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FIELD DETECTION OF PEACH X-DISEASE MYCOPLASMALIKE ORGANISM IN SPECKLED LEAFHOPPER, Paraphlepsius irroratus (Say) (Homoptera: Cicadellidae) USING A DNA PROBE

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ABSTRACT

FIELD DETECTION OF PEACH X-DISEASE MYCOPLASMALIKE ORGANISM IN SPECKLED LEAFHOPPER, *Paraphlepsius irroratus* (Say) (Homoptera: Cicadellidae) USING A DNA PROBE

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X-disease in stone fruit is caused by a mycoplasmalike organism (MLO). C6c, a fragment of pWX1 (an X-disease specific probe derived from infected *Colladonus montanus* (Van Duzee)), was used to hybridize with DNA from several procaryotic and eucaryotic organisms that are associated with leafhoppers are close relation to the X-disease agent. C6c gave a positive reaction with only Eastern X-disease infected leafhoppers.

X-diseased *P. irroratus* seasonallity was observed in Fennville and East Lansing, MI during 1988. Infected adult leafhopper were detected in the first emergence flight. The percentage of diseased adult leafhoppers in the first generation was higher than in the second generation.

The extensive occurrence of X-diseased leafhoppers was also monitored at Fennville, East Lansing, Lawrence, Ludington and Suttons Bay during 1988. X-diseased *P. irroratus* were observed at Fennville, East Lansing and Lawrence but not at Ludington and Suttons Bay.

DEDICATIONS

To my father and mother

who introduced me to the beautiful and interesting creature,

the insect

The fear of the LORD
is the beginning of wisdom,
and knowledge of the Holy One
is understanding

Proverbs 9: 10

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I also benefited from the cooperation of the Michigan stone fruit growers whose farm were used for this study.

I gratefully thank Yong Tang Yan for introducing me the 'dot blot' technique and to Scott Douglas for his assistance in the field.

Without the cooperation of my co-worker, Carlos Garcia-Salazar, this research would not have been possible. His support, courage and patience in spending many late nights and early mornings working with ^{32}P is gratefully acknowledged.

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CHAPTER I

GENERAL LITERATURE REVIEW

INTRODUCTION

X-disease, a serious disease of stone fruit caused by mycoplasma like organism (MLO), has been causing extensive tree loss in Michigan. Berrien County, the most intense peach growing area in Michigan, had approximately 1.4% infection of 238,602 trees inspected in 1987. This infection rate was down from 1986 (2.10%) which was up over the previous year (1.65%) (Dreves 1985, 1986, and 1987).

This disease was first reported and named X-disease in 1933 in Connecticut (Stoddard 1933). By 1938 it had become a major peach disease in most of the northern United States (Stoddard 1938, 1941). In Michigan, X-disease was first reported in 1941 (Cation 1941, Stoddard 1947).

The causative organism of X-disease of stone fruit (*Prunus* spp) in the Northeastern and Western areas of the United States is believed to be a mycoplasma-like organism (MLO). The causal agent was first thought to be a virus until studies demonstrated that the agent had similar ultrastructural characteristics to mycoplasma. The X-disease agent was found in infectious leafhoppers and in the phloem sieve tube elements of diseased trees, yet this MLO

has never been cultured (Nasu *et al.* 1970, Granet and Gilmer 1971, Jones *et al.* 1974, Agrios 1978). Transmission of MLOs occurs during feeding of infected leafhoppers on a susceptible host. The leafhopper injects the MLO into the plant phloem where it multiplies and spreads through the host-plant vascular tissue (Agrios 1978, Rosenberger 1982). Stoddard (1947) first hypothesized with support from McCoy (1979) that the MLO overwinters in the root and is translocated up the tree during spring time. Meanwhile, Rosenberger and Jones (1977) concluded that the X-disease pathogen overwinters both in buds and roots of diseased plants even though the percentage of infected buds was low and remained low until mid-June. During cold weather, the MLO agent apparently remains in the aerial portion of the tree and in the roots with high titer. Recent unpublished work by Dr. Bruce Kirkpatrick at the University California at Davis supports both views (personal communication).

Leafhoppers are the only known vectors of X-disease in commercial orchards. From 70 leafhopper species collected on yellow sticky board traps placed in and around several X-diseased peach and sour cherry orchards in Southwest Michigan, *Paraphlepsius irroratus* (Say) and *Scaphytopius acutus* (Say) were the most abundant (Taboada *et al.* 1977). *P. irroratus* is considered the most important X-disease vector in Michigan because of its abundance and high transmission efficiency (Rosenberger and Jones 1977, Larsen and Whalon 1987).

Nymphs of *P. irroratus* generally stay in the orchard ground cover where they feed on herbaceous plants (Rosenberger and Jones 1978). Many herbaceous and grass host have now been identified as alternate host for X-disease (Table I.1). Adults appear to feed on woody plants during the evening after a crepuscular activity period (Larsen and Whalon 1987). To be able to transmit the MLO from plant to plant, the leafhoppers have to acquire the MLO by feeding on infected plants. They begin to transmit the pathogen after an incubation period of 10 to 45 days depending on temperature. The shortest incubation occured at about 30 ^{o}C and the longest at 10 ^{o}C (Agrios 1978). In the vector, the incubation period involves the MLO's circulation through the hemolymph and infection of other tissues including eventually the salivary glands before the leafhopper is competent to transmit the MLO (Agrios 1978, Chiykowski and Sinha 1988).

In the past, several researchers have independently reported the identification of mycoplasmas from plants or insects infected with X-disease (Granet and Gilmer 1971, Nasu *et al*. 1974, Chiykowski and Sinha 1980). A few researchers, furthermore, have tried to diagnose the occurrence of X-disease both in plants and its vectors (Sinha and Chiykowski 1984, 1986, Kirkpatrick 1987). Identification of X-disease in plants has been based on symptomatology, immunology, host range, vector-pathogen relationship and characteristic DNA.

Rosenberger and Jones (1978), Chiykowski and Sinha (1980), Suslow and Purcell (1982), all used symptomatology to identify the MLO in its vector and host. There are a number of descriptive symptoms used for MLO diagnosis, e.g. virescence, phyllody, stunt, proliferation ('witches' broom), etc (Table I.2 and I.3). But symptomatology is an unreliable and therefore often unsatisfactory means of detecting the presence of MLO.

Immunosorbent electron microscopy (ISEM) techniques have been introduced to detect and identify plant mycoplasma (Derrick and Brlansky 1976). The recent advances in immuno-gold labelling have demonstrated the specificity and sensitivity of this technique in detecting the occurrence of *Spiroplasma citri* (Mowry *et al.* 1985). However, this technique requires considerable effort, time and is too expensive to be applied for routine field detection. It is clear that a rapid, simple technique to detect the occurrence of X-disease in vectors and plants is desirable.

More rapid and specific methods than electron microscopy have been developed. Polyclonal and monoclonal sera have been used against MLO antigens through enzyme linked immunoassays (Sinha and Chiykowski 1984, 1986, Lin and Chen 1985). Purified and partially purified preparation from celery leaves infected with peach X-disease did not give a specific reaction in ELISA with antiserum against the MLO. Detection of MLO from a single infected leafhopper by ELISA was unsuccessful even with high

concentration of antigen. Several limitations arose associated with these methods. Polyclonal antibodies showed cross reactions to other MLO preparations and apparently share the same common antigen possessed by the aster yellow (AY) MLO agent and thus lack specificity. Monoclonal antibodies have been developed but are too specific and thus less usefull in evaluating the general presence of X-disease.

Recent advances in molecular biology may prove to be useful in developing detection systems for MLO disease in vectors and plants. The use of specific DNA probes has proved highly effective for assaying mycoplasma. This approach was developed to detect the occurrence of X-disease MLO in both purified and partially purified vector and plant samples (Kirkpatrick 1987).

The overall goal of this research was to evaluate several DNA probes as diagnostic tool for detecting Michigan X-disease MLOs in leafhopper vectors sampled throughout Michigan from 1987-1988. A secondary goal was to attempt to relate the results of this work with the epidemiology of X-disease of stone fruit in Michigan. This research was done based on the following objectives and hypotheses.

OBJECTIVES

1. To evaluate several DNA probes for detecting Michigan X-disease.
2. To develop the necessary laboratory protocols for use of the DNA probe(s).
3. To preliminary determine the seasonal and geographical incidence of X-disease in field collected *Paraphlepsius irroratus* (Say) samples .

HYPOTHESIS

H_1. A DNA probe can be used as a rapid, specific detection system of X-disease MLO in leafhopper vector samples.

H_2. The seasonal incidence of X-disease in leafhoppers and the geographical distribution of X-disease leafhopper can be established from field frozen leafhopper samples.

Table I.1. Experimentally inoculated orchard herbaceus plants (A) and symptom for plants from orchard tested positive for X-disease pathogen (B).

Common name	Scientific Name
A. Assay test : ELISA and indicator plant[1]	
Blueweed	*Echium vulgare* L.
Bladder campion	*Silene cucubalus* Wibel
Spinach	*Spinacia oleracea* L.
	Matricaria chamomilla L.
Pineappleweed	*Matricaria matricariodes* (Less.) Porter
Dandelion	*Taracacum officinale* Weber
Meadow goat's beard	*Tragopogan pratensis* L.
Shepperd's purse	*Capsella bursa-pastoris* (L.) Medic.
Stinkweed	*Thlaspi arvense* L.
Pin-clover	*Erodium cicutarium* (L.) L'Her
Barley cv. Vanier	*Hordeum vulgare* L.
Flat pea	*Lathyrus sylvestris* L.
	Lapinus leucophyllus Dougl.
Black medic	*Medicago lupulina* L.
Yellow sweet clover	*Melilotus officinalis* (L) Lam.
Alsike clover	*Trifolium hybridum* L.
B. Assay test : ELISA and Dot hybridization[2]	
Filaree	
Plantago L	*Plantago lanceolata*
Ansinkia	
Burr Clover	*Trifolium* sp
Convolvulus	*Convolvulus* sp
Pigged[3]	*Amaranthus* sp
Mustard[3]	*Brassica mustum*
Plantago M[3]	*Plantago* sp

[1]Each test plant was inoculated using 8 *Paraphlepsius irroratus* (Say) that had acquired X-disease MLO from infected celery. MLO-free leafhoppers were caged for 14 days on plants showing symptoms and were tested singly on celery seedlings (Chiykowski and Sinha 1988). Assessment based upon classical transmission study and immunological test.

[2]The orchard weeds were inoculated experimentally by means of the leafhopper *Colladonus montanus* (Van Duzee). Leaf and root tissue were used for assay (Kirkpatrick personal communication). Assessment based on immunological test and cdna hybridization technique.

[3]Only roots tested positive

Table I.2. Mycoplasmalike Organism Symptomatology Chart

DISEASE	White clover			Celery		Aster			Cherry	Periwinkle	
	Leave	Flower	Plant	Leave	Root	Leave	Flower	Shoot	Fruit	Tip	Flower
Aster yellows Western Strain Eastern Strain	not smaller reddening propeller leaves	virescence phyllody	general stunting	Twisting petiole progressively pronounced	Possible but not rapid root destruction	Severe vein clearing (14 days)	Phyllody virescence	Stunted main shoot axillary growth in shoots (if any) profuse axillary growth		with small leaves, shorter internodes.	Green flower
Clover Club Leaf	chlorotic leaf margins leaf stunting curling of leaflet	virescence phyllody				not known					
Clover Phyllody (Strawberry Green Petal) Probably not found in Michgan	Propeller leaves chlorotic leaf margins leaf stunting lamina grows not as severe as X-disease					mild vein clearing (30 days)					virescence phyllody
Clover Yellow-edge	chlorotic leaf margins and eventually necrosis (29 days) , lamina does not grow	stop flowering or are smaller				mild vein clearing (33.2 days)	reduced size , faded color				flowers greatly reduced and faded
Clover Proliferation probably not in MI but found out west											green flowers
Spiroplasma citri (Brittleroot Strain)											flowers reduced
X-disease	chlorotic leaf margins lamina necrosis small and propeller leaf old leaves die clumps of dwarf leaves at axial (60 days) reddening	stops flowering small if flowering	definite stanting of plant premature death	inward rolling of leaves early chlorotic new growth marginal chlorosis reduced leaf lamina	rapid root destruction	mild vein clearing (35 days) reduced leaf size	reduced size, faded color	necrosis at center stuntet branches	small, bitter delayed ripening		flowers reduced and faded

Sources : McGrew and Posnette 1970, Markham and Townsen 1979, Markham 1982.

Table I.3. X-disease Symtomatology Chart

Host Plant	Root	Tip	Leave	Flower	Plant	Fruit
White Clover			chlorotic leaf margins, lamina necrosis, small propeler leaves, old leaves die, clump and dwarf leabes at axial (60 days), reddening	stop flowering small if flowering	definite stunting premature of death	
Celery	root destruction		inward rolling of leaves early chlorosis, new growth marginal chlorosis and reduced leaf lamina		stunted	
Aster		necrosis at center stunted branches		reduced size faded color		
Cherry						small and bitter delayed ripening, no seeds
Chokecherry		rosetted leaves	turns bright red and yellow during mid-summer			
Peach		foliage remain on tips only	random discolorod pattern, stiff and brittle, 'shot hole' appearence upward rolling, leaves frequantly fall	normal		premature ripen, unpleasent taste, abortive seed, dry up and drop on infected branch
Periwinkle				flower reduced and faded		

Sources : Stodard 1947, Whalon unpublished, Agrios 1978.

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CHAPTER II

Development and evaluation of a DNA hybridization technique for detection of X-disease mycoplasmalike organism in *Paraphlepsius irroratus* (Say) in Michigan

INTRODUCTION

X-disease has been a major problem for Michigan peach growers. Ten Michigan peach orchards were surveyed annually from 1973 to 1976. The percentage of X-diseased trees observed ranged from 2 to 75% (Rosenberger and Jones 1977). Observations conducted by the Michigan Department of Agriculture (MDA) in Bainbridge Township showed that the percentage of X-diseased peach trees fluctuated annually but generally increased during a 6 year period from 1980 to 1986 (1.09 to 1.80%) and decreased the following year (1987) to 1.25%. This decrease was presumably due to removal of the infected trees but environmental and vector population density may also have contributed. In three years (1985-1987), an average of 15.48% of inspected trees has been removed annually (Dreves 1985, 1986 and 1987).

The causal agent of X-disease was first assumed to be a virus until researchers demonstrated that the agent had similar ultrastructural characteristics to mycoplasma and named X-disease mycoplasmalike organism (MLO) because it has yet to be classified (Nasu et al. 1970, Granet and Gilmer 1971, Jones et al. 1974). MLO was observed in infectious leafhoppers and in the phloem sieve tube elements of diseased trees (Agrios 1978), yet this MLO has never been

cultured (Sinha 1988). Since MLOs are uncultivable, they can not be detected or classified by conventional media preparations as with bacteria; therefore, there is a need for developing a detection system for X-disease MLO. Traditionally, X-disease MLO diagnosis has been developed based on host symptomatology, and plant host-vector-MLO relationship but these approach are time consuming and expensive. Three major techniques have been developed to diagnose MLO in plants and insects: 1) immunosorbent assay (ELISA) with polyclonal or monoclonal antibody, 2) immunosorbent electron microscopy (ISEM), and 3) nucleic acid hybridization.

Experimentally, ELISA has been used for detecting X-disease MLO (Chiykowski and Sinha 1984, 1986). But it failed to give detectable specific reactions with experimentally infected celery. The progress in the production of monoclonal antibodies against X-disease is underway (Chiykowski, personal communication) although monoclonals produced to aster yellow were not usefull (Lin and Chen 1986) because the antibodies were too epitopes conferring only subspecies specificity.

Nucleic acid hybridization techniques have been widely used for various purposes. With this technique, single stranded DNA is fixed on a membrane substrate (filter) and is hybridized with ^{32}P labelled single stranded DNA probes. Complementary base pairing of the homologous strands results in a probe-sample ligation. This hybrid molecule remains on

the membrane while the unpaired labelled DNA is washed away during the washing step. The hybrid molecule or positive samples can be visualized using autoradiography (Strickberger, 1985).

A number of DNA probes have been developed for the detection of procaryotic organisms (Amikam 1985, Kirkpatrick 1987). However, none of these probes have been used in extensive field studies, primarily because of the long, complicated DNA extraction procedures. For routine diagnostic use, a probe should be specific with known hybridization potential against other procaryotes, contaminants and possible strains of the target pathogen. The most promising X-disease probes (Kirkpatrick 1987) were not evaluated with Michigan X-disease or with crude preparations from field sampled leafhopper vectors. Nor were these probes evaluated with symbiotic or contaminating procaryotic DNA associated with Michigan vectors. Exploration of possible false positive reaction are critical since leafhopper vector might carry several symbiotic procaryotic and simple eucaryotic organisms (e.g. yeast). In this study, C6c DNA probe derived from the pWX1 X-disease probe (Kirkpatrick 1987) was evaluated against several procaryotic and simple eucaryotic organisms using nucleic acid hybridization techniques.

MATREIALS AND METHODS

Probe source

C6c DNA probe is a 1.9 kb EcoRI-Hind III fragment of pWX1 (Figure I.1). pWX1, provided by Dr. Bruce Kirkpatrick (Davis, CA), is a Western X-disease-specific plasmids consisting of a 4.4 kb DNA fragment from HindIII and EcoRI partially digested DNA of X-diseased *Colladonus montanus* (Van Duzee) (Kirkpatrick 1976, 1987). pWX1 was cloned in pUC8 using *Escherichia coli* strain JM 83.

Leafhopper samples

Healthy *P. irroratus* were maintained in a caged plastic pots (10 cm diameter, 17.5 cm height). Four males and four females were put on three week old clover. To provide food for nymphs that feed on barley, barley seeds were planted in the same pots and the same time when putting the adults. The second or third leafhopper instar were transferred onto seven days old barley planted in caged plastic pots and maintain them until they become adults. The diseased leafhoppers were provided by Dr. L.N. Chiykowski (Chiykowski and Sinha 1984).

Procaryotic and eucaryotic DNA samples

C6c DNA probe was evaluated for hybridization against various procaryotic and eucaryotic DNA that might be extracted from leafhoppers in routine sampling. These DNA

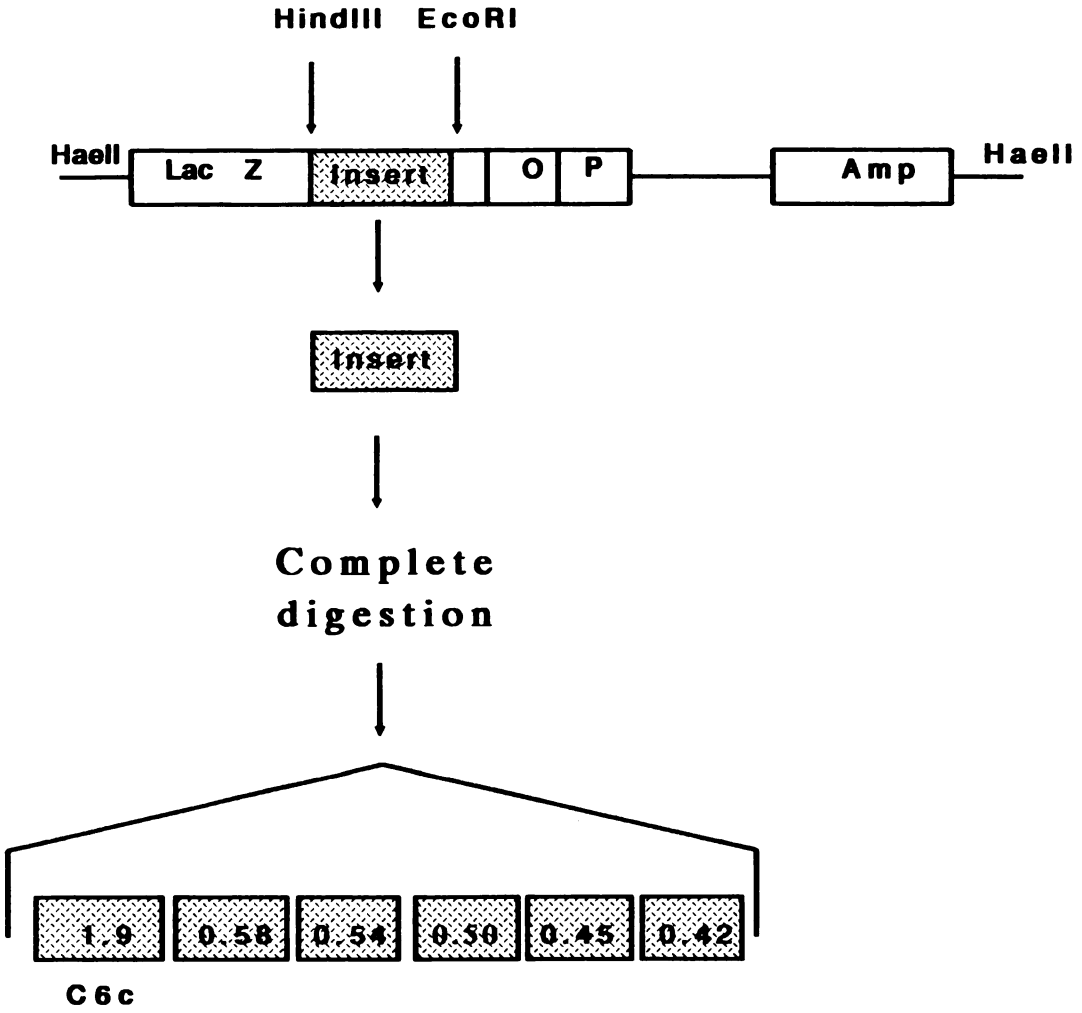

Figure I.1. pWX1, a pUC8 plasmid with insertion of a DNA fragment from *Colladonus montanus* (van Duzee). Double complete digestion with HindIII and EcoRI resulted 6 fragments 1.9, 0.58, 0.54, 0.5, 0.45 and 0.42 kb.

sources are various MLOs, bacteria, yeast and healthy and infected leafhoppers (Table II.1).

Mycoplasma alkalescens and *Mycoplasma californicum* were cultured in broth medium described by ATCC (American Type Culture Collection, Rockville, MD, 1982) that contained Pleuropneumonialike organism (PPLO) broth (Difco) with 20% horse serum and 10% (v/v) of 25% (w/v) fresh baker yeast extract (Difco). Although these were not mycoplasma that were likely to be in insect guts, they represented easily obtained, and cultivable organisms which are closely related to X-disease MLO.

Frozen bacteria cell cultures obtained from ATCC were grown in a rich broth media containing 10 g tryptone, 5 g yeast extract and 10 g NaCl. Media LD8 containing *a*-Ketoglutaric acid and pyruvic acid as described by Lee and Davis (1984) was used to culture *Spiroplasma citri*.

A standard colony amplification protocol was used for obtaining enough cells for DNA extraction. Briefly, freeze dried cells obtained from ATCC were streaked onto the appropriate agar medium plates. Single colonies were then selected, transferred to 5 ml liquid media and grown up in 1 liter quantities before DNA extraction.

DNA extraction was carried out following Carle *et al.* (1983), Maniatis (1982), Sherman *et al.* (1970) for *S. citri* and mycoplasma, bacteria and yeast respectively. The basic procedure was to separate cells using differential centrifugation, lyse cells, separate the DNA through

phenol/isoamylalcohol extraction procedure and precipitate the DNA.

MLO extraction from leafhoppers

MLO was extracted from leafhoppers according to Kirkpatrick (1987) with some modifications. Twenty healthy or diseased leafhoppers were ground in 8 ml of MLO enriched buffer (0.1 M Na_2HPO_4, 10% sucrose, 50 mM ascorbic acid, and 1% polyvinylpyrollidone pH 7.6), centrifuged at 3.000xg in a Sorvall SS-34 rotor at 4 ^{o}C for 20 min and the supernatant was harvested and centrifuged once more at 12.000xg for 30 min at 4 ^{o}C. The resulting pellet was resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and stored at -70 ^{o}C until later use.

Probe radiolabelling

Nick translation of C6c DNA probe with ^{32}P was carried out using kit from Amersham (Arlington Heights, IL). Unincorporated nucleotides were separated by passing the reaction mixture through a 1 ml Sephadex G-75 fine column which was previously saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). Labelled probe was harvested as a fraction of the first column peak to ensure only ^{32}P incorporated probe. The specific activity of each nick translation was assayed by removing two 1.0 *u*l samples before and two 1.0 *u*l samples after ^{32}P incorporation. These samples were blotted onto a nylon membrane and air

dried. One from each sample were washed according to the first step of washing protocol. The specific activity of the samples were measured using a scintillation counter (Shelby 1987).

Detection technique: Dot Blot Hybridization

The samples were blotted onto the nylon membrane, hybridized and autoradiographed according to the instruction provided by the company (Method III, GeneScreen^{TM}, New England Nuclear, Dupont, Boston, MA). Before being blotted the nylon membrane was soaked in 2X SSC buffer (diluted appropriately from a stock of 20X SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0) and air dried. MLO DNA was alkaline denatured with 0.5 M NaOH for 10 min and chilled on ice for another 10 min. After denaturation, the solution was diluted with 10X SSC to a final volume of 400 *u*l. This dilution was blotted onto the membrane fixed into the dot-blot apparatus (BioRad, Richmond, CA) and then washed carefully with 2X SSC for 2 min. The nucleic acid was fixed onto the membrane by laying the wet membrane face up on a clean glass plate and irradiated for 5 min with a 254 nm shortwave UV light sources (UVG-11, Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 15 cm.

The wet membrane was then prehybridized in a plastic bag with 10 ml of prehybridization solution (50% deionized formamide, 0.2% polyvinyl-pyrrolidone, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M Tris-HCl (ph 7.5), 1.0 M NaCl,

0.1% sodium pyrophosphate, 1.0% sodium dedoxyl sulfate, 10% dextran sulfate and denatured salmon sperm DNA (≥100 ug/ml). The plastic bag was sealed off and incubated for 6 hr at 42 ^{o}C.

Hybridization was accomplished by cutting one corner of the plastic bag and adding 2.5 ml of hybridization solution (prepared as prehybridization solution but without 1 M NaCl) containing the radioactive labelled probe. Prior to use, the probe and salmon sperm DNA were denatured by being boiled for 10 min followed by immediately chilling on ice for 5 min. The plastic bag was resealed and incubate for 16 hr at 42 ^{o}C. Before autoradiography, the membrane was washed twice for 5 min each with 100 ml of a washing solution (0.3 M sodium chloride, 0.06 M Tris-HCl pH 8.0 and 0.002 M EDTA) at room temperature with gentle rocking. A second wash procedure was done twice for 30 min each at 60 ^{o}C using 100 ml of the above washing solution containing 1% SDS. The final wash was performed by washing the membrane twice for 30 min at room temperature with 100 ml ten fold diluted washing solution.

Autoradiography was carried out by exposing X-ray film (Kodak XAR) for 24-48 hr with an intensifying screen (Sigma) at -70 ^{o}C. X-ray film was developed in a dark room. The Integrated Optical Density (IOD) of the hybrid dots were quantified using 2-D/1-D soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Ca).

Evaluation of C6c DNA probe

To evaluate C6c DNA probe against several nucleic acids, a 200 ng probe per 10 ml hybridization solution to hybridize the various samples (see Table II.1), and the reaction membrane was exposed for 24 hr. A serial dilution of C6c was blotted and hybridized with C6c itself to determine the detection limit of the probe. These experiments were performed twice with hybridization solution with specific activity at the range of of $2x10^8$ and $2x10^9$ cpm/*ug*.

Characterization of C6c DNA probe with Eastern X-disease MLO

This experiment attempted to estimate the number of X-disease MLO cells in leafhopper vector. Three membranes were prepared to establish a standard curve for estimating the amount of X-disease MLO DNA in leafhopper vector by blotting a dilution series of healthy and diseased *P. irroratus* extraction. The serial dilution was as follow: 1, 1/2, 1/4, 1/8 and 1/16 leafhopper extract containing DNA. Each of these dilutions was further diluted with 7, ten fold dilutions (see Figure II.2A). A serial dilution of C6c DNA was blotted on the same membrane as a positive control. The serial dilution was as follow: 1.0, 0.5, 0.25, and 0.125 ngs. Each of these dilutions were further diluted with 7, ten fold dilutions (see Figure II.2A). The number C6c DNA copies in each dilutions blotted on the membrane were estimated based on the molecular weight of single copy.

One ng of purified C6c DNA was estimated contain 480,390,461 copies of DNA (Stryer, 1988). The use of C6c DNA to estimate the number of X-disease MLO cells is based on the following assumptions: 1) C6c DNA from Western X-disease is homologous to the MLO DNA causing Eastern X-disease carry by *P. irroratus*, 2) assuming the MLO cells are diploid, copy of specific dsDNA represents a single cell.

Table II.1. Sources of various DNA used for C6c evaluation.

Species	Source/Origin/Maintenance
Mycoplasma californicum	ATCC* 33461
Mycoplasma alkalescens	ATCC* 29103
Spiroplasma citri	(HRSC)C.E. Eastman, Illinois. Natural History Survey, University Illinois, Champaign, Il.(1982).
Paraphlepsius irroratus (Say)	Maintained in greenhouse cultures at 20±8 °C and 16L:8D.
Frozen infected *P. irroratus*	L.N. Chiykowski, Agriculture, Ottawa, Ontario, Canada (1984).
Bacillus subtilis	ATCC* 6051-U
Pseudomonas aeriginosa	ATCC* 10145-U
Staphylococcus aureus	ATCC* 12600-U
Saccharomyces cereviceae	ATCC* 2601-U
Candide albicans	ATCC* 14053-U

*ATCC: American Type Culture Collection, Rockville, Md, 1982

RESULTS

Evaluation of C6c DNA Probe

No hybridization reactions were observed between C6c DNA and DNA from *Spiroplasma citri*, *Mycoplasma californicum*, *Mycoplasma alkalescens*, *Bacillus subtilis*, *Pseudomonas aeriginosa*, *Staphylococcus aureus*, *Saccharomyces cereviceae*, *Candide albicans* and healthy *Paraphlepsius irroratus* (Say) (Figure II.1). C6c DNA probe hybridized to DNA from infected leafhoppers which carried Eastern X-disease (Figure II.2).

The probe was able to detect up to 0.0005 pg dilution of C6c or equal to 240 copies of C6c (Figure II.3).

Estimation of the presence of X-disease MLO

The Integrated Optical Density (IOD) values resulted from dots initially increased proportionally with the corresponding dilutions of the C6c DNA or *P. irroratus*. After reaching a certain dilution, the IOD values did not consistently proportionally increase with the corresponding dilutions. These values were from dots which were not perfectly blotted on the membrane possibly due to the prevention of proper DNA denaturation and fixation to the filter by protein deposited in crude homogenate.

To develop the equation to estimate the number of X-disease MLO in single *P. irroratus*, the reliable IOD (which

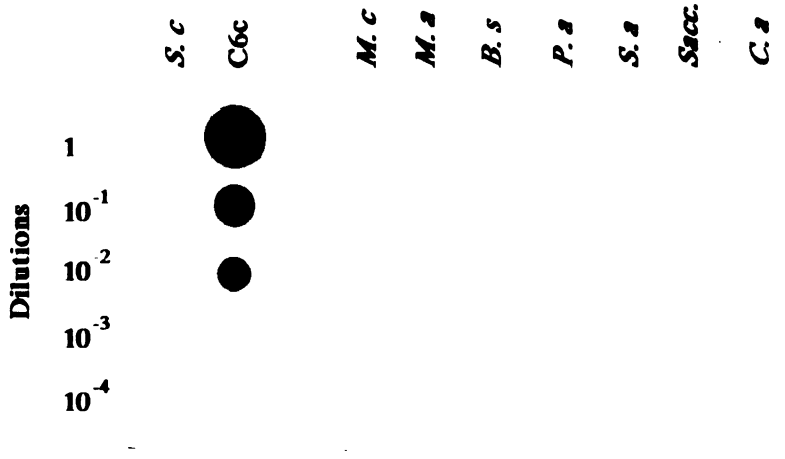

Figure II.1. Autoradiogram of C6c with specific activity of 2.7×10^8 cpm/ul hybridization with several procaryotic and eucaryotic DNA samples. B.s: *Bacillus subtilis*; C.a: *Candide albicans*; M.a: *Mycoplasma alkalscens*; M.c: *Mycoplasma californicum*; P.a: *Pseudomonas aeriginosa*; Sacc.: *Saccharomyces cereviceae*; S.c: *Spiroplasma citri*; S.a: *Staphylococcus aureus*.

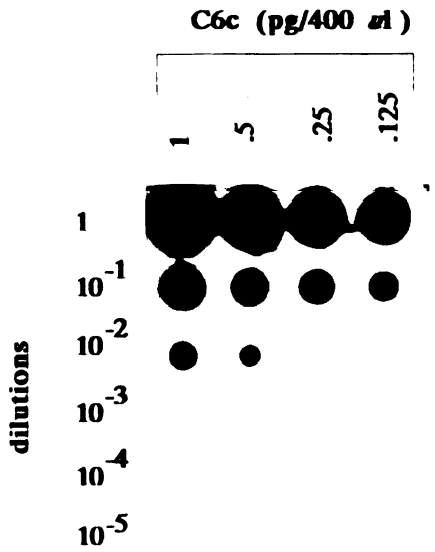

Figure II.2. Detection limit of C6c with specific activity of 5.1 x 10^8 cpm/ul of hybridization solution.

decreased with the dilutions) from autoradiographs of C6c DNA and *P. irroratus* samples hybridized against C6c DNA probe were selected and plotted against the number of C6c and the leafhoppers homogenate dilutions respectivelly (Figure II.4).

The resulting equations which best fit the actual IOD of C6c DNA from three different dot blots using probes with three different specific activities were :

$y = 0.003\ x$ at $5x10^7$ cpm/ug, 24 h, r=.99, P<0.001,

$y = 0.0016\ x$ at $2.6x10^8$ cpm/ug, 24 h, r=.91, P=0.004,

and

$y = 0.0003\ x$ at $5x10^8$ cpm/ug, 24 h, r=.92,P=0.003,

where 'y' was the OD at 'x' dilutions of C6c DNA. Based on these equations, the IOD of one C6c DNA copy were: 0.003, 0.0016, and 0.0003 (Table II.2). These values were used to estimate number of MLO cells in experimentally infected leafhoppers.

The resulting equations which best fit the actual IOD of *P. irroratus* from three different dot blots using probes with three different specific activities were :

$Y = 2{,}152{,}030\ X$ or $2x10^6\ X$,

at $5x10^7$ cpm/ug, r=.99, P<.001,

$Y = 9{,}487{,}810\ X$ or $9x10^6\ X$

at $2.6x10^8$ cpm/ug, r=.99, P<.001, and

$Y = 19{,}893{,}400\ X$ or $2x10^7\ X$

at $5x10^8$ cpm/ug, r=.99, P<.001,

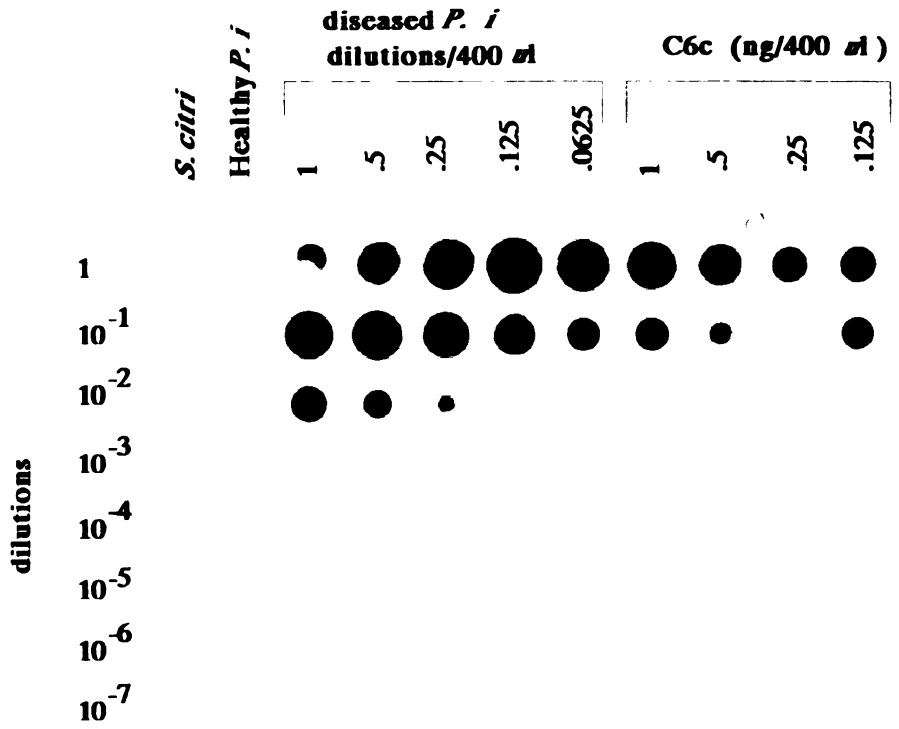

Figure II.3. Autoradiogram of diseased *Paraphlepsius irroratus* (Say) with specific activity of 5 x 10^8 cpm/ul of hybridization solution.

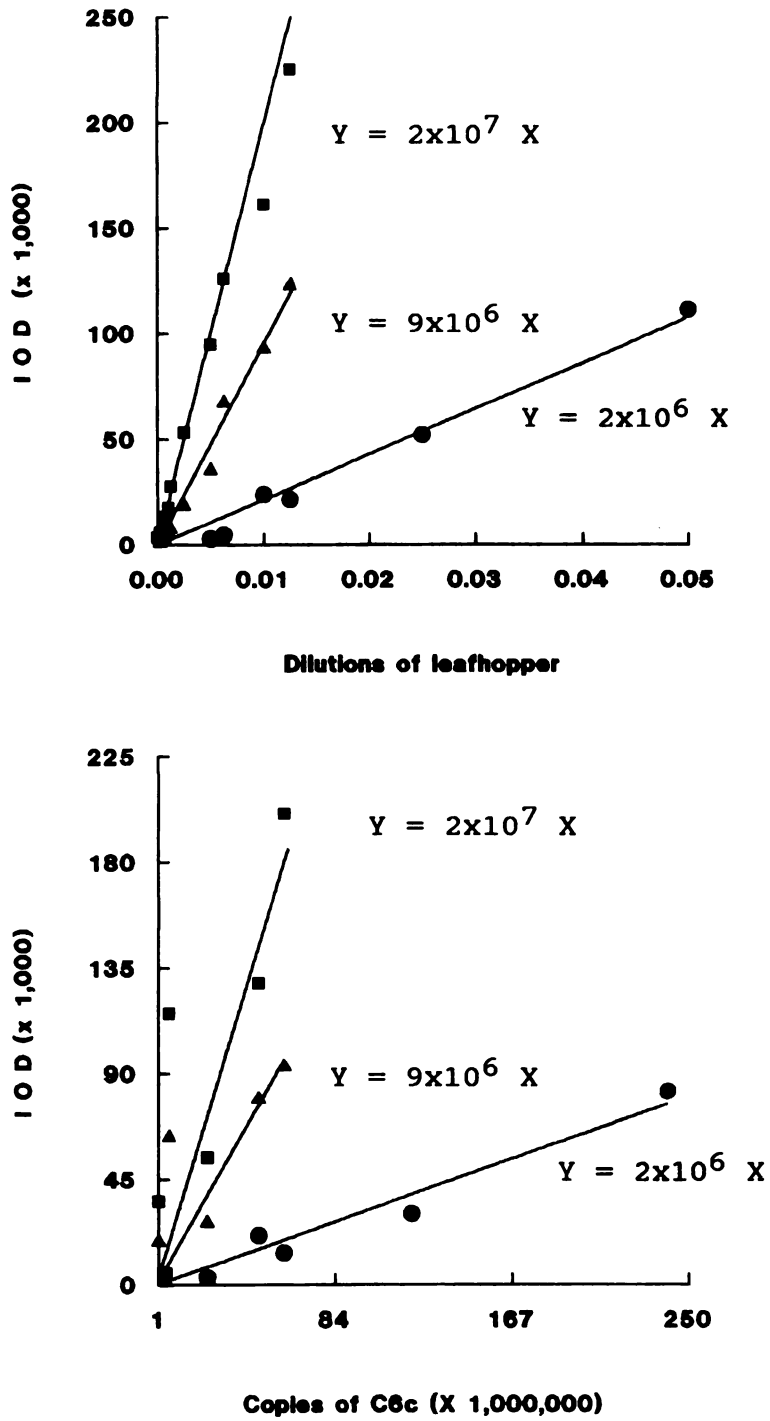

Figure II.4. Estimated lines of the integrated optical density values of diseased *Paraphlepsius irroratus* (Say) and C6c.

where 'y' was the IOD at 'x' dilutions of diseased leafhopper. The resulting equations were used to estimate the number of X-disease MLO cells in one leafhopper. The estimated number of X-disease MLO cells was: $7x10^9$; $6x10^9$ and $6.6x10^9$ (Mean $\pm$ S.E = $6.6x10^9 \pm 2x10^8$) (Table II.2).

The detection limit of X-disease MLO in *P. irroratus*

With the given conditions, the smallest dilution that can be used to detect MLO DNA was between one to the 400^{th} to $166,666^{th}$ dilutions of one diseased leafhopper or approximately equal to 1 among 400 to 166,666 leafhoppers (see Appendix 1).

Table II.2. The number of X-disease MLO cells on experimentally infected *Paraphlepsius irroratus* (Say).

Experiment No.[a]	IOD of C6c[b]	IOD of one *P.i*[c]	Number of MLO per *P.i*[d]
1	0.0003	2,152,030	7,173,433,335
2	0.0016	9,487,810	5,929,881,250
3	0.0030	19,893,400	6,631,133,334
	Mean		**6,578,149,306**

[a] The specific activity were $5x10^7$, $2.6x10^8$ and $5x10^8$ cpm/ug respectively.
[b] IOD of one C6c DNA copy
[c] IOD of one *P. irroratus*
[d] The number of X-disease MLO cells in *P. irroratus*: (c/b)/2

DISCUSSION

The nucleic acid hybridization techniques make use of the fact that a hybrid molecule can be made between the two hydrogen bonded strands of homologous nucleic acids. A previous report describing the use of nucleic acid hybridization for the detection of mycoplasma (Taylor 1984) suggested that this method would be more specific than any other means. The high sensitivity, speed and simplicity of nucleic acid hybridization techniques make these approaches very useful not only for basic research but also for clinical diagnosis. This conclusion is confirmed by the observation that C6c DNA probe detects only X-disease MLO and not the other procaryotic and eucaryotic flora and fauna that might be internally or externally present in leafhopper samples.

Since the C6c DNA probe derived from Western X-disease MLO infected *C. montanus* strongly hybridized under high stringency with DNA from Eastern X-disease MLO infected *P. irroratus*, this indicates that both strains of X-disease MLO have high degree of DNA homology. Further studies need to be done, including sequence analysis of the C6c fragment from Eastern and Western X-disease MLO, to determine the degree of similarity of these organisms.

Laboratory detection with C6c DNA probe showed high sensitivity in detecting the occurrence of MLO in *P. irroratus*. However, the fixation of MLO nucleic acid in

a crude extract of leafhoppers in a single dot blot well by UV cross-link was partially blocked by protein (Figure I.2.A). The observation suggests that a serial dilution of leafhopper samples is needed to determine the optimum hybridization result. The quantification of hybridization results indicates that the C6c DNA probe under the reported conditions can detect X-disease MLO in dilutions of total DNA extracted from one diseased leafhopper. Thus only a portion of the DNA extracted from a leafhopper needs to be utilized for evaluation. This approach allows for multiple testing on a single leafhopper's DNA with many different probes.

The detection limit of C6c, with the given conditions, was 240 copies of C6c. Laboratory detection limit of this probe in detecting X-disease MLO in *P. irroratus* was approximately 1 among 400 to 166,666 leafhoppers.

The sensitivity of the detection of X-disease MLO using C6c DNA probe may be due to the specificity of the C6c DNA probe fragment. The specificity allows the probe to hybridized a unique site of the genomic DNA samples.

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CHAPTER III

Seasonal distribution of X-diseased *Paraphlepsius irroratus* (Say) field samples using DNA probe

INTRODUCTION

Peach X-disease is one of the most serious mycoplasmalike organism (MLO) disease of stone fruit in Michigan. Observation done by the Michigan Department of Agriculture (MDA) showed that the percentage of X-diseased trees increased during 6 years period (1980-1986) that resulted with the decrease of the production (Dreves 1985, 1986, 1987). It has been estimated that Michigan peach growers suffer between $1.5 and $3.9 million annually in tree losses from this disease (M.E. Whalon personal communication).

The increased incidence of the peach X-diseases in Michigan has attract more research interest in recent years. Stoddard (1938) reported that infected peach trees grew normally until about the middle of June when small, yellowish areas appeared on the leaves. Rosenberger and Jones (1978) using chockecherry as an indicator plant, demonstrated that the symptoms of X-diseases were observed from mid June to late July. Larsen and Whalon (1987) observed the phenology of X-disease symptomatology in chockecherry from southwest to Northwest Michigan. This study demonstrated the same incidence of X-disease from

Southwest to Northwest Michigan, although symptom expression was progressively later in the North. Peach X-disease transmission patterns by *Paraphlepsius irroratus* (Say) (Rosenberger and Jones 1978, Chiykowski and Sinha 1988), indicated that leafhoppers have the capability to transmit the disease over a period of several weeks. These observations were made on peach, chockecherry and celery. Transmission of X-disease from cherry to indicator plants by *Colladonus montanus* (Van Duzee) has been recorded by Suslow and Purcell (1982). However, these symptomatology were done under laboratory conditions, thus the environmental factors that might affect the transmission patterns and development of the X-disease MLO in the field were not considered.

Throughout these studies the occurrence of the MLO in its vectors was determined solely by symptomatology. There are a number of symptoms caused by MLOs such as virescence, phyllody, stunt, proliferation ('witches' broom), etc. But symptomatology alone is not sufficient to determine the presence of MLOs since many of these symptoms are caused by other pathogens, water stress and poor nutrition. Because symptomatology is very an unreliable method of establishing the presence of X-disease MLO in its vector, a better method with high specificity and sensitivity is needed.

The previous study (Chapter II) demonstrated the utility of a dot blot hybridization technique to determine the presence of the X-disease MLO in its vector. The

sensitivity and efficiency of this technique allows study of the occurrence of the X-disease MLO in its leafhopper vectors under field conditions. This approach allows direct study of X-disease epidemiology. This chapter reports the use of the dot blot hybridization technique to preliminary establish the seasonal distribution of peach X-disease in field populations of *P.irroratus*, as well as a crude classification of the geographical distribution of X-diseased leafhoppers. This understanding is critical to the development of an integrated pest management program for X-disease and its vectors.

MATERIALS AND METHODS

Leafhopper sample

Paraphlepsius irroratus (Say) were collected by sweepnet, yellow sticky boards and light traps along a transect from Southwest (Benton Harbor) to Northwest (Northport), Michigan during the 1987 and 1988 growing seasons. In each of the sites, 50 sweep net samples were taken from the orchard ground cover and 50 in the surrounding vegetation. Six yellow boards coated with bird-tanglefoot (The Tanglefoot Co, Grand Rapid, MI) were placed in each orchard site. The yellow sticky traps were replaced every week. Light trapping was carried out in each site by locating the three 1 m^2 light trap boxes (Larsen and Whalon 1987) to capture the adult leafhoppers during their

crepuscular flight period which started at sunset and ended approximately 45-75 min later. All the leafhoppers sampled were transported on ice to the laboratory where they were sorted, identified, counted and stored at -70 ^{o}C in dated vials.

Leafhopper assay for seasonal and crude distribution study

The occurrence of X-disease in the leafhopper was determined weekly by submitting three groups of 10 leafhoppers per light trap in two sites (Trevor Nicols Research Station, Fennville and Collins Rd. Entomology Research Station at East Lansing, MI) to dot blot hybridization analysis. For weekly samples with fewer than 30 leafhoppers, the total number of leafhoppers were a equally in three groups and analyzed. All of the light trapped leafhoppers from Sottun Bay, Ludington and Lawrence, MI, were assayed by date.

MLO extraction and blotting

X-disease MLO from frozen leafhoppers was extracted according to the method of Kirkpatrick (1987) with some modifications. Leafhoppers were placed individually into 1.5 ml microtubes containing 400 *u*l of MLO enrichment buffer (0.1M Na_2HPO_4, 10% sucrose, 1% polyvinylpyrolliodine and 50mM ascorbic acid added prior used, pH 7.6) and mascerated with siliconized glass rod by rotating the rod 6 to 8 times. The mixture was centrifuged at 5,000 *g* for 20 min. The

supernatant was decanted in to a second 1.5 ml microtube and centrifuged at 16,000 *g* for 30 min. The resulting pellet was resuspended with 100 *u*l of TE buffer pH 8.00 prior to storage at -70^{o}C or resuspended in 10X SSC (1.5M sodium chloride, 1.5M sodium citrate) for immediately blotting.

The sample was blotted onto a nylon membrane, hybridized and visualized according to the instruction provided by the company (Method III, GeneScreen^{TM}, New England Nuclear, Dupont, Boston, MA). Before blotting the nylon membrane was soaked in 2X SSC buffer (diluted from 10X SSC) and air dried. DNA was alkaline denatured by treating the extraction solution with 0.5M NaOH for 10 min and chilled on ice for another 10 min. After denaturation, the solution was diluted with 10X SSC to the final volume of 400 *u*l. The 400 *u*l dilution was then blotted onto the membrane with a dot-blot apparatus (BioRad, Richmond, CA). After blotting, the membrane was washed carefully with 2X SSC for 2 min. The nucleic acids were fixed onto the membrane by laying it face up on a clean glass plate for irradiation for 5 min with 254 nm shortwave UV light source (UVG-11, Ultraviolet Products, Inc., San Gabriel, CA) at a distance of 15 cm.

DNA sample hybridization

The prepared membrane with sample dots was prehybridized in a plastic bag by treating with 10 ml of a mixture of 50% deionized formamide, 0.2% polyvinyl-

pyrrolidone, 0.2% bovine serum albumin, 0.2% ficoll, 0.05M Tris-HCl (ph 7.5), 1.0M NaCl, 0.1% sodium pyrophosphate, 1.0% sodium dedoxyl sulfate, 10% dextran sulfate and denatured salmon sperm DNA (≥100 *ug*/ml). The plastic bag containing the prehybridization solution and the membrane was sealed and incubated for 6 hr at 42 ^{o}C.

Hybridization was carried out by opening the bag, adding 2.5 ml of prehybridization solution minus NaCl containing the radioactive labelled probe, and resealing the bag before incubation for 16 hr at 42 ^{o}C. Based on the previous study, *P. irroratus* field samples were hybridized by 200 ng/10 ml of C6c DNA probe at a specific activity of 0.5 to 2x10^{8} cpm/*ug*. The autoradiographs were exposed for 24 hr. Before autoradiography, the membrane was washed twice for 5 min each with 100 ml of a washing solution containing 0.3M sodium chloride, 0.06M Tris-HCl (pH 8.0) and 0.002 EDTA at room temperature with gentle rocking. The second washing was repeated 2X for 30 min each at 60 ^{o}C using 100 ml fresh washing solution containing 1% SDS. The last washing was also performed twice for 30 min at room temperature with a 100 ml ten fold dilution of the washing solution.

Autoradiography was carried out by exposing X-ray film (Kodak XAR) for 24-48 hr with an intensifying screen (Sigma) at -70 ^{o}C. X-ray film was developed in a dark room. The hybridized dots were quantified using 2-D/1-D soft laser

scanning densitometer (Biomed Instruments, Inc., Fullerton, Ca).

RESULTS

P. irroratus population trends

1987 and 1988 East Lansing and Fennville leafhopper trapped (Figure III.1) confirmed already established seasonal population trends and voltinism for *P. irroratus* (Taboada *et al.* 1975, Rosenberger and Jones 1978, Mowry 1982, Larsen and Whalon 1987). At both sites, population peaked in both generations at approximately 500-1,000 DD (Base 55 ^{O}F) and 2,000-2,200 DD respectively. Total number of leafhopper trapped in Fennville both year was smaller comparing with total number trapped in E. Lansing. The smaller number in Fennville was may be due to the insectiside applications during the seasons. The number of leafhopper trapped by light traps was averagelly higher comparing with the number of leafhopper trapped by yellow sticky board per day.

X-diseased *P. irroratus* seasonallity

The percentage of diseased leafhopper ranged from 0 to 43.33% (Table III.1). In the first generation, early in season the percentage of diseased adult leafhopper was already high. The percentage of X-diseased leafhopper peaked at the same time with the peak of total number that

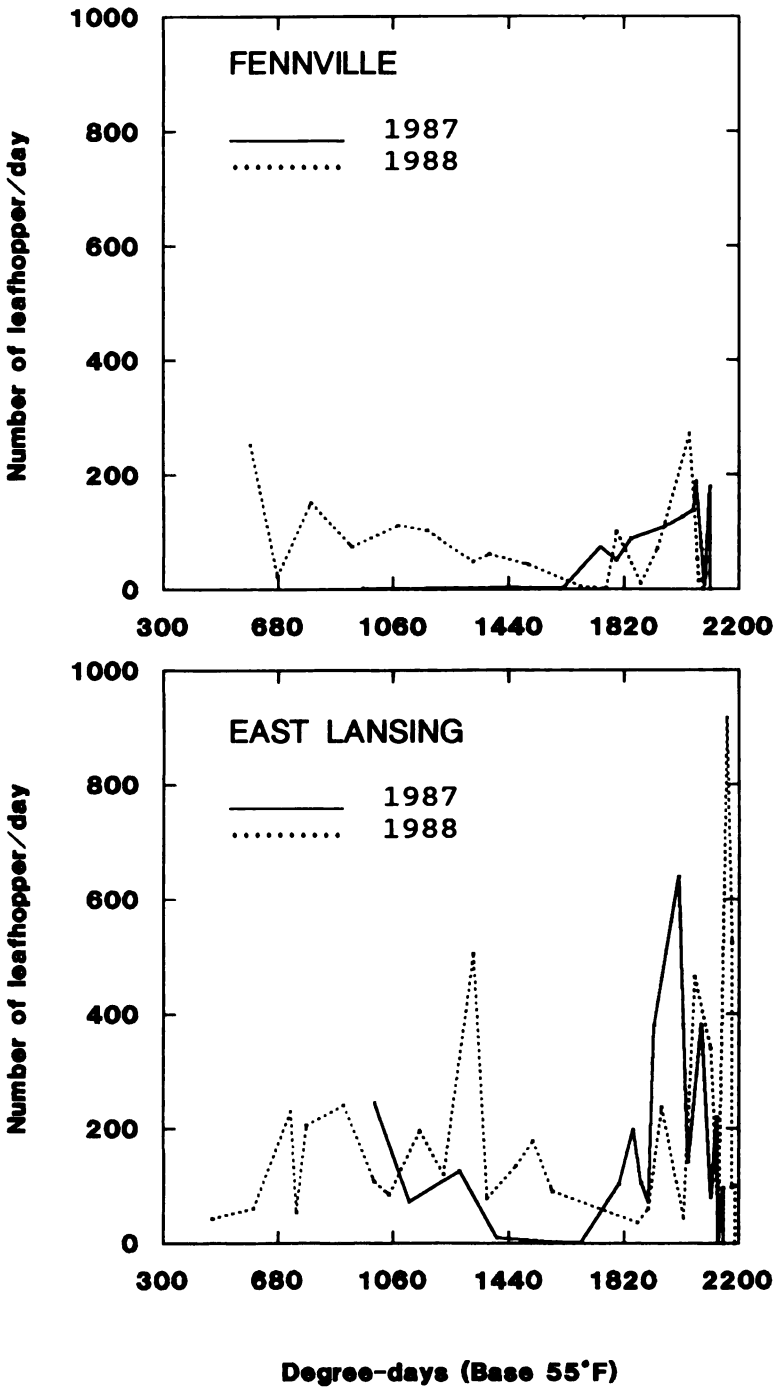

Figure III.1. Total number of light trap and yellow sticky board trapped *Paraphlepsius irroratus* (Say) per day at Fennville and East Lansing, Michigan 1987-1988

Table III.1. Total number of adult *Paraphlepsius irroratus* (Say) and diseased leafhoppers observed at Fennville and East Lansing, Michigan 1988.

Jdate	+/tested[a]	% diseased[b] (mean±SE)	Total trapped[c]	Total diseased[d]
A. Fennville				
175	11/30	36.67 ± 8.98	231	85
189	4/30	13.33 ± 8.89	120	16
196	1/30	3.33 ± 3.33	34	1
204	2/30	6.67 ± 3.39	91	6
210	0/30	0	55	0
216	2/30	6.67 ± 6.67	48	3
224	2/28	7.14 ± 3.89	28	2
243	0/30	0	94	0
257	0/30	0	51	0
271	1/30	3.33 ± 3.33	175	6
287	2/14	14.28 ± 6.79	14	2
295	0/0	0	0	0
B. East Lansing				
165	9/30	30 ± 15.27	43	13
172	10/30	33.3 ± 14.53	60	20
179	6/30	20 ± 5.77	229	46
185	13/30	43.33 ± 12.02	205	89
195	8/30	26.67 ± 8.82	107	28
201	0/30	0 ± 0	41	0
206	0/30	0 ± 0	119	0
213	4/30	13.33 ± 6.67	77	10
216	0/30	0 ± 0	134	0
221	4/27	14.81 ± 14.81	27	4
235	5/30	16.67 ± 8.82	36	6
244	3/30	10 ± 10	32	3
254	6/30	20 ± 10	44	9
259	3/13	23.33 ± 1.67	13	3
265	6/30	20 ± 5.77	30	6
270	7/30	23.33 ± 8.82	33	8
274	1/30	3.33 ± 3.33	141	5
295	0/0	0 0	0	

[a]+/tested : total number leafhopper with positive signal/total number of 3 groups of 10 *P. irroratus* tested. Samples with leafhoppers less than 30 were all tested

[b]Mean percentage diseased leafhopper from 3 groups of *P. irroratus* tested·

[c]Total number of adult leafhopper trapped in three light traps.

[d]Estimated from percentage of diseased leafhoppers observed.

occurred between the end of June and early July when the accumulated degree day were 500-1,000 DD which was in (Figure III.2). In the middle of season the percentage of diseased leafhopper was almost zero and then rebound in the late season with lower total percentage than the early season. The average percentage of X-diseased leafhopper per week observed in Fennville and E. Lansing were (x ± S.E) 7.6 ± 3% and 16.56 ± 2.99% respectively.

There appeared to be a temperature effect on the percentage of diseased leafhopper observed (Figure III.3) As mean temperature increased beyond 75 ^{o}F (after 1,000 DD) the percentage of diseased leafhoppers declined. Conversely, at the end of the season, when the mean temperature dropped below 75 ^{o}C, the percentage of diseased leafhoppers increased slowly until a killing frost occurred.

At both sites, there was a correlation between the number of leafhoppers trapped with the estimated number of diseased leafhoppers ($r = 0.71$ and 0.7 with $P = 0.0001$ and 0.002 respectively) (Figure III.2, Table III.1).

X-diseased *P. irroratus* distribution

The X-disease MLO occurred in trapped leafhoppers in all sites except Ludington and Suttons Bay (Table III.2). This observation is consistent with X-disease orchard surveys which indicates that the disease is much less frequent the further North samples were taken. Only an average of 10 leafhoppers per sample were captured in

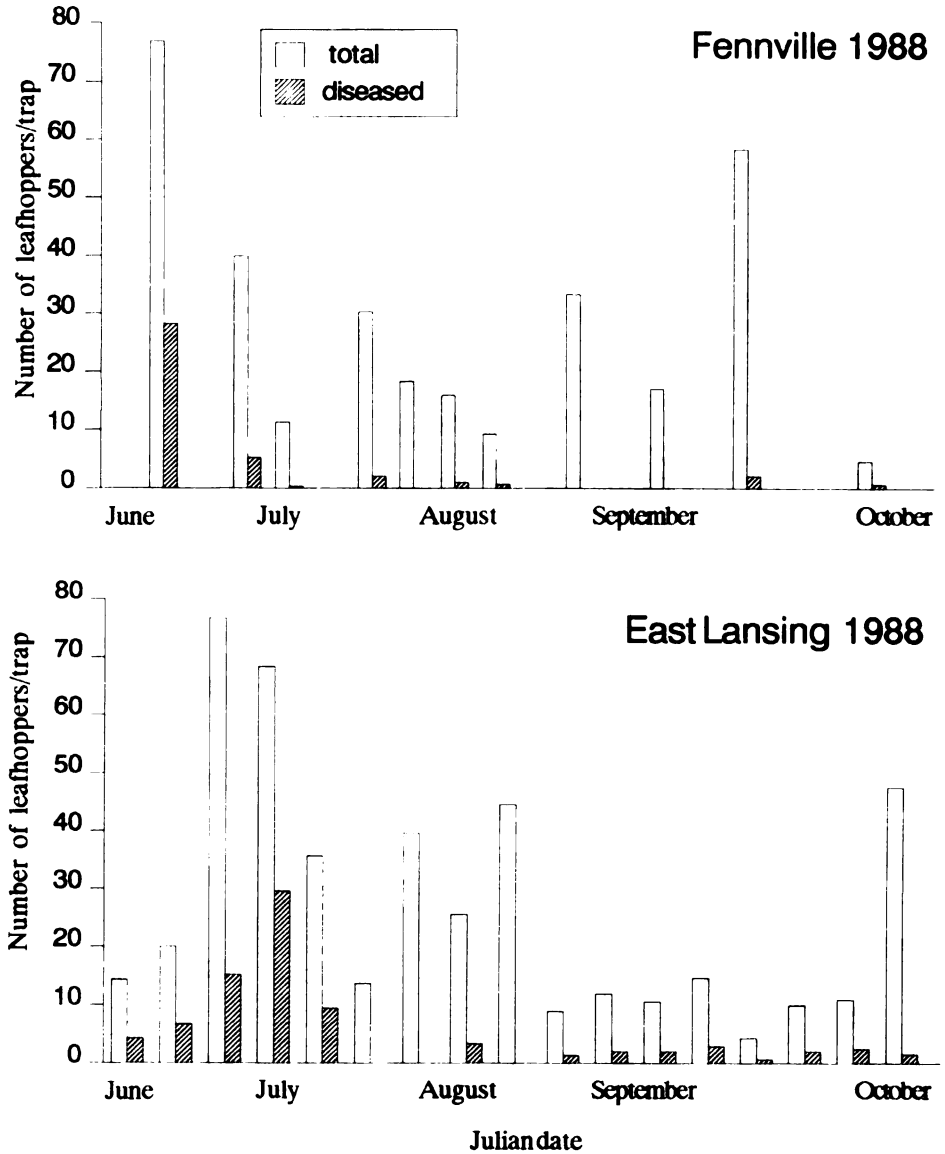

Figure III.2. Total number of diseased *Paraphlepsius irroratus* (Say) caught and the number of leafhoppers at Fennville and East Lansing, Michigan 1988.

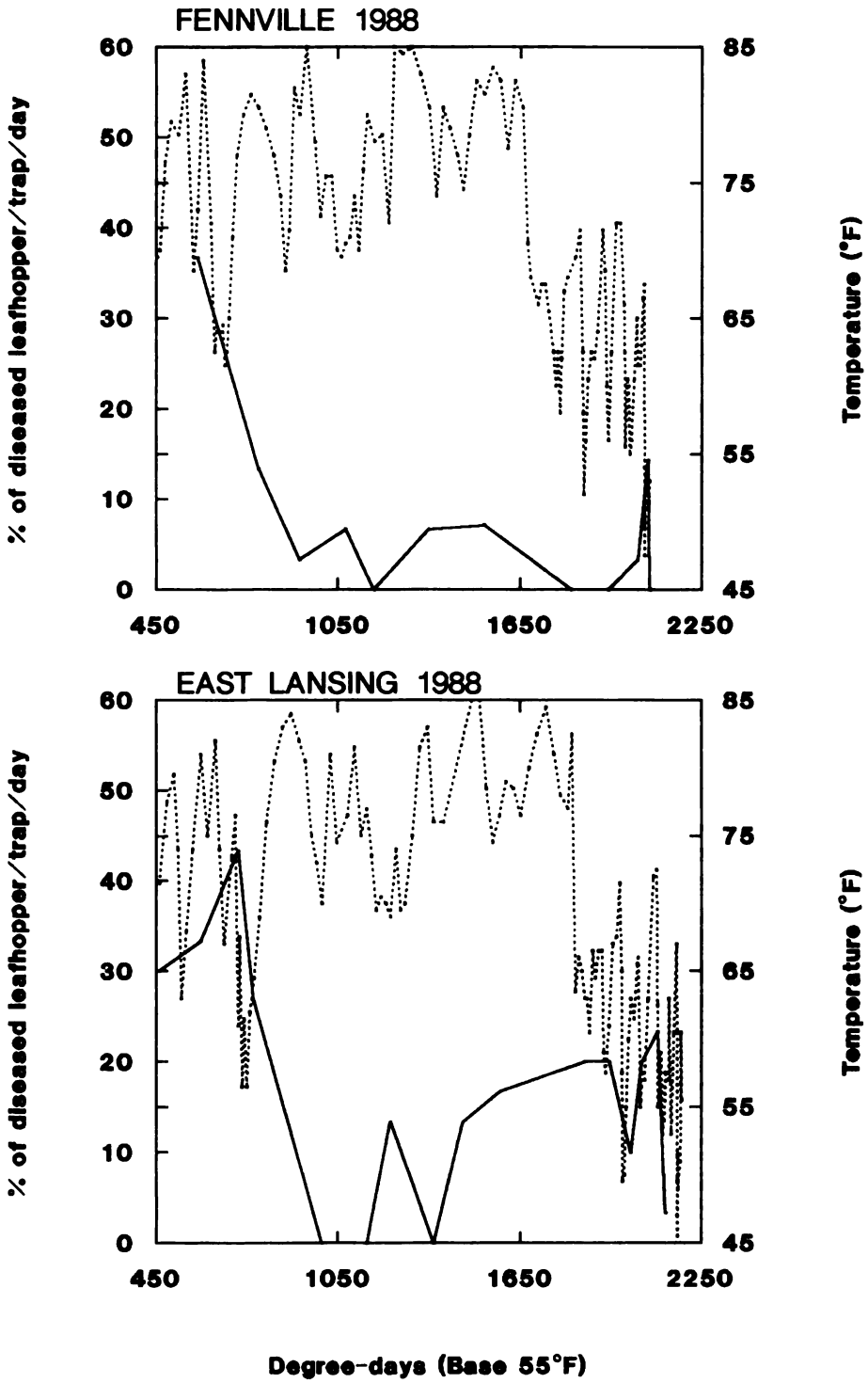

Figure III.3. The relationship between diseased *Paraphlepsius irroratus* (Say) and mean temperature at Fennville and East Lansing, Michigan 1988. (_____ diseased *P. irroratus*, ... Mean temperature)

Table III.2. The seasonal occurrence of X-diseased *Paraphlepsius irroratus* (Say) in several stone fruit orchards Southern to Northern Michigan 1988[1].

Date	Fennville	E. Lansing	Lawrence	Ludington	Suttons Bay
June					
Week 2	+				
Week 3	+				
Week 4	+	+	+		
July					
Week 1	+	+	+	-	-
Week 2	+	+	+	-	-
Week 3	+	-			
Week 4	-	+	+		-
August					
Week 1	+	-	+		
Week 2	+	+	+		-
Week 3	+				
Week 4	+				
September					
Week 1	-	+			
Week 2	-	+			
Week 3	+	+			
Week 4	+	+	+		-

[1] + : the presence of X-disease MLO was observed
- : the X-disease MLO was not observed

Northern Michigan while an average of 110 leafhoppers per sample were captured in Central and Southern Michigan. Diseased leafhoppers in Fennville did not occur in the fourth week of July or the first and second weeks of September. The X-diseased leafhoppers were not observed in East Lansing during the third week of July or the first week of August. The Lawrence site yielded diseased leafhopper every sample date. Incidentally, this site has a long history of X-diseased orchards.

DISCUSSION

The results from field sampling support earlier reports (Taboada *et al.* 1975, Rosenberger & Jones 1978, Larsen and Whalon 1987) that *P. irroratus* has two generations each season. The adult leafhopper populations reached their peaks during mid June and September at 500 to 1,000 and 2,000 to 2,200 DD base 55 ^{O}F respectively.

Two hypothesis explaining the mechanism of leafhopper acquisition of X-disease MLO has been postulated (Rosenberger and Jones 1978). The first states that adult leafhoppers acquire the MLO while feeding on woody host. Therefore, X-diseased competent leafhoppers will begin to transmit after 20 to 30 day incubation period. With this type of acquisition, we would expect that emerging adult leafhoppers would initially be uninfected then, as time passes, the percentage of diseased adults should increase.

The second hypothesis states that leafhoppers acquire the X-disease MLO during their nymphal development while feeding in the ground cover. Since nymphs feeds primary on grasses (Rosenberger and Jones 1978, Chiykowski and Sinha 1988), the hypothesis suggests that grasses within orchard ground cover may be an important X-disease alternate host at least for the first generation. My data demonstrate that first generation adults are infected at emergence with peak infection early in the adult period followed by declining percentage of diseased adults during the peak population. The second hypothesis is further supported with the study by Chiykowski and Sinha (1988) which showed that nymphs can acquire X-disease MLO as efficiently as adults. These authors also demonstrated the susceptibility of a grass *species, Hordeum vulgare* (Graminae), to X-disease. The presence of this and other susceptible grasses (Chiykowski, personal communication) in orchards or nearby ground cover would provide a good source of inoculum for nymphs. Further research is needed on the detection of X-disease in ground cover plants, particularly grasses.

The percentage of diseased leafhoppers was surprisingly high considering the low rate of X-disease transmission to orchard trees (Purcell and Elkinton 1980, Purcell 1987, Whalon, unpublished data). However, the percentage of diseased peaches and cherry in the study orchard and surround area, as judged by symptomatology, was high (> 60% in nearby Fennville peach site and 22% in the East Lansing

cherry site). There are many factors that contribute to the competence of leafhoppers to vector the X-disease MLO. For example, the pathological effect of X-disease on its Western leafhopper vector has been studied in *Colladonus montanus* (Van Duzee) (Jensen 1958, 1959, 1972). These works demonstrated that the X-disease MLO causes premature death and that temperature effects the longevity of infected leafhoppers. A similar study on *P. irroratus* has yielded similar results (Garcia-Salazar *et al.* 1990). The average longevity of X-diseased adult *P. irroratus* at 25 ^{o}C (mean temperature) significantly shortened ($x \pm S.E = 13 \pm 5.8$ days) when compared to X-diseased leafhoppers reared at 15^{o}C ($x \pm S.E = 35 \pm 21.3$ days). Another study indicated that temperature does effect the incubation period from acquisition to the transmission (Chiykowski and Sinha 1988). The average incubation period of leafhoppers that acquired X-disease MLO by feeding at 28^{o}C was significantly shorter (32 days) than at 21^{o}C (53.9 days).

The occurrence of X-diseased leafhoppers in East Lansing, Fennville and Lawrence were similar. In Ludington and Suttons Bay, occurrence of X-diseased leafhoppers could not be ruled out since there were too few leafhoppers captured. The population of the leafhopper vectors was very low and leafhoppers were not always trapped during the sampling periods.

These results suggest several future research needs including: 1) the relationship between X-disease, ground

cover, leafhopper population density and resulting diseased trees, 2) a search for ground cover host for the leafhopper vector and the X-disease MLO, 3) the relationship between temperature, X-disease infection, diseased leafhopper longevity and transmission competence.

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GENERAL CONCLUSIONS

This research has developed the protocols and controls necessary to evaluate a DNA probe for X-disease detection in *P. irroratus* field collected samples using a dot blot hybridization techniques. This information is important in developing an understanding of the epidemiology of leafhopper vectored X-disease in order to develop the bestü strategy to manage the X-disease of stone fruit in Michigan.

The evaluation of the C6c DNA probe, a restriction fragment of pWX1 was a more specific probe for detecting the occurence of X-disease MLO in leafhopper vectors than pWX1. As reported for many cDNA probes, the sensitivity of this probe and signal stripping techniques allows for multiple testing of a single leafhopper, as well as the estimation of the number of cells of X-disease MLO present in individual leafhoppers. This approach opens the possibility of studying X-disease etiology and epidemiology.

Further DNA hybridization and sequencing studies using other Mollicutes that are closely related to the X-disease MLO will give more precision in estimating the number of X-disease MLO in its leafhopper vectors. Development of a nonradioactive diagnosis system is needed to overcome the laborious and laboratory limited work of radio-detection of field samples.

Leafhopper monitoring confirmed that *P. irrotratus* has

two generations per season. The X-disease was observed in leafhoppers from Fennville, East Lansing, and Lawrence, but not from sites farther North. The seasonal occurence of the X-disease MLO in its leafhopper vector, *P. irrotratus* occurres throughout most of the season. It appears that the leafhoppers acquire the X-disease MLO during the nymphal stage when they live and feed in the ground cover. Apparently, temperature also effects the presence of the MLO in its vectors because the percentage of diseased leafhoppers decreased astemperature increased.

Further research is needed to identify the ground cover that nymphs acquire X-disease from. Perhaps the best managment approach will involve the development of ground cover plants that are either resistant to X-disease or leafhoppers or both.

APPENDIX A

LEAFHOPPER VOUCHER SPECIMENS PLACED IN THE MICHIGAN STATE UNIVERSITY ENTOMOLOGY MUSEUM

APPENDIX C

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.: 1990-01

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

Field detection of peach X-disease mycoplasmalike organism in speckled leafhopper, Paraphlepsius irroratus (Say) (Homoptera: Cicadellidae) using a DNA probe

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

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name (s) (typed)
Utami Rahardja

Date 1/10/1990

*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 C.1

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
Paraphlepsius irroratus (Say)	Van Buren Co, MI Lawerence 6/27/1988						3		MSU
	7/04/1988					5			MSU
	Mason Co, MI Ludington 7/06/1988					6	4		MSU
	Ingham Co, MI East Lansing 9/05/1987					3	2		MSU
	7/31/1988					3	2		MSU
	Allegan Co, MI Fennville 6/23/1988					5	5		MSU
	Leelanau Co, MI Suttons Bay 8/09/1988						1		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Utami Rahardja

Date 1/10/1990

Voucher No. 1990-01

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

Curator

10 January 1990

Date

APPENDIX B

FIELD MONITORING DATA

Table 1. Number of *Paraphlepsius irroratus* (Say) caught at East Lansing and Fennville, Michigan 1987-1988.

Jdate	DD55[1]	Light[2]	Yellow[3]	Total
East Lansing 1987				
190	998.9	244		244
197	1113.4	72		72
205	1276.4	125		125
210	1399.9		10	10
217	1549.9		3	3
225	1676.3		1	1
232	1802.2		103	103
238	1849.1		198	198
242	1876.6	104		104
246	1899.6	72		72
248	1919.5	380		380
254	2000.5	132	507	639
258	2032.1	142		142
264	2076.2		381	381
270	2107.5	40	39	79
276	2127.6		219	219
283	2132.6	0	2	2
304	2148.6		95	95
311	2148.6		0	0
Fennville 1987				
190	961.1		1	1
212	1381.1		3	3
217	1468.8		2	2
225	1618.9		2	2
232	1742.1		73	73
238	1794.5		50	50
245	1842.1		88	88
254	1953.3		109	109
261	2013.3		127	127
269	2048.5		139	139
276	2058.6		188	188
286	2086.8		8	8
304	2105.3		179	179
305	2105.3		0	0
East Lansing 1988				
165	461.5	43		43
172	597.2	60		60
179	720.2	229		229
182	739.9		54	54
185	772.7	205		205
190	895.7		240	240

Table 1. (Cont')

Jdate	DD55[1]	Light[2]	Yellow[3]	Total
				195
998.7	107		107	
197	1047.2		84	84
201	1147.2	41	153	195
206	1225.7	119		119
211	1321.4		509	504
213	1366.9	77		77
216	1461.9	134		134
218	1517.9		178	178
221	1583.4	27	63	90
235	1864.2	36		36
239	1900.1		60	60
244	1944.2	32	205	237
254	2017.1	44		44
259	2054.1	13	451	464
265	2107.1	30	309	339
270	2132.7	33		33
274	2160.0	141	774	917
281	2176.7	0	524	524
288	2176.7		97	97
298	2185.9		100	100
304	2185.9		0	0
Fennville 1988				
175	586.7	231	21	252
182	677.0		22	22
189	788.2	120	33	152
196	924.3	34	40	74
204	1076.3	91	20	111
210	1171.6	55	52	102
216	1322.6	48		48
218	1376.1		61	61
224	1506.1	28	14	42
231	1675.1		5	5
238	1760.6		1	1
243	1794.4	94	6	100
252	1874.8		10	10
257	1930.7	51	20	71
271	2036.2	175	96	271
281	2062.5		52	52
287	2066.8	14		14
290	2078.8	0	16	16
302	2080.5		14	14
311	2080.5		0	0

[1] Degree-days base 55 ^{o}F
[2] Total number of three traps
[3] Total number of six traps

APPENDIX C

X-DISEASE DETECTION DATA

Table 1. Number of total and diseased of *Paraphlepsius irroratus* (Say) at East Lansing 1988

Jdate	Rep.	Number tested	Number positive
165	1	10	2
	2	10	6
	3	10	1
172	1	10	1
	2	10	3
	3	10	6
179	1	10	1
	2	10	2
	3	10	3
185	1	10	2
	2	10	5
	3	10	6
195	1	10	4
	2	10	1
	3	10	3
201	1	10	0
	2	10	0
	3	10	0
206	1	10	0
	2	10	0
	3	10	0
213	1	10	2
	2	10	2
	3	10	0
216	1	10	0
	2	10	0
	3	10	0
221	1	9	4
	2	9	0
	3	9	0
235	1	10	3
	2	10	0
	3	10	2
244	1	10	0
	2	10	3
	3	10	3
254	1	10	1
	2	10	1
	3	10	4
259	1	5	1
	2	4	1
	3	4	1
265	1	10	3
	2	10	1
	3	10	2

Table 1 (Con't)

Jdate	Rep.	Number tested	Number positive
270	1	10	4
	2	10	2
	3	10	1
274	1	10	1
	2	10	0
	3	10	0

Table 2. Number of total and diseased of *Paraphlepsius irroratus* (Say) at Fennville 1988.

Jdate	Rep.	Number tested	Number positive
175	1	10	5
	2	10	2
	3	10	4
189	1	10	0
	2	10	3
	3	10	1
196	1	10	0
	2	10	1
	3	10	0
204	1	10	0
	2	10	1
	3	10	1
210	1	10	0
	2	10	0
	3	10	0
216	1	10	0
	2	10	0
	3	10	2
224	1	10	0
	2	10	1
	3	8	1
243	1	10	0
	2	10	0
	3	10	0
257	1	10	0
	2	10	0
	3	10	0
271	1	10	1
	2	10	0
	3	10	0
287	1	5	1
	2	5	1
	3	4	0

Table 3. Integrated optical densities of C6c and diseased *Paraphlepsius irroratus* (Say).

No.	Exposure time (hr)	Specific[a] activity cpm/ug	C6C (ng)	IOD[b]	*P.i*[c]	IOD[b]
1	24	$5x10^8$	0.05	3195	0.005	2871
			0.10	21304	0.00625	4702
			0.125	13822	0.01	23920
			0.25	30480	0.0125	21634
			0.5	82329	0.025	51998
			1	4623	0.05	111222
					0.0625	100844
					0.1	107642
					1	7475
2	24	$2x10^9$	0.0025	18516	0.00025	1160
			0.01	1226	0.0005	1806
			0.0125	62964	0.000625	2176
			0.05	26554	0.001	5655
			0.1	79296	0.00125	7439
			0.125	93104	0.0025	18516
			0.25	87815	0.005	35501
			0.5	140879	0.00625	67382
			1	202876	0.01	93107
					0.0125	122867
					0.025	162171
					0.05	197812
					0.0625	220887
					0.1	178746
					0.125	256120
					0.25	168931
					0.5	96107
					1	40501
3	48	$2x10^9$	0.0025	35835	0.000006	3606
			0.01	4837	0.00025	6141
			0.0125	115799	0.0005	8818
			0.05	54384	0.000625	13087
			0.1	128662	0.001	17620
			0.125	200837	0.00125	27898
			0.25	183938	0.0025	53319
			0.5	242223	0.005	95094
			1	341086	0.00625	126130
					0.01	161246
					0.0125	225287
					0.025	284168
					0.05	343535
					0.0625	385833

Table 3. (Cont')

No.	Exposure time (hr)	Specific[a] activity cpm/ug	C6C (ng)	IOD[b]	*P.i*[c]	IOD[b]
					0.1	321926
					0.125	427715
					0.25	327067
					0.5	231257
					1	124641

a) Specific activity of probe
b) Optical density were quantified using 2-D/1-D Soft Laser Scanning Densitometer (Biomed Instruments, Inc., Fullerton, Ca).
c) The data represent the fractions or dilutions of diseased leafhopper

Table 4. Number of *Paraphlepsius irroratus* (Say) caught by light traps at Lawrence, Ludington and Suttons Bay 1988.

Jdate	Number[1] caught	Number[2] tested	Signal[3]
Lawrence			
6/27	78	20	+
7/4	140	20	+
7/11	99	20	+
7/25	36	20	+
8/1	73	20	+
8/8	25	20	+
9/29	90	20	+
Ludington			
7/1	0	0	
7/5	46	20	-
7/16	67	20	-
8/1	0	0	
8/23	0	0	
9/30	0	0	
10/21	0	0	
Suttons Bay			
7/1	0	0	
7/8	1	1	-
7/19	3	3	-
7/28	8	8	-
8/8	5	5	-
9/30	7	7	-
10/21	0	0	

1 Number of *P. irroratus* caught by three light traps
2 Number of *P. irroratus* subjected to dot hybridization
3 + signal indicates the presence of X-disease MLO
- signal indicates the absence of X-disease MLO