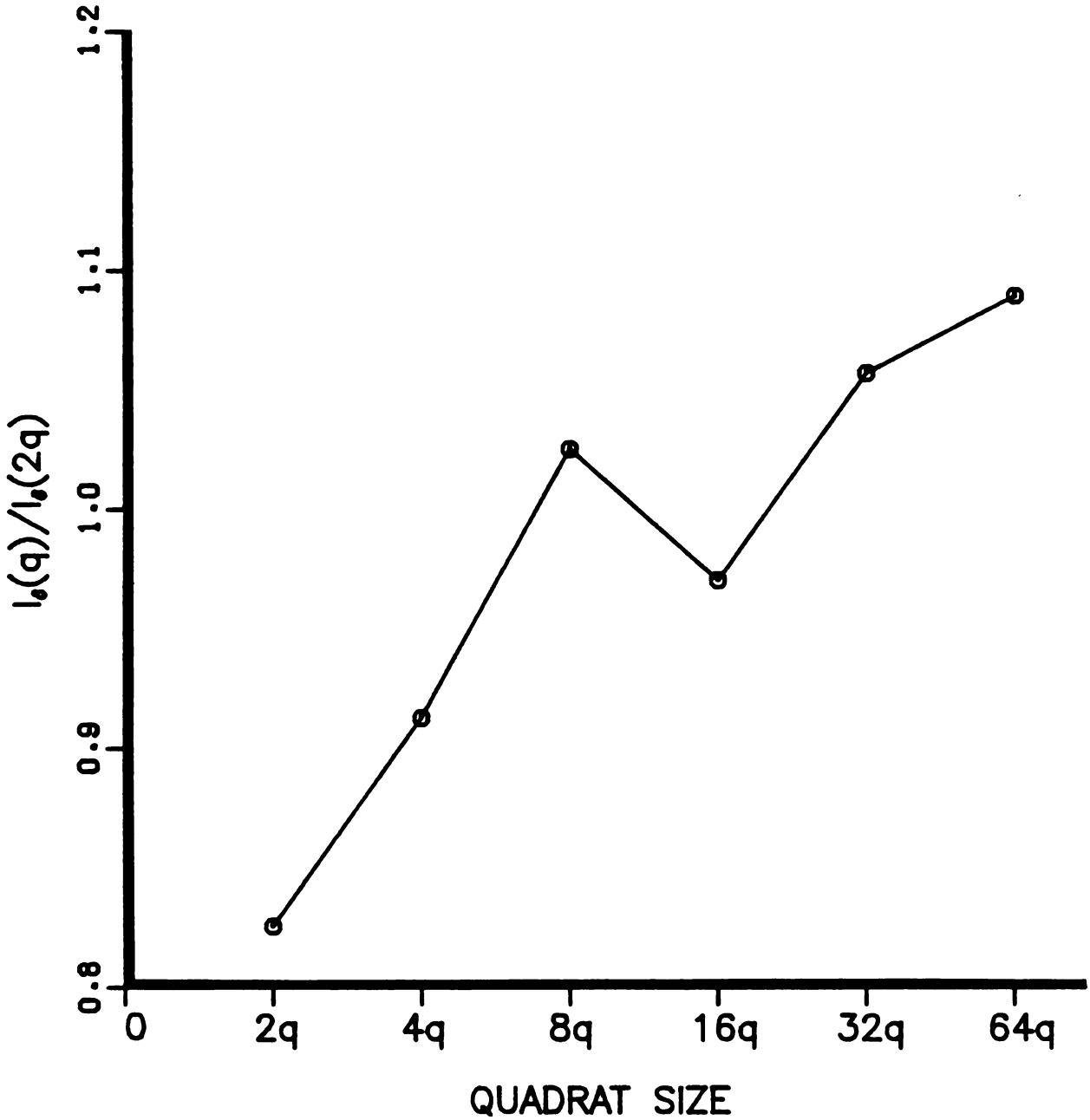


Figure 25.--Morisita's index of dispersion used to estimate the in-field clump size of X-diseased trees for the commercial sample site.

The mean clump size of the X-diseased trees was used as a basis for comparing the spatial patterns of diseased trees and leafhoppers. One hundred random sample locations were located on the map in Figure 23 and these were sampled as described above, but using only the 8q quadrat. For each sample, the presence or absence of X-diseased trees, along with the year of symptom expression, and leafhoppers were recorded. This data was arranged into 2 x 2 contingency tables for each year of X-disease symptoms expression and for all years combined (Table 16). The χ^2 test for independence was applied to the data in these tables and the resulting X^2 values were compared to the tabular value at $\alpha = 0.05$ with one degree of freedom (Sokal and Rohlf 1969).

The spatial patterns of X-diseased trees with symptom expression in the years 1978 and 1980 occur in a dependent manner with the leafhopper spatial distribution, while those for 1979, 1981 and all trees occur independently. Figure 26 shows the increase in percent of X-disease symptom expression from 1978 to 1981. The largest increase in symptom expression occurred in 1979, which was independent of the leafhopper spatial pattern. The 1981 symptom expression, which was the lowest, was also independent of the leafhopper spatial pattern. Two reasons are seen for this. First, there were too few diseased trees in 1981 (25) to allow for effective analysis and, second, 14 of the trees showing symptoms in 1981 occurred immediately adjacent to trees showing symptoms in 1979 with only 1 and 5 trees being adjacent to those showing symptoms in 1978 and 1980, respectively.

If it is assumed that the population of S. confinis sampled here was a resident one, which seems a reasonable assumption, then it can be postulated that a within-orchard spread of X-disease in the range of 2-6% occurs annually

Table 16.--Two-way contingency tables for the presence or absence sampling of X-diseased trees and leafhoppers captured at the commercial sample site.

X-DISEASED TREES

LEAFHOPPERS

1978 Symptoms

	Present	Absent	Totals
Present	18	50	68
Absent	15	17	32
Totals	33	67	100

$X^2 = 4.097$

1979 Symptoms

	Present	Absent	Totals
Present	60	8	68
Absent	26	6	32
Totals	86	14	100

$X^2 = 0.882$

1980 Symptoms

	Present	Absent	Totals
Present	47	21	68
Absent	14	18	32
Totals	61	39	100

$X^2 = 5.886$

1981 Symptoms

	Present	Absent	Totals
Present	23	45	68
Absent	11	21	32
Totals	34	66	100

$X^2 = 0.003$

All Symptoms

	Present	Absent	Totals
Present	64	4	68
Absent	32	0	32
Totals	96	4	100

$X^2 = 1.961$

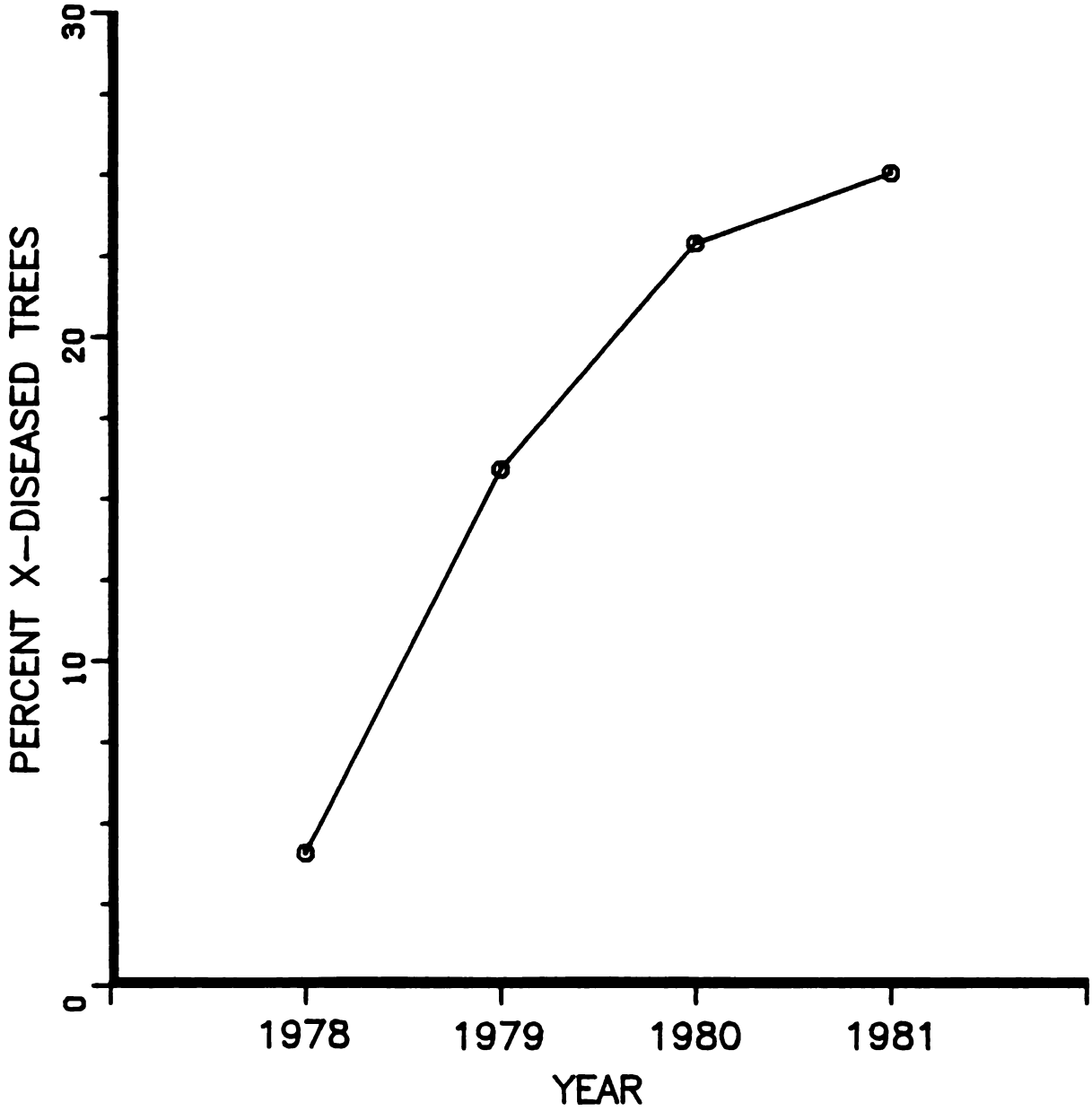


Figure 26.--Percent of X-diseased trees for each year from 1978 to 1981 for the commercial sample site.

once the disease has been established within the orchard. The largest increase in symptom expression may be due to a migrating vector population the previous year. This may very well represent the situation in light of the fact that no known vectors were trapped in the 1980 samples. Unfortunately, no previous leafhopper population data are available for this location to incorporate in this analytical approach. This points out the need to monitor leafhopper populations in a spatial manner over a period of years so that comparisons with the incidence of X-disease may be made.

In 1979, X-diseased chokecherry was essentially eradicated within at least one mile surrounding the commercial orchard. If chokecherry alone were the most important factor in X-disease spread one would expect more than 4.08% diseased trees through 1978. It seems apparent that vector movement is extremely important in the spread of the disease from inoculum sources outside the orchard. Once the disease is established within the orchard, however, there appears to be a low level of within-orchard spread possibly attibutable to resident leafhopper populations. Larger increases than this low level, which would probably vary with different orchards, are likely the result of immigrating leafhoppers carrying the pathogen from outside inoculum sources.

Leafhopper Testing with ELISA

From the ground samples, 720 leafhoppers and delphacids were tested individually with ELISA. An additional 970 insects were tested in groups of 10. In none of these tests was a positive result obtained. As none of these insects were known vectors of X-disease pathogen, it may be that none were carrying the pathogen. As discussed earlier (see ELISA Specificity), this ELISA technique probably is not capable of detecting the X-disease pathogen. The organism used

to generate the antiserum for this technique is indistinguishable from Spiroplasma citri and there was some doubt about it being the X-disease pathogen from the beginning because it was never able to produce X-disease symptoms in laboratory experiments. This demonstrates the importance of isolating, in pure culture, the X-disease pathogen. This should be the first goal in future X-disease research.

Plant Testing with ELISA

Field Collected Plant Tissue

Of all the field-collected plants tested with ELISA, only two gave possible positive results. These were an apparently healthy milkweed (Asclepias syriaca L.) sample and an X-diseased peach (Prunus persica L. Batsch) leaf sample taken from the same location in southwest Michigan (location: T35 R15W Sec 20 NW 1/4). These samples had A_{405} readings of 0.13 and 0.12, respectively. These readings were the mean of four wells and were considered positive when compared to the healthy peach and culture medium controls that both had A_{405} readings of 0.06 in this test. Of more than 350 tests involving 28 different plant species, only these two gave positive results, indicating that the X-disease pathogen was not detected due to very low pathogen titers and/or the inability of this ELISA to detect the actual pathogen. The positive results may indicate the possibility of an occasional dual infection of S. citri in X-diseased peach tissue or its presence in other plants, viz., milkweed, in this area of the country. None of the attempts to localize the X-disease pathogen in plant tissue was successful.

The effects of plant homogenates on ELISA results can be seen in Table 17. A number of plant homogenates signficantly reduced the A_{405} reading relative

Table 17.--Effects of field-collected plant homogenates on ELISA results.

Plant	Absorbance (405 nm)[a] Uninoculated	Inoculated	Variance
Black Cherry - fruit *Prunus serotina* Ehrh.	0.05	1.54	0.0015
Black Cherry - leaves	0.05	1.58	0.0006
Black Cherry - stems	0.05	1.61	0.0004
Peach - leaves *Prunus persica* L. Batsch	0.12	1.50	0.0002
Peach - stems	0.06	1.62	0.0001
Tart Cherry - fruit *Prunus cerasus* L.	0.05	1.51	0.0048
Tart Cherry - leaves	0.06	1.34	0.0017
Tart Cherry - stems	0.05	1.59	0.0001
Common Milkweed *Asclepias syriaca* L.	0.13	1.26	0.0012
Curled Dock *Rumex crispus* L.	0.05	1.61	0.0002
Dandelion *Taraxacum officinale* L.	0.07	1.59	0.0001
Field Goldenrod *Solidago nemoralis* Ait.	0.06	1.59	0.00003
Lamb's Quarter *Chenopodium album* L.	0.06	1.58	0.0009
Motherwort *Leonurus cardiaca* L.	0.06	1.60	0.0007
Quackgrass *Agropyron repens* (L.) Beauv.	0.06	1.53	0.00003
Red Clover *Trifolium pratense* L.	0.08	1.42	0.0002
Rough Pigweed *Amaranthus retroflexus* L.	0.06	0.58	0.00003
Roundleaved Mallow *Malva neglecta* L.	0.06	1.16	0.0002
Tumbleweed *Amaranthus albus* L.	0.06	0.43	0.0004
White Clover *Melilotus alba* Desr.	0.06	1.59	0.0001

Table 17.--(cont'd.).

Plant	Absorbance (405 nm)[a] Uninoculated	Inoculated	Variance
Wild Strawberry *Fragaria virginiana* Duch.	0.05	1.47	0.00003
Antigen Control	--	1.60	0.0002
Medium Control	0.06	--	--
Healthy Peach Leaves	--	0.06	--

[a]Mean of four wells.

to the antigen control. Rough pigweed and tumbleweed homogenates had the most pronounced effects. Of particular interest are the results from peach, tart cherry and milkweed homogenates. The inoculated treatments are all less than the antigen control. (The analysis of variance shows all these to be significantly different from the antigen control, but no suitable transformation could be found that would make the data meet the assumption of homogeneity of variances. It is unknown, therefore, at which significance level this test can be considered valid.) This means that the positive results obtained to peach leaves and milkweed are probably depressed by plant constituents and more antigen was present than the A_{405} reading indicates. While tart cherry fruit depressed the A_{405} reading somewhat, the greatest effect was from the leaves. Leaf tissue is the most often tested plant material in ELISA techniques. It is necessary, then, to access the effect of plant constituents on ELISA results to insure that possible positive results are not masked.

These homogenate tests were performed with clarified extracts, not with preparations made according to the method of Raju and Nyland (1981) (see preparation of Plant Tissue for ELISA in Methods and Materials). This more rigorous preparation method may eliminate the effects of plant constituents. In either case, the effects still need to be known before results can be presented with confidence.

It is not clear from these tests at what point plant constituents depress the ELISA results. They may have been active against the spiroplasma antigen, the coating γ-globulin, or both. One would expect the antibody proteins to be more sensitve to plant constituents than the spiroplasma antigen because they have no history of combatting plant compounds as does the latter. The culturing of some

spiroplasmas from homogenated plant tissue is known to be inhibited by plant compounds in the homogenate (Liao and Chen 1977) and this adverse effect may be responsible for depressed ELISA results. Again, for whatever reason, the effect of plant constituents cannot be overlooked.

The ELISA test results of the celery tissue from California are listed in Table 18. These are in some conflict with the results of the ELISA specificity test (Table 9). The suspected S. citri infected celery samples (A7 and A12) produced negative results as did the aster yellows infected sample (A11). All of these produced strong positive results in the ELISA specificity test. The only positive results in this test were obtained from samples of the mild strains of X-disease (A4,A6,A8 and A10) with the well known PYLR strain producing negative results. A possible explanation for this apparent conflict is that all the organisms producing positive results in the ELISA specificity test were actually S. citri as well as those considered mild X-disease strains in this test. This seems a reasonable conclusion in light of the fact that California researchers cannot distinguish these mild strains from S. citri. The indication is that the organisms causing X-disease and aster yellows are serologically distinct from S. citri and are not yet in laboratory culture. The negative results from the corn stunt infected celery confirms that this ELISA does not react with the corn stunt spiroplasma (see ELISA specificity test).

Table 18.--ELISA test results for celery (Apium graveolens L.) tissue from California.

Code	Infecting Agent	No. 1 g Samples Tested	Replicates (plate wells)	A_{405} Range	A_{405} Mean
A1	PYLR[a] - Berkeley Strain	7	28	0.05-0.09	0.063
A2	Healthy Control	6	24	0.05-0.07	0.055
A3	PYLR[a] - Berkeley Strain	6	24	0.04-0.07	0.061
A4	Mild X-disease Strain (young leaves)	13	52	0.05-0.44	0.130
A5	Corn Stunt	13	52	0.06-0.08	0.065
A6	Mild X-disease Strain (old leaves)	9	36	0.90-1.60	1.214
A7	Possible S. citri	10	40	0.05-0.07	0.060
A8	Mild PYLR[a] Strain	7	28	1.62-1.65	1.630
A9	Healthy Control	7	28	0.06-0.09	0.070
A10	Mild PYLR[a] Strain (different from A8)	11	44	1.56-1.64	1.608
A11	Aster Yellows - Tule Lake	6	24	0.05-0.07	0.060
A12	Possible S. citri	13	52	0.03-0.08	0.061
Antigen Control (1:10)		1	12	1.61-1.67	1.638
Antigen Control (1:100)		1	12	1.03-1.43	1.311
Medium Control (1:10)		1	12	0.05-0.07	0.060
Medium Control (1:100)		1	12	0.05-0.08	0.059
Conjugate Buffer		1	12	0.05-0.06	0.052

[a]Peach Yellow Leaf Roll strain of X-disease.

CONCLUSION

This research points up the necessity of isolation and propagation in pure culture of the X-disease pathogen. This, along with the fulfillment of Koch's postulates, should be the immediate focus of future research on X-disease. The development of a bioassay technique, e.g., ELISA, is critical to the monitoring of actual vector populations and the sure identification of X-diseased trees and possible herbaceous hosts of the pathogen. Without the in vitro cultivation of the X-disease pathogen, this type of bioassay is impossible. A great deal of time and effort was invested in this project that reaped near total negative results in the serological investigation of plant and insect tissue. Because of this, future efforts cannot be jusitified along these lines until the X-disease pathogen is, without doubt, in laboratory culture.

Without the information supplied by an effective bioassay technique, recommendations to check the spread of X-disease, especially in regard to leafhopper vector control, cannot be made with high levels of confidence for success. For example, insecticide sprays aimed at the ground cover or applied later in the season after equipment is normally put away for the year may not depress population levels below the low levels observed in the commercial orchard in this resarch. More importantly, those sprays may be directed at populations that contain no pathogen-carrying insects, even if those populations consist of known leafhopper vector species. In addition, given the rather wide variety of hosts of the X-disease pathogen identified in the laboratory and greenhouse, it may be that herbaceous plants in and around orchards play a significant role in the spread of X-disease, particularly if these plants are suitable hosts for leafhopper vectors. Again, control recommendations in this

area must be based upon positive identification of possible pathogen hosts through a sound bioassay technique.

The application of spatial pattern analysis techniques may prove enlightening in the epidemiology of X-disease. Correlation of the spatial pattern of X-diseased trees, and even X-diseased orchards on the regional level, with leafhopper populations and concurrent alternative host plants should help in understanding the pattern of spread from year to year in various locations. This type of approach on a regional basis coupled with an effective bioassay technique, may reveal migrating leafhopper populations with a higher percentage of pathogen carriers than indigenous populations as well as possible regional foci of inoculum. It would also help explain the apparently regional importance of X-disease, e.g., the severe X-disease problem in southwest Michigan as opposed to little, if any, disease in the cherry growing areas of the northwest part of the state.

APPENDICES

APPENDIX A

RECIPES FOR MEDIA AND REAGENTS USED IN CULTURING SPIROPLASMAS AND PERFORMING ELISA

APPENDIX A

RECIPES FOR MEDIA AND REAGENTS USED IN CULTURING SPIROPLASMAS AND PERFORMING ELISA

Modified C-3G Medium

1.5 g PPLO broth (Difco Laboratories, Detroit, MI)

12.0 g sucrose

1.0 ml 0.2% phenol red

56.0 ml distilled water

Mix thoroughly and autoclave at 120°C for 20 minutes. After cooling to room temperature, aseptically add the following:

20.0 ml rabbit serum (GIBCO) heat inactivated at 56°C for one hour and cooled to room temperature before adding.

1.7 ml 1M HEPES (N'-2-hydroxyethyl piperazine-N-2 ethanosulfonic acid) buffer

10.0 ml 25% fresh yeast extract (Microbiological Associates, Inc., Bethesda, Maryland)

10.0 ml 0.1% yeastolate (Difco) filter sterilized through 0.22 μm filter before adding.

0.3 ml penicillin (250,000 units/ml) (GIBCO)

M1A Medium

65.0 ml distilled water

1.2 ml 0.5% phenol red

0.1 g glucose

0.1 g fructose

1.0 g sucrose

1.0 g tryptone

7.0 g sorbitol

2.1 g PPLO broth (Difco)

0.8 g Peptone

Mix thoroughly, adjust pH to 7.8 with 1N NaOH and autoclave at 120°C for 20 minutes. After cooling to room temperature, aseptically add the following:

160.0 ml Schneider's Drosophila medium (GIBCO)

50.0 ml fetal bovine serum (GIBCO)

10.0 ml 25% fresh yeast extract (Microbiological Associates)

30.0 ml 0.1% yeastolate (Difco) filter sterilized through 0.22 μm filter before adding

1.0 ml penicillin (250,000 units/ml) (GIBCO)

Phosphate Buffered Saline (PBS)

8.0 g NaCl

0.2 g KH_2PO_4

2.17g $Na_2PO_4 \cdot 7H_2O$

0.2 g KCl

0.2 g NaN_3

Make up to 1 liter with distilled water and adjust pH to 7.4 with NaOH or HCl

Siliconizing Solution

1.5 ml dimethyldichlorosilane (Sigma Chemical Co., St. Louis, MO)

98.5 ml chloroform

Coating Buffer

1.59g Na_2CO_3

2.93g $NaHCO_3$

0.2 g NaN_3

Make up to 1 liter with distilled water and adjust pH to 9.6 with NaOH of HCl

PBS-Tween

Add 0.5 ml Tween-20 (polyoxyethylenesorbitan monolaurate) (Sigma) per liter of phosphate buffered saline. Mix thoroughly.

Conjugate Buffer

To 1 liter of PBS-Tween, add:

20.0 g polyvinylpyrrolidone, MW = 10,000 (Sigma)

2.0 g ovalbumin (Sigma)

Completely dissolve polyvinylpyrrolidone before adding ovalbumin. Store at 5°C and use within 1 month.

Saturated Ammonium Sulfate Solution

91.0 g $(NH_4)_2SO_4$

100.0 ml distilled water

Mix extensively. May have to be filtered to remove undissolved salt.

Substrate Buffer

97.0 ml diethanolamine (Sigma)

0.2 g NaN_3

Make up to 1 liter with distilled water and adjust pH to 9.8 with HCl. Store at 5°C and check pH just prior to use.

APPENDIX B

FORTRAN COMPUTER PROGRAM TO GENERATE RANDOM SAMPLE LOCATIONS IN THE PEACH TREE CANOPY

APPENDIX B

FORTRAN COMPUTER PROGRAM TO GENERATE RAMDOM SAMPLE LOCATIONS IN THE PEACH TREE CANOPY

```
      PROGRAM RANSAMT (INPUT,OUTPUT,TAPE 2)
C  THIS PROGRAM GENERATES RANDOM SAMPLE LOCATIONS IN THE
C  PEACH TREE CANOPY ACCORDING TO THE NUMBER OF THE TREE AND
C  THE OCTANT WITHIN THE TREE.
      REWIND 2
C  THIS FIRST WRITE STATEMENT PRINTS OUT THE HEADING ON THE
C  FIRST PAGE OF THE SAMPLE NUMBERS.
      WRITE(2,900)
      DO 30 I=1,30
      DO 40 J=1,4
C  THERE ARE SIX TREES SELECTED IN EACH ITERATION OF THE LOOP,
C  ONE FOR EACH PEACH BLOCK, FOR A TOTAL OF 24 SAMPLES.
      NTREE1=RANF(-1)*434+1
      NDIV1=RANF(-1)*8+1
      NTREE2=RANF(-1)*181+1
      NDIV2=RANF(-1)*8+1
      NTREE3=RANF(-1)*168+1
      NDIV3=RANF(-1)*8+1
      NTREE4=RANF(-1)*300+1
      NDIV4=RANF(-1)*8+1
      NTREE5=RANF(-1)*201+1
      NDIV5=RANF(-1)*8+1
      NTREEJ=RANF(-1)*57+1
      NDIVJ=RANF(-1)*8+1
C  THIS WRITE PRINTS OUT THE TREE NUMBER ALONG WITH THE
C  OCTANT WITHIN THE TREE TO BE SAMPLED.
40    WRITE(2,102)I,J,NTREE1,NDIV1,NTREE2,NDIV2,NTREE3,NDIV3,
     +NTREE4,NDIV4,NTREE5,NDIV5,NTREEJ,NDIVJ
      WRITE(2,101)
30    CONTINUE
101   FORMAT(*0*)
102   FORMAT(6X,I5,I6,1X,6(I6,*-*,I1)
900   FORMAT(*1*,41X,*BLOCK*,/,7X,*SAMPLE*,7X,49(1H-),/,
     +8X,*DATE*,3X,*REP*,5X,*1*,7X,*2*,7X,*3*,7X,*4*,
     +7X,*5*,5X,*JOLLY*,/,7X,62(1H-))
      STOP
      END
```

APPENDIX C

FORTRAN COMPUTER PROGRAM TO GENERATE RANDOM SAMPLE LOCATIONS IN THE ORCHARD GROUND COVER

APPENDIX C

FORTRAN COMPUTER PROGRAM TO GENERATE RANDOM SAMPLE LOCATIONS IN THE ORCHARD GROUND COVER

```
      PROGRAM RANSAMG(INPUT,OUTPUT,TAPE 1)
C  THIS PROGRAM GENERATES RANDOM SAMPLE LOCATIONS IN THE
C  GROUND COVER OF THE SIX PEACH ORCHARD BLOCKS SAMPLED.
      REWIND 1
C  THE FIRST WRITE STATEMENT PRINTS OUT THE HEADING ON THE
C  FIRST PAGE OF THE SAMPLE LOCATION NUMBERS.
      WRITE(1,900)
      DO 10 I=1,30
      DO 20 J=1,4
C  THE SIX BLOCKS ARE DESIGNATED AS LOCATIONS 1 THROUGH 6
C  AND J.  THE RANF FUNCTION GENERATES A RANDOM NUMBER
C  FOR EACH VARIABLE.
      NLOC1=RANF(-1)*774+1
      NLOC2=RANF(-1)*384+1
      NLOC3=RANF(-1)*280+1
      NLOC4=RANF(-1)*532+1
      NLOC5=RANF(-1)*324+1
      NLOCJ=RANF(-1)*140+1
C  THIS WRITE STATEMENT PRINTS OUT THE APPROPRIATE SAMPLE
C  LOCATION IN THE PROPER BLOCK COLUMN.
20    WRITE(1,100)I,J,NLOC1,NLOC2,NLOC3,NLOC4,NLOC5,NLOCJ
      WRITE(1,101)
10    CONTINUE
100   FORMAT(6X,I5,4X,I2,6I8)
101   FORMAT(*0*)
900   FORMAT(*1*,41X,*BLOCK*,/,7X,*SAMPLE*,7X,49(1H-),/,
     +8X,*DATE*,3X,*REP*,5X,*1*,7X,*2*,7X,*3*,7X,*4*,7X,
     +*5*,5X,*JOLLY*,/,7X,62(1H-))
      STOP
      END
```

APPENDIX D

FORTRAN COMPUTER PROGRAM TO CALCULATE UNWEIGHTED AND WEIGHTED ESTIMATES OF COMMON K FOR THE NEGATIVE BINOMIAL DISTRIBUTION

APPENDIX D

FORTRAN COMPUTER PROGRAM TO CALCULATE UNWEIGHTED AND WEIGHTED ESTIMATES OF COMMON K FOR THE NEGATIVE BINOMIAL DISTRIBUTION

```
      PROGRAM COMMONK(INPUT,OUTPUT,TAPE 1,TAPE 2=OUTPUT)
C  ****************************************************************
C  ARRAYS ARE ESTABLISHED TO STORE ENTERED AND
C  COMPUTED DATA FOR FUTURE USE AND OUTPUT.
C  ****************************************************************
      DIMENSION N(50)
      DIMENSION XBAR(50)
      DIMENSION VAR(50)
      DIMENSION XPRIM(50)
      DIMENSION YPRIM(50)
      DIMENSION AKINV(50)
      DIMENSION AK1(50)
      DIMENSION W(50)
      DIMENSION WXPRIM(50)
      DIMENSION WYPRIM(50)
      DIMENSION WXY(50)
      DIMENSION WXSQ(50)
      REWIND 1
      REWIND 2
C  ****************************************************************
C  THE SAMPLE SIZE, SAMPLE MEAN AND SAMPLE VARIANCE MUST
C  BE ENTERED FOR COMPUTATION OF THE COMMON K.
C  ****************************************************************
1     PRINT*,"ENTER NUMBER OF SAMPLES--"
      READ*,J
      IF(J.GT.50)GOTO 2
      GOTO 3
2     PRINT*,"SORRY, NUMBER OF SAMPLES LIMITED TO 50"
      PRINT*,"DO YOU WANT TO CONTINUE? Y OR N--"
      READ 100,A
      IF(A.EQ.1HY)GOTO 1
      GOTO 8
3     PRINT*,"ENTER SAMPLE SIZE, SAMPLE MEAN AND SAMPLE"
      PRINT*,"VARIANCE FOR EACH SAMPLE IN THE FORM:"
      PRINT*,"N,XBAR,VAR"
      DO 10 I=1,J
      PRINT*,I,"*"
      READ*,N(I),XBAR(I),VAR(I)
10    CONTINUE
      PRINT*,"DO YOU WISH TO ENTER A K VALUE? Y OR N--"
      READ 100,B
```

```
      IF(B.EQ.1HN)GOTO 9
      PRINT*,"ENTER K VALUE--"
      READ*,XK1
      XKINV=1/XK1
C  ****************************************************************
C  THE PROGRAM NOW COMPUTES VALUES OF X', Y', 1/K AND
C  K FOR EACH SAMPLE AS WELL AS THE SUMMATIONS OF X'
C  AND Y', 1/KC AND KC AS AN UNWEIGHTED ESTIMATE OF
C  THE COMMON K ACCORDING TO THE FORMULAS GIVEN IN:
C  ELLIOTT, J.M.  1977.  METHODS FOR THE STATISTICAL
C  ANALYSIS OF SAMPLES OF BENTHIC INVERTEBRATES.
C  FRESHWATER BIOL. ASSOC. #25.  160 PP.
C  ****************************************************************
      SUMX=0.0
      SUMY=0.0
      DO 20 I=1,J
      XPRIM(I)=(XBAR(I)**2)-(VAR(I)/N(I))
      YPRIM(I)=VAR(I)-XBAR(I)
      AKINV(I)=YPRIM(I)/XPRIM(I)
      AK1(I)=XPRIM(I)/YPRIM(I)
      SUMX=SUMX+XPRIM(I)
      SUMY=SUMY+YPRIM(I)
20    CONTINUE
      IF(B.EQ.1HY)GOTO 11
      XKINV=SUMY/SUMX
      XK1=SUMX/SUMY
C  ****************************************************************
C  THE PROGRAM NOW COMPUTES THE WEIGHTING FACTOR, W,
C  FOR EACH SAMPLE, THE NECESSARY WEIGHTED STATISTICS
C  WX', WY', WX'Y', WX'SQ, THE SUMMATIONS OF WX'Y' AND
C  WX'SQ AND THE WEIGHTED COMMON K ACCORDING TO THE
C  FORMULAS GIVEN IN:  BLISS, C.I. AND A.R.G. OWEN.
C  1958.  NEGATIVE BINOMIAL DISTRIBUTIONS WITH A
C  COMMON K.  BIOMETRIKA 45:  37-58.
C  ****************************************************************
11    SUMWX=0.0
      SUMWY=0.0
      SUMWXY=0.0
      SUMWX2=0.0
      DO 30 I-1,J
      W(I)=(0.5*(N(I)-1)*(XK1**4))/((XK1*(XK1+1)-(2*XK1-1)
     +/N(I)-3/N(I)**2)*(XPRIM(I)*(XBAR(I)+XK1)**2))
      WXPRIM(I)=W(I)*XPRIM(I)
      WYPRIM(I)=W(I)*YPRIM(I)
      WXY(I)=WXPRIM(I)*YPRIM(I)
      WXSQ(I)=WXPRIM(I)*XPRIM(I)
      SUMWX=SUMWX+WXPRIM(I)
      SUMWY=SUMWY+WYPRIM(I)
      SUMWXY=SUMWXY+WXY(I)
      SUMWX2=SUMWX2+WXSQ(I)
30    CONTINUE
      YKINV=SUMWXY/SUMWX2
      YK2=SUMWX2/SUMWXY
```

```
C  ****************************************************************
C  ALL DATA IS NOW WRITTEN ONTO TAPES FOR OUTPUT.
C  TAPE 1 OUTPUTS TO THE CYBER 750 AND TAPE 2
C  OUTPUTS TO THE INTERACTIVE TERMINAL.
C  ****************************************************************
       WRITE(1,200)
       WRITE(1,300)(N(I),XBAR(I),VAR(I),XPRIM(I),YPRIM(I),
      +AKINV(I),AK1(I),W(I),WXPRIM(I),WYPRIM(I),WXY(I),
      +WXSQ(I),I=1,J)
       WRITE(1,400)SUMX,SUMY,XKINV,XK1,SUMWX,SUMWY,
      +SUMWXY,SUMWX2,YKINV,YK2
       GOTO 5
4      WRITE(2,500)
       WRITE(2,600)(N(I),XBAR(I),VAR(I),XPRIM(I),YPRIM(I),
      +AKINV(I),I=1,J)
       WRITE(2,700)SUMX,SUMY,XKINV
       WRITE(2,750)
       WRITE(2,800)(AK1(I),W(I),WXPRIM(I),WYPRIM(I),
      +WXY(I),WXSQ(I),I=1,J)
       WRITE(2,900)XK1, SUMWX,SUMWY,SUMWXY,SUMWX2,YKINV,YK2,
       GOTO 7
C  ****************************************************************
C  OPTIONS ARE NOW GIVEN TO THE USER FOR THE
C  DISPOSITION OF THE OUTPUT.
C  ****************************************************************
5      PRINT*,"COMPUTATIONS ARE NOW COMPLETE.  BECAUSE OF THE LENGTH"
       PRINT*,"OF THE OUTPUT, YOU MAY HAVE IT PRINTED AT THE"
       PRINT*,"TERMINAL OR ON THE CYBER 750 PRINTER.  THE OUTPUT"
       PRINT*,"IS PRESENTED MORE EFFICIENTLY ON THE LARGE CYBER"
       PRINT*,"750 PAPER.  ENTER 1 FOR THE TERMINAL OUTPUT OR 2"
       PRINT*,"FOR THE CYBER 750 OUTPUT."
       READ*,K
       IF(K.EQ.1HY)GOTO 4
6      PRINT*,"WHEN PROGRAM TERMINATES, ENTER 'DISPOSE,"
       PRINT*,"TAPE 1,PA.'.  COPY DOWN SEQUENCE NUMBER AND"
       PRINT*,"PICK-UP OUTPUT AT THE COMPUTER CENTER."
       GOTO 8
7      PRINT*,"WOULD YOU LIKE CYBER 750 OUTPUT ALSO? Y OR N--"
       READ 100,C
       IF(C.EQ.1HY)GOTO 6
100    FORMAT(A1)
200    FORMAT(* *,136(1H-),/,3X,*N*,7X,*MEAN*,5X,*VARIANCE*,
      +7X,*X'*,10X,*Y'*,10X,*1/K*,10X,*K*,11X,*W*,10X,*WX'*,
      +9X,*WY'*,8X,*WX'Y'*,7X,*WX'SQ*,/,* *,136(1H-),/)
300    FORMAT(* *,I4,11F12.5)
400    FORMAT(*0*,*TOTALS*,22X,4F12.5,12X,4F12.5,///,
      +36X,*1/KC = *,F12.5,24X,*KC = *,F12.5,///)
500    FORMAT(* *,70(1H-),/,3X,*N*,7X,*MEAN*,5X,*VARIANCE*,
      +7X,*X'*,10X,*Y'*,10X,*1/K*,/,* *,70(1H-))
600    FORMAT(* *,I4,5F12.5)
700    FORMAT(*0*,*TOTALS*,22X,3F12.5)
750    FORMAT(* *,//,70(1H-),/,5X,*K*,11X,*W*,10X,*WX'*,9X,
      +*WY'*,7X,*WX'Y'*,7X,*WX'SQ*,/,* *,70(1H-))
```

```
800     FORMAT(* *,F10.5,5F12.5)
900     FORMAT(*0*,F10.5,12X,4F12.5,//,15X,*1/KC = *,
       +F12.5,10X,*KC = *,F12.5,//)
        STOP
        END
```

APPENDIX E

ESTIMATION OF OPTIMUM SAMPLE SIZE FOR USE WITH FIXED-AREA GROUND SAMPLING TECHNIQUES

APPENDIX E

ESTIMATION OF OPTIMUM SAMPLE SIZE FOR USE WITH FIXED-AREA GROUND SAMPLING TECHNIQUES

An aggregated spatial pattern frequently describes the dispersion of many insect species in the field. Large variations are associated with sampling these aggregated populations which render small samples, and the data they generate, statistically unreliable. However, to obtain acceptable statistical reliability often requires sample sizes that are inordinately large and impossible to take with the practical constraints of the time and money allocated to a particular research project. This is almost always the case unless the sampling system is exceptionally efficient in detecting individuals from the population of interest.

The researcher must, at the outset, decide upon the degree of reliability necessary to fulfill the goals of the research, e.g., the accuracy of mean prediction necessary to make valid biological conclusions from sample data. While it is often done, the degree of reliability obtained from a certain sample size cannot be so low as to render meaningless any conclusions made from the sample data, even if those conclusions are qualified by stating the sample reliability. Meaningless conclusions, even if so stated before they are made, are, nonetheless, meaningless. The researcher is not free, therefore, to choose any level of reliability but only a minimum level necessary to render valid any conclusions made. Conversely, insisting upon an unnecessarily high degree of reliability may render the sample effort impossible to accomplish. Optimum sample size has been defined as the smallest sample size necessary to obtain the

desired level of reliability. This definition might be modified to state the smallest sample size necessary to obtain the necessary level of reliability to validly fulfill the goals of the research.

Once the necessary, rather than desired, level of reliability is determined, optimum sample size can be computed. The cost of a sample unit, in terms of time and money, can then be applied to this optimum figure and a determination made whether or not it is feasible to actually take the sample. If it is not, reducing the reliability to lower the sample size can only be done if the research goals are modified to take this reduction into account. Apart from this, the alternatives are to make the sampling system more efficient or increase the amount of time and/or money. It must be emphasized that automatic reduction in sample reliability is unacceptable simply to meet practical considerations. If sample cost is fixed, then the research must be redefined to allow for reduced sample reliability and the subsequent sample size reduction.

When the spatial pattern of the population to be sampled is aggregated with the random variable following the negative binomial distribution (NBD), the optimum sample size can be calculated as

$$n = (t^2/D^2)(1/x+1/k)$$

where t is found in Student's t-distribution, D is the half width of a 95% confidence interval, x is the sample mean and k is the dispersion parameter of the NBD (Elliott 1977). In this equation, D is the measure of reliability and must be chosen to allow the fulfillment of the research objectives. If the sample estimate of the population parameter, e.g., the mean, can be tolerated to lie within $\pm$20% of the true value, then D = 0.2. Obviously, the formula can be rearranged so that the reliability of a particular sample can be determined, which produces

$$D = (t^2/n)(1/x+1/k).$$

Table E1 shows the reliability of each of the 10 lumped samples from the commercial orchard sampling data for Streptanus confinis along with the sample size necessary to estimate population parameters within ±10% of the true value.

From a purely statistical point of view, one might be tempted to reject the entire set of samples as being unreliable. However, the unreliability might be considered a "statistical artifact" given the very low mean values of these samples (all less than one). With means in this range, statistical reliability can never be obtained as evidenced by the sample sizes (n) necessary to estimate the true mean with ±10%. Each ground cover sample takes 10-12 minutes, not including sorting time in the laboratory, making these sample sizes impossible to accomplish. It is also reasonable to assume that these sample means are not gross underestimates of the true means because of 508 total samples taken, only 81 produced specimens of S. confinis, indicating the true aggregated spatial pattern and low density of this leafhopper. The fact that the sampling scheme detected S. confinis at all is to its credit and the spatial analysis cannot be rejected due to apparent sample unreliability. However, if the goal of this sampling had been to compare leafhopper species populations with a rigorous statistical analysis, e.g., analysis of variance, then the sample reliability becomes much more significant and these samples may not be valid. Even in this case, the sample reliability cannot be judged soley upon its computed value, as, again, populations of a very low density will never demonstrate statistical reliability even if the sample means are reasonably accurate.

Table E2 lists the sample sizes necessary to obtain certain levels of reliability with sample means for the four common k values calculated from the

Table E1.--Application of optimum sample size equation to lumped sample data for *Streptanus confinis*.

Sample Number	n	$\bar{x}$	s^2	k	D	n(D=0.1)
1	60	0.583	3.162	0.063	1.00	6757
2	40	0.600	2.554	0.212	0.81	2452
3	52	0.404	0.991	0.190	0.77	2973
4	40	0.275	0.615	0.172	0.98	3630
5	56	0.214	0.535	0.222	0.81	3526
6	60	0.433	1.368	0.100	0.91	4729
7	40	0.325	0.892	0.192	0.92	3183
8	40	0.325	0.892	0.192	0.92	3183
9	60	0.233	0.318	0.700	0.62	2198
10	60	0.300	1.773	0.101	0.94	5084

Table E2.--Sample size estimates for Streptanus confinis using four estimates of common k.

	k_{c1} = 0.135			k_{c2} = 0.128			k_{c3} = 0.214			k_{c4} = 0.137		
Mean	0.1[a]	0.2[a]	0.4[a]	0.1[a]	0.2[a]	0.4[a]	0.1[a]	0.2[a]	0.4[a]	0.1[a]	0.2[a]	0.4[a]
0.2	4766	1192	298	4922	1230	308	3716	929	232	4725	1181	295
0.5	3614	903	226	3770	942	236	2563	641	160	3572	893	223
1.0	3230	807	202	3385	846	212	2179	545	136	3188	797	199
5.0	2922	731	183	3078	770	192	1872	468	117	2881	720	180
10.0	2884	721	180	3040	760	190	1834	458	115	2843	711	178
15.0	2871	718	179	3027	757	189	1821	455	114	2830	707	177
20.0	2864	716	179	3020	755	189	1814	454	113	2823	706	176
40.0	2855	714	178	3011	753	188	1805	451	113	2814	703	176
60.0	2852	713	178	3008	752	188	1802	450	113	2810	703	176
80.0	2850	713	178	3006	752	188	1800	450	112	2809	702	176
100.0	2849	712	178	3005	751	188	1799	450	112	2808	702	175

[a]Half-width of a 95% confidence interval, i.e., D, in the equation:

$$n = (t^2/D^2)(1/\bar{x} + 1/k_c).$$

S. confinis data from the commercial sample site. Clearly, none of these sample sizes can possibly be accomplished. With populations showing this high degree of aggregation, which is not unusual for many insect populations, sample sizes that produce high levels of statistical reliability are practically inoperable, even at very high densities. Furthermore, sample size seems to stabilize at higher mean values, indicating that aggregation as reflected in the k value is the limiting factor in sample size estimation. Therefore, in sampling populations of this type, a rethinking of sampling design might be in order. Preliminary sampling might be directed at determining the foci of aggregation, e.g., favorable habitat or climate. The sampling scheme can then be designed to sample in these areas thus maximizing chances of capturing individuals, particularly in low density populations, and increasing the accuracy of population parameter estimation. This is, of course, biased sampling, but most statistical procedures are robust enough to handle this providing the sample is still random, e.g., a random sample of predetermined habitats.

The marriage of statistically reliable sample sizes with those that are practically obtainable remains an impossibility with the current definitions of reliability. Presently, the one will almost always be sacrificed for the other. A redefinition of sample reliability must be put forth that will allow for data generated from reasonable sample sizes to be applied confidently and validly to research goals and conclusions. In practical fact, most data from research is treated as if this were already the case because sample reliability is seldom examined. Sampling theory as it addresses this area should be restructured to allow for valid statistical analyses and conclusions to be applied to data from practically obtained samples.

APPENDIX F

LEAFHOPPER VOUCHER SPECIMENS PLACED IN THE MICHIGAN STATE UNIVERSITY ENTOMOLOGICAL MUSEUM

APPENDIX F

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 1982-1

Title of thesis or dissertation (or other research projects):

Leafhopper Sampling in Michigan Peach Orchards and Serological Detection of a Spiroplasma Associated with X-disease in Plant and Insect Tissue

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

none

Investigator's Name (s) (typed)
Thomas M. Mowry

Date January 18, 1982

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24:141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.
Copies: Included as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX F

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♂	Adults ♀	Other	Museum where deposited
1. *Amblyaellus curtisii* (Fitch)	Ingham Co. MICH.: East Lansing					1			MSU
2. *Aphrodes bicinota* (Schrank)	Ingham Co. MICH.: East Lansing						4		MSU
3. *Aphrodes flavostrigata* (Don.)	Ingham Co. MICH.: East Lansing					1	3		MSU
4. *Aphrodes fuscofaciata* (Goeze)	Ingham Co. MICH.: East Lansing					3	1		MSU
5. *Athysanus argentarius* Metc.	Ingham Co. MICH.: East Lansing					1	1		MSU
6. *Commellus comma* (Van D.)	Ingham Co. MICH.: East Lansing						1		MSU
7. *Dicraneura mali* (Prov.)	Ingham Co. MICH.: East Lansing					2	3		MSU
8. *Doratura stylata* (Boh.)	Ingham Co. MICH.: East Lansing					1	3		MSU
9. *Draeculacephala antica* (Walk.)	Ingham Co. MICH.: E.Lan./Haslett					1	4		MSU
10. *Endria inimica* (Say)	Ingham Co. MICH.: East Lansing					1	1		MSU
11. *Latalus sayi* (Fitch)	Ingham Co. MICH.: East Lansing					2	2		MSU
12. *Macrosteles fascifrons* (Stal)	Ingham Co. MICH.: East Lansing					3			MSU
13. *Paraboloratus viridis* Uhl.	Ingham Co. MICH.: East Lansing						1		MSU
14. *Paraphlepsius irroratus* (Say)	Ingham Co. MICH.: Haslett						1		MSU
15. *Psammotettix farratus* (DeL&Dav)	Ingham Co. MICH.: East Lansing					1	3		MSU
16. *Psammotettix lividellus* (Zett.)	Ingham Co. MICH.: East Lansing						1		MSU
17. *Streptanus confinis* (Raut.)	Ingham Co. MICH.: E.Lan./Haslett						4		MSU
18. *Tyloaygus bifidus* (Say)	Ingham Co. MICH.: East Lansing					2			MSU
19. *Philaenus spumarius* L.	Ingham Co. MICH.: East Lansing						2		MSU
20. *Graminella nigrifrons* (Forbes)	No specimens available; used in research.								
21. *Xestocephalus pulicarius* Van D.	No specimens available; used in research.								

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Thomas M. Mowry

Date January 18, 1982

Voucher No. 1982-1

Received the above listed specimens for deposit in the Michigan State University Entomology Museum.

Curator

Date 18 Jan. 1982

LITERATURE CITED

LITERATURE CITED

Anthon, E.W. and H.R. Wolfe. 1951. Additional insect vectors of western X-disease. Plant Dis. Rep. 35: 345-346.

Ashton, W.D. 1972. The LOGIT Transformation with Special Reference to its Uses in Bioassay. Hafner Publishing Co., New York. 88 pp.

Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry 6: 43-52.

Beirne, B.P. 1956. Leafhoppers (Homoptera: Cicadellidae) of Canada and Alaska. Can. Ent. 88, Supp. 2. 180 pp.

Bliss, C.I. and A.R.G. Owen. 1958. Negative binomial distributions with a common k. Biometrika 45: 37-58.

Bove, J.M. and C. Saillard. 1979. Cell biology of spiroplasmas. Pp. 83-153 In: The Mycoplasmas. III. Plant and Insect Mycoplasmas. R.F. Whitcomb and J.G. Tully, eds. Academic Press, New York. 351 pp.

Bove, J.M., G. Moutous, C. Saillard, A. Fos, J. Bonfils, J.C. Vignault, A. Nhami, M. Abassi, K. Kabbage, B. Hafidi, C. Mouches and G. Viennot-Bourgin. 1979a. Mise en evidence de Spiroplasma citri, l'agent causal de la maladie du (stubborn) des agrumes dans 7 cicadelles du Maroc. C.R. Hebd. Seances Acad. Sci. Ser. D. 288: 335-338.

Bove, J.M., A. Nhami, C. Saillard, J.C. Vignault, C. Mouches, M. Garnier, G. Moutous, A. Fos, J. Bonfils, M. Abassi, K. Kabbage, B. Hafidi and G. Viennot-Bourgin. 1979b. Presence au maroc de Spiroplasma citri, l'agent causal de la maladie du (stubborn) des agrumes dans les Pervenches (Vinca rosea L.) implantees en bordure d'orangeraies maladies, et contamination probable du chiendent (Cynodon dactylon (L.) Pers.) par le spiroplasma. C.R. Hebd. Seances Acad. Sci. Ser. D. 288: 399-402.

Clark, M.F. and A.N. Adams. 1976. Laboratory notes on the ELISA technique. East Malling Res. Stn., Maidstone, Kent. 6 pp.

Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483.

Clark, M.F., A.N. Adams and D.J. Barbara. 1976a. The detection of plant viruses by enzyme-linked immunosorbent assay (ELISA). Acta Hortic. 67: 43-49.

Clark, M.F., A.N. Adams, J.M. Thresh and R. Casper. 1976b. The detection of plum pox and other viruses in woody plants by enzyme-linked immunosorbent assay (ELISA). Acta Hortic. 67: 51-57.

Clark, M.F., C.L. Flegg, M. Bar-Joseph and S. Rottem. 1978. The detection of Spiroplasma citri by enzyme-linked immunosorbent assay (ELISA). Phytopathol. Z. 92: 332-337.

Clarke, R.G., R.H. Converse and M. Kojima. 1980. Enzyme-linked immunosorbent assay to detect potato leafroll virus in potato tubers and viruliferous aphids. Plant. Dis. 64: 43-65.

Dempster, J.P. 1961. A sampler for estimating populations of active insects upon vegetation. J. Anim. Ecol. 30: 425-427.

Doi, Y., M. Teranaka, K. Yora and H. Asuyama. 1967. Mycoplasma or PLT group-like microorganisms found in the phloem elements of plants infected with mulberry dwarf, potato witches' broom, aster yellows, or Paulownia witches' broom. Ann. Phytopathol. Soc. Japan 33: 259-266.

Elliott, J.M. 1977. Some methods for the statistical analysis of samples of benthic invertebrates. Fresh. Biol. Assn. No. 25. 160 pp.

Engvall, E., K. Jonsson and P. Perlman. 1971. Enzyme-linked immunosorbent assay. I. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochim. Biophys. Acta 251: 427-434.

Engvall, E. and P. Perlmann. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8: 871-874.

Engvall, E. and P. Perlmann. 1972. Enzyme-linked immunosorbent assay (ELISA). III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol. 109: 129-135.

Fletcher, J., G.A. Schultz, R.E. David, C.E. Eastman and R.M. Goodman. 1981. Brittleroot disease of horseradish: Evidence for an etiological role of Spiroplasma citri. Phytopathology 71: 1073-1080.

Freund, J. and K. McDermott. 1942. Sensitization to horse serum by means of adjuvants. Proc. Soc. Exp. Biol. Med. 49: 548-553.

Gera, A., G. Loebenstein and B. Raccah. 1978. Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay. Virology 86: 542-545.

Gilmer, R.M. 1954. Insect transmission of X-disease virus in New York. Plant Dis. Rep. 38: 628-629.

Gilmer, R.M. 1960. Recovery of X-disease virus from naturally infected milkweeds. Phytopathology 50: 636 (abstr.).

Gilmer, R.M. and E.C. Blodgett. 1976. X-disease. Pp. 145-155 In: Virus Diseases and Non-infectious Disorders of Stone Fruits in North America. USDA Handbook 437. 433 pp.

Gilmer, R.M., J.D. Moore and G.W. Keitt. 1954. X-disease virus: I. Host range and pathogenesis in chokecherry. Phytopathology 44: 180-185.

Gilmer, R.M., D.H. Palmiter, G.A. Schaefers and F.L. McEwen. 1966. Insect transmission of X-disease virus of stone fruits in New York. N.Y. State Agric. Exp. Sta. (Geneva) Bull. 813. 22 pp.

Granett, A.L. and R.M. Gilmer. 1971. Mycoplasmas associated with X-disease in various Prunus species. Phytopathology 61: 1036-1037.

Huang, J. and G. Nyland. 1970. The morphology of a mycoplasma associated with peach X-disease. Phytopathology 60: 1534. (Abstr.).

Heikinheimo, O. and M. Raatikainen. 1962. Comparison of suction and netting methods in population investigations concerning the fauna of grass leys and cereal fields, particularly in those concerning the leafhopper, Calligypona pellucide (F.). Valt. Maatal. Koetoim. Julk. 191: 1-29.

Hildebrand, E.M. and D.H. Palmiter. 1938. Yellow-red virosis (X-disease) of peach and chokecherry. Plant Dis. Rep. 22: 394-396.

Holmes, F.O. 1941. Peach X-disease virus. Pp. 82-83 In: Handbook of Phytopathogenic Viruses. F.O. Holmes, ed. Burgess Publishing Co., Minneapolis, Minn. 221 pp.

Ishiie, T., Y. Doi, K. Yora and H. Asuyama. 1967. Suppressive effects of antibiotics of tetracycline group on symptom development of mulberry dwarf disease. Ann. Phytopathol. Soc. Jpn. 33: 267-275.

Jensen, D.D. 1953a. Leafhopper-virus relationships of peach yellow leaf roll. Phytopathology 43: 561-564.

Jensen, D.D. 1953b. Longevity of the leafhoppers Colladonus geminatus (Van Duzee) on peach. J. Econ. Entomol. 46: 1120-1121.

Jensen, D.D. 1955. Evidence that celery is a host of peach yellow leaf roll virus. Phytopathology 45: 694. (abstr.).

Jensen, D.D. 1956. Insect transmission of virus between tree and herbaceous plants. Virology 2: 249-260.

Jensen, D.D. 1957a. Transmission of peach yellow leaf roll virus by Fieberiella florii (Stal) and a new vector, Osbornellus borealis DeL. & M. J. Econ. Entomol. 50: 668-672.

Jensen, D.D. 1957b. Differential transmission of peach yellow leaf roll virus to peach and celery by the leafhopper, Colladonus montanus. Phytopathology 47: 575-578.

Jensen, D.D. 1958. Reduction in longevity of leafhoppers carrying peach yellow leaf roll virus. Phytopathology 48: 394. (abstr.).

Jensen, D.D. 1959. A plant virus lethal to its insect vector. Virology 8: 164-175.

Jensen, D.D. 1969. Comparative transmission of western X-disease virus by Colladonus montanus, C. geminatus, and a new leafhopper vector, Euscelidius variegatus. J. Econ. Entomol. 62: 1147-1150.

Jensen, D.D. 1971. Vector fecundity reduced by western X-disease. J. Invert. Pathol. 17: 389-394.

Jensen, D.D., N.W. Frazier and H.E. Thomas. 1952. Insect transmission of yellow leaf roll virus of peach. J. Econ. Entomol. 45: 335-337.

Jensen, D.D. and H.E. Thomas. 1954. Leafhopper transmission of the Napa strain of cherry buckskin virus from cherry to peach. Phytopathology 44: 494. (abstr.).

Jensen, D.D., R.F. Whitcomb and J. Richardson. 1967. Lethality of injected peach western X-disease virus to its leafhopper vector. Virology 31: 532-538.

Johnson, C.G., T.R.E. Southwood and H.M. Entwistle. 1958. A new method of extracting arthropods and molluscs from grassland and herbage with a suction apparatus. Bull. Ent. Res. 48: 211-218.

Jones, A.L., G.R. Hooper and D.A. Rosenberger. 1974. Association of mycoplasmalike bodies with little peach and X-disease. Phytopathology 64: 755-756.

Jones, A.L. and D.A. Rosenberger. 1977. X-disease of peach and cherry: A guide to chokecherry identification. Mich. St. Univ. Ext. Bull. E-842. 4 pp.

Jones, A.L., R.F. Whitcomb, D.L. Williamson and M.E. Coan. 1977. Comparitive growth and primary isolation of spiroplasmas in media based on insect tissue culture formulations. Phytopathology 67: 738-746.

Kaloostian, G.H. 1951a. Transmission of western X little cherry virus from sour and sweet cherry to peach by Colladonus geminatus (Van D.). Plant Dis. Rep. 35: 348.

Kaloostian, G.H. 1951b. Transmission of western X-disease virus from chokecherry to peach by Colladonus geminatus (Van D.). Plant Dis. Rep. 35: 347.

Kelly, D.C., M.L. Edwards and J.S. Robertson. 1978. The use of enzyme-linked immunosorbent assay to detect, and discriminate between, small iridescent viruses. Ann. Appl. Biol. 90: 369-374.

Kelly, D.C., M.L. Edwards, H.F. Evans and J.S. Robertson. 1978. The use of enzyme-linked immunosorbent assay to detect a nuclear polyhedrosis virus in Heliothis armigera larvae. J. Gen. Virol. 40: 465-469.

Kloepper, J.W. and D.G. Garrott. 1980. Relation of in vitro morphology to isolation of plant spiroplasmas. Curr. Microbiol. 4: 365-370.

Kunkel, L.O. 1944. Transmission of virus from X-diseased peach trees to herbaceous plants. Phytopathology 34: 1006. (abstr.).

Lacy, G.H., M.S. McClure and T.G. Andreadis. 1979. Reducing populations of vector leafhoppers is a new approach to X-disease control. Frontiers of Plant Sci. 32: 2-4.

Lee, P.E. and D.D. Jensen. 1963. Crystalline inclusions in Colladonus montanus (Van Duzee), a vector of western X-disease virus. Virology 20: 328-332.

Liao, C.H. and T.A. Chen. 1977. Culture of corn stunt spiroplasma in a simple medium. Phytopathology 67: 802-807.

Longworth, J.F. and G.P. Carey. 1980. The use of an indirect enzyme-linked immunosorbent assay to detect baculovirus in larvae and adults of Oryctes rhinoceros from Tonga. J. Gen. Virol. 47: 431-438.

Lott, T.B. 1947. "Small bitter cherry", a fruit abnormality of the bing cherry variety. Sci. Agric. 27: 260-262.

Lukens, R.J., P.M. Miller, G.S. Walton and S.W. Hitchcock. 1971. Incidence of X-disease of peach and eradication of chokecherry. Plant Dis. Rep. 55: 645-647.

MacBeath, J.H., G. Nyland and A.R. Spurr. 1972. Morphology of mycoplasma-like bodies associated with peach X-disease in Prunus persica. Phytopathology 62: 935-937.

Morisita, M. 1959. Measuring the dispersion of individuals and analysis of the distributional patterns. Mem. Fac. Sci. Kyushu Univ. Ser. E. Biol. 2: 215-235.

McClure, M.S. 1980a. Role of wild hosts plants in the feeding, oviposition, and dispersal of Scaphytopius acutus (Homoptera: Cicadellidae), a vector of peach X-disease. Environ. Entomol. 9: 265-274.

McClure, M.S. 1980b. Spatial and seasonal distributions of leafhopper vectors of peach X-disease in Connecticut. Environ. Entomol. 9: 668-672.

Michigan Statistical Abstract. 13th ed. D.I. Verway, ed. Division of Research, Graduate School of Business Administration, Michigan State University.

Mowry, T.M., M.E. Whalon and A.L. Jones. 1981. Selected bibliography of the enzyme-linked immunosorbent assay and its application to entomology. Bull. Entomol. Soc. Am. 27: 113-116.

Nasu, S., D.D. Jensen and J. Richardson. 1970. Electron microscopy of mycoplasma-like bodies associated with insect and plant hosts of peach western X-disease. Virology 41: 583-595.

Nasu, S., D.D. Jensen and J. Richardson. 1974a. Extraction of western X mycoplasmalike organism from leafhoppers and celery infected with peach western X-disease. Appl. Ent. Zool. 9: 53-57.

Nasu, S., D.D. Jensen and J. Richardson. 1974b. Primary culturing of the western X mycoplasma-like organism from Colladonus montanus leafhopper vectors. Appl. Ent. Zool. 9: 115-126.

Nielson, M.W. 1968. The leafhopper vectors of phytopathogenic viruses (Homoptera: Cicadellidae): Taxonomy, biology and virus transmission. USDA Tech. Bull. No. 1382. 386 pp.

Nielson, M.W. and L.S. Jones. 1954. Insect transmission of western-X-little-cherry virus. Phytopathology 44: 218-220.

Nyland, G. 1955. Interference between the viruses or virus strains causing yellow leaf roll and western X-disease of peach. Phytopathology 45: 694-695.

Nyland, G. 1971. Remission of symptoms of pear decline in pear and peach X-disease in peach after treatment with a tetracycline. Phytopathology 61: 904-905.

Oman, P.W. 1951. The nearctic leafhoppers: A generic checklist. Mem. Ent. Soc. Wash. No. 3. 252 pp.

Palmiter, D.H. and E.M. Hildebrand. 1943. The yellow-red virosis of peach: Its identification and control. N.Y. Agric. Exp. Sta. (Geneva) Bull. 704. 17 pp.

Palmiter, D.H., W.J. Coxeter and J.A. Adams. 1960. Seasonal history and rearing of Scaphytopius acutus (Say) (Homoptera: Cicadellidae). Ann. Ent. Soc. Amer. 53: 843-846.

Parker, K.G., D.H. Palmiter, R.M. Gilmer and K.M. Hickey. 1963. X-disease of peach and cherry trees and its control. N.Y. State Agric. Ext. Bull. 1100. 12 pp.

Pielou, E.C. 1977. Mathematical Ecology. John Wiley and Sons, New York. 138 pp.

Purcell, A.H. 1979. Transmission of X-disease agent by the leafhoppers Scaphytopius nitridus and Acinopterus angulatus. Plant Dis. Rep. 63: 549-552.

Purcell, A.H., G. Nyland, B.C. Raju and M.R. Heringer. 1981. Peach yellow leaf roll epidemic in northern California: Effects of peach cultivar, tree age, and proximity to pear orchards. Plant Dis. 65: 365-368.

Ragsdale, D.W. 1980. Quantitative assessment of predation of Nezara viridula L. using ELISA. Ann. Meetings Entomol. Soc. Am. (Atlanta, 1980) Presentation No. 432.

Raju, B.C. and G. Nyland. 1981. Enzyme-linked immunosorbent assay for the detection of corn stunt spiroplasma in plant and insect tissue. Curr. Microbiol. 5: 101-104.

Raju, B.C., G. Nyland, E.A. Backus and D.L. McLean. 1981. Association of a spiroplasma with brittle root of horseradish. Phytopathology 71: 1067-1072.

Rawlins, T.E. and W.T. Horne. 1931. "Buckskin", a destructive graft-infectious disease of the cherry. Phytopathology 21: 331-335.

Rawlins, T.E. and H.E. Thomas. 1941. The buckskin disease of cherry and other stone fruits. Phytopathology 31: 916-925.

Reeves, E.L. and L.M. Hutchins. 1941. A progress report on western X-disease, a virus disease of peaches. Wash. State Hort. Assoc. Proc. 37: 27-30.

Richards, B.L. 1945. The red-leaf chokecherry virus as a possible cause of wilt in the sweet cherry and of dieback in the sour cherry. Utah Acad. Sci., Arts and Letters Proc. 22: 9. (abstr.).

Richards, B.L., L.M. Hutchins and E.L. Reeves. 1949. The western "X" virus a cause of "little cherry" in Utah. Phytopathology 39: 19. (abstr.).

Richards, B.L., E.L. Reeves and L.M. Hutchins. 1946. Wilt and decline, a virus disease of sweet and sour cherries in Utah. Phytopathology 36: 409. (abstr.).

Richards, B.L., B.N. Wadley and G.W. Cochran. 1948. Little cherry, a virus disease of sweet and sour cherries in Utah. Utah Farm and Home Sci. 9: 10-12.

Rosenberger, D.A. 1977. Leafhopper vectors, epidemiology and control of peach X-disease. Ph.D. Diss. Mich. St. Univ., East Lansing, MI. 95 pp.

Rosenberger, D.A. and A.L. Jones. 1977a. Spread of X-disease in Michigan peach orchards. Plant Dis. Rep. 61: 830-834.

Rosenberger, D.A. and A.L. Jones. 1977b. Symptom remission in X-diseased peach trees as affected by date, method, and rate of application of oxytetracycline-HCl. Phytopathology 67: 277-282.

Rosenberger, D.A. and A.L. Jones. 1978. Leafhopper vectors of the peach X-disease pathogen and its seasonal transmission from chokecherry. Phytopathology 68: 782-790.

Saillard, C., J. Dunez, O. Garcia-Jurado, A. Nhami and J. Bove. 1978. Detection de Spiroplasma citri dans les agrumes et les Pervenches par le technique immuno-enzymatique (ELISA). C.R. Hebd. Seances Acad. Sci. Ser. D. 286: 1245-1248.

Schlocker, A. and G. Nyland. 1951. Yellow leaf roll of peach. Calif. Dept. Agric. Bull. 40: 39-42.

Sokal, R.R. and F.J. Rohlf. 1969. Biometry. W.H. Freeman and Co., San Francisco. 776 pp.

Stoddard, E.M. 1934. Progress report of investigations on a new peach trouble. Conn. Pomol. Soc. Proc. 43: 115-117.

Stoddard, E.M. 1938. The "X-disease" of peach. Conn. Agric. Exp. Stn. Circ. 122: 53-60.

Taboada, O., D.A. Rosenberger and A.L. Jones. 1975. Leafhopper fauna of X-diseased peach and cherry orchards in southwest Michigan. J. Econ. Entomol. 68: 255-257.

Taylor-Robinson, D., R.H. Purcell, D.C. Wong and R.M. Chanock. 1966. A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. J. Hyg. 64: 91-104.

Thomas, H.E., T.E. Rawlins and K.G. Parker. 1940. A transmissible leaf-casting yellows of peach. Phytopathology 30: 322-328.

Thomas, M., J. Neibauer, H. Belter and R. Earl. 1981. Southwest Michigan horticultural crop growers' needs for extension, demonstration and research.

Thomson, S.V., D.G. Garrott, B.C. Raju, M.J. Davis, A.H. Purcell and G. Nyland. 1978. A spiroplasma consistently isolated from western X-infected plants. Proc. 4th Int. Conf. Plant Path. Bact., Angers, France. P. 475. (abstr.).

Van Weemen, B.K. and A.H.W.M. Schuurs. 1971. Immunoassay using antigen-enzyme conjugates. FEBS Lett. 15: 232-236.

Voller, A., A. Bartlett, D.E. Bidwell, M.F. Clark and A.N. Adams. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33: 165-167.

Voller, A., D.E. Bidwell and A. Bartlett. 1979. The Enzyme-linked Immunosorbent Assay (ELISA). A Guide with Abstracts of Microplate Applications. Dynatech Europe, Borough House, Guernsey, G.B. 129 pp.

Voller, A., D.E. Bidwell, G. Huldt and E. Engvall. 1974. A microplate method of ELISA and its application to malaria. Bull. W.H.O. 51: 209-221.

Whitcomb, R.F. 1980. The genus Spiroplasma. Ann. Rev. Microbiol. 34: 677-709.

Whitcomb, R.F. 1981. The biology of spiroplasmas. Ann. Rev. Entomol. 26: 397-425.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1966a. The infection of leafhoppers by western X-disease virus. I. Frequency of transmission after injection or acquisition feeding. Virology 28: 448-453.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1966b. The infection of leafhoppers by western X-disease virus. II. Fluctuation of virus concentration in the hemolymph after injection. Virology 28: 454-458.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1967. The infection of leafhoppers by western X-disease virus. III. Salivary, neural, and adipose histopathology. Virology 31: 539-549.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1968a. The infection of leafhoppers by western X-disease virus. IV. Pathology in the alimentary tract. Virology 34: 69-78.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1968b. The infection of leafhoppers by western X-disease virus. VI. Cytopathological interrelationships. J. Invert. Pathol. 12: 202-221.

Whitcomb, R.F., D.D. Jensen and J. Richardson. 1979. Pathogenicity of mycoplasmas for arthropods. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 245: 200-221.

Williamson, D.C., J.G. Tully and R.F. Whitcomb. 1979. Serological relationships of spiroplasmas as shown by combined deformation and metabolism inhibition tests. Int. J. Syst. Bacteriol. 29: 345-351.

Wolfe, H.R. 1955a. Transmission of the western X-disease virus by the leafhopper, Colladonus montanus (Van D.). Plant Dis. Rep. 39: 298-299.

Wolfe, H.R. 1955b. Relation of leafhopper nymphs to the western X-disease virus. J. Econ. Entomol. 48: 588-590.

Wolfe, H.R. and E.W. Anthon. 1953. Transmission of the western X-disease virus from sweet and sour cherry to peach by two species of leafhoppers. J. Econ. Entomol. 46: 1090-1092.

Wolfe, H.R., E.W. Anthon and L.S. Jones. 1950. Transmission of western X-disease of peaches by the leafhopper, Colladonus geminatus (Van D.) Phytopathology 40: 971. (abstr.).

Wolfe, H.R., E.W. Anthon, G.H. Kaloostian and L.S. Jones. 1951. Leafhopper transmission of western X-disease. J. Econ. Entomol. 44: 616-619.

U.S. Bureau of the Census, Statistical Abstract of the United States: 1978. 99th ed. Washington, D.C.

```
      IF(B.EQ.1HN)GOTO 9
      PRINT*,"ENTER K VALUE--"
      READ*,XK1
      XKINV=1/XK1
C  ****************************************************************
C  THE PROGRAM NOW COMPUTES VALUES OF X', Y', 1/K AND
C  K FOR EACH SAMPLE AS WELL AS THE SUMMATIONS OF X'
C  AND Y', 1/KC AND KC AS AN UNWEIGHTED ESTIMATE OF
C  THE COMMON K ACCORDING TO THE FORMULAS GIVEN IN:
C  ELLIOTT, J.M.  1977.  METHODS FOR THE STATISTICAL
C  ANALYSIS OF SAMPLES OF BENTHIC INVERTEBRATES.
C  FRESHWATER BIOL. ASSOC. #25.  160 PP.
C  ****************************************************************
      SUMX=0.0
      SUMY=0.0
      DO 20 I=1,J
      XPRIM(I)=(XBAR(I)**2)-(VAR(I)/N(I))
      YPRIM(I)=VAR(I)-XBAR(I)
      AKINV(I)=YPRIM(I)/XPRIM(I)
      AK1(I)=XPRIM(I)/YPRIM(I)
      SUMX=SUMX+XPRIM(I)
      SUMY=SUMY+YPRIM(I)
20    CONTINUE
      IF(B.EQ.1HY)GOTO 11
      XKINV=SUMY/SUMX
      XK1=SUMX/SUMY
C  ****************************************************************
C  THE PROGRAM NOW COMPUTES THE WEIGHTING FACTOR, W,
C  FOR EACH SAMPLE, THE NECESSARY WEIGHTED STATISTICS
C  WX', WY', WX'Y', WX'SQ, THE SUMMATIONS OF WX'Y' AND
C  WX'SQ AND THE WEIGHTED COMMON K ACCORDING TO THE
C  FORMULAS GIVEN IN:  BLISS, C.I. AND A.R.G. OWEN.
C  1958.  NEGATIVE BINOMIAL DISTRIBUTIONS WITH A
C  COMMON K.  BIOMETRIKA 45:  37-58.
C  ****************************************************************
11    SUMWX=0.0
      SUMWY=0.0
      SUMWXY=0.0
      SUMWX2=0.0
      DO 30 I-1,J
      W(I)=(0.5*(N(I)-1)*(XK1**4))/((XK1*(XK1+1)-(2*XK1-1)
     +/N(I)-3/N(I)**2)*(XPRIM(I)*(XBAR(I)+XK1)**2))
      WXPRIM(I)=W(I)*XPRIM(I)
      WYPRIM(I)=W(I)*YPRIM(I)
      WXY(I)=WXPRIM(I)*YPRIM(I)
      WXSQ(I)=WXPRIM(I)*XPRIM(I)
      SUMWX=SUMWX+WXPRIM(I)
      SUMWY=SUMWY+WYPRIM(I)
      SUMWXY=SUMWXY+WXY(I)
      SUMWX2=SUMWX2+WXSQ(I)
30    CONTINUE
      YKINV=SUMWXY/SUMWX2
      YK2=SUMWX2/SUMWXY
```

```
C  ****************************************************************
C  ALL DATA IS NOW WRITTEN ONTO TAPES FOR OUTPUT.
C  TAPE 1 OUTPUTS TO THE CYBER 750 AND TAPE 2
C  OUTPUTS TO THE INTERACTIVE TERMINAL.
C  ****************************************************************
       WRITE(1,200)
       WRITE(1,300)(N(I),XBAR(I),VAR(I),XPRIM(I),YPRIM(I),
      +AKINV(I),AK1(I),W(I),WXPRIM(I),WYPRIM(I),WXY(I),
      +WXSQ(I),I=1,J)
       WRITE(1,400)SUMX,SUMY,XKINV,XK1,SUMWX,SUMWY,
      +SUMWXY,SUMWX2,YKINV,YK2
       GOTO 5
4      WRITE(2,500)
       WRITE(2,600)(N(I),XBAR(I),VAR(I),XPRIM(I),YPRIM(I),
      +AKINV(I),I=1,J)
       WRITE(2,700)SUMX,SUMY,XKINV
       WRITE(2,750)
       WRITE(2,800)(AK1(I),W(I),WXPRIM(I),WYPRIM(I),
      +WXY(I),WXSQ(I),I=1,J)
       WRITE(2,900)XK1, SUMWX,SUMWY,SUMWXY,SUMWX2,YKINV,YK2,
       GOTO 7
C  ****************************************************************
C  OPTIONS ARE NOW GIVEN TO THE USER FOR THE
C  DISPOSITION OF THE OUTPUT.
C  ****************************************************************
5      PRINT*,"COMPUTATIONS ARE NOW COMPLETE.  BECAUSE OF THE LENGTH"
       PRINT*,"OF THE OUTPUT, YOU MAY HAVE IT PRINTED AT THE"
       PRINT*,"TERMINAL OR ON THE CYBER 750 PRINTER.  THE OUTPUT"
       PRINT*,"IS PRESENTED MORE EFFICIENTLY ON THE LARGE CYBER"
       PRINT*,"750 PAPER.  ENTER 1 FOR THE TERMINAL OUTPUT OR 2"
       PRINT*,"FOR THE CYBER 750 OUTPUT."
       READ*,K
       IF(K.EQ.1HY)GOTO 4
6      PRINT*,"WHEN PROGRAM TERMINATES, ENTER 'DISPOSE,"
       PRINT*,"TAPE 1,PA.'.  COPY DOWN SEQUENCE NUMBER AND"
       PRINT*,"PICK-UP OUTPUT AT THE COMPUTER CENTER."
       GOTO 8
7      PRINT*,"WOULD YOU LIKE CYBER 750 OUTPUT ALSO? Y OR N--"
       READ 100,C
       IF(C.EQ.1HY)GOTO 6
100    FORMAT(A1)
200    FORMAT(* *,136(1H-),/,3X,*N*,7X,*MEAN*,5X,*VARIANCE*,
      +7X,*X'*,10X,*Y'*,10X,*1/K*,10X,*K*,11X,*W*,10X,*WX'*,
      +9X,*WY'*,8X,*WX'Y'*,7X,*WX'SQ*,/,* *,136(1H-),/)
300    FORMAT(* *,I4,11F12.5)
400    FORMAT(*0*,*TOTALS*,22X,4F12.5,12X,4F12.5,///,
      +36X,*1/KC = *,F12.5,24X,*KC = *,F12.5,///)
500    FORMAT(* *,70(1H-),/,3X,*N*,7X,*MEAN*,5X,*VARIANCE*,
      +7X,*X'*,10X,*Y'*,10X,*1/K*,/,* *,70(1H-))
600    FORMAT(* *,I4,5F12.5)
700    FORMAT(*0*,*TOTALS*,22X,3F12.5)
750    FORMAT(* *,//,70(1H-),/,5X,*K*,11X,*W*,10X,*WX'*,9X,
      +*WY'*,7X,*WX'Y'*,7X,*WX'SQ*,/,* *,70(1H-))
```