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EPIDEMIOLOGICAL STUDIES ON THE BLUEBERRY STUNT DISEASE

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by

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ABSTRACT

EPIDEMIOLOGICAL STUDIES ON THE BLUEBERRY STUNT DISEASE

by

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Population changes of Scaphytopius spp., possible vectors of Blueberry Stunt Disease (BBSD), were monitored during 1989, 1990 and 1991 using yellow sticky traps and a D-Vac^{R} aspirator. Scaphytopius magdalensis, S. frontalis and S. acutus showed two population peaks, one after petal fall stage and a larger second peak in late summer to early fall. Healthy, cv. Bluecrop highbush blueberry plants were placed under stunt-diseased bushes in the field for 2-wk periods during 1989 and 1990. These plants and some of the leafhoppers trapped during 1990 and 1991 were tested for MLO infection with a DNA probe (pAY22) that detected BBSD-MLO. The percentage of plants and the number of Scaphytopius spp. that were MLO-positive tended to follow the same bimodal distribution found in the population studies. BBSD transmission tests were performed with Scaphytopius spp. collected from the field. MLO transmission was achieved with S. magdalensis, S. acutus, and S. frontalis.

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INTRODUCTION

Blueberry stunt caused by a mycoplasma-like organism (MLO) is an important disease of highbush blueberry (Vaccinium corymbosum L.) in many regions of the United States.

The affected plants show a yellowing of the leaf margins and between lateral veins; the midribs and lateral veins retain their normal color. Leaves are often cupped downward and reduced in size; bushes are smaller with shorter internodes and their shape is distorted. Fruit set and berry size are reduced and bush life is shortened.

The most common method of controlling blueberry stunt is to spray insecticides to prevent transmission by killing the leafhoppers that vector the MLO. More information on the epidemiology of the disease is required to properly time insecticide applications, minimizing their number and to relate them to the periods when the leafhoppers are more efficient in transmitting the stunt MLO.

The objectives of this research were: a) to determine the population dynamics of Scaphytopius magdalensis and related species during the blueberry growing season in Michigan, b) to identify periods when the disease is more efficiently transmitted in the field and c) to determine if

other Scaphytopius spp. besides S.magdalensis can transmit the disease.

LITERATURE REVIEW

Blueberry Stunt Disease

The Blueberry Stunt Disease (BBSD), observed since 1928, was first reported in 1942 as a virus disease because of its graft transmissibility, the absence of an apparent pathogenic organism, the failure of chemicals to correct the condition and the character of the natural spread in the field (Wilcox, 1942). Its mycoplasma-like nature was later determined by transmission electron microscopy; pleomorphic bodies were observed similar to those mycoplasma-like bodies observed in other yellows-infected plants (Chen, 1971; Hartman et al., 1972; Dale and Moore, 1978; Gocio and Dale, 1982).

Since its discovery in New Jersey, BBSD is now known to exist in Canada, Maine, Massachusetts, New Hampshire, New York, Michigan, North Carolina, Pennsylvania, Maryland, Virginia and most recently in Arkansas (Ramsdell and Stretch, 1987). The disease is most economically important in all cultivars of highbush blueberries (*Vaccinium corymbosum* L. and *V. australe* Small) but can also affect other blueberries (*V. vacillans* Torr., *V. atrococcum* Helbr., *V. stamineum* L. and *V. myrtilloides* Michx.) (Ramsdell and Stretch, 1987).

Graft transmission to V. amoenum Ait, V. altomontanum Ashe and V. elliotii Chap (Hutchinson et al., 1960) and V. ashei Reade (Dale and Mainland, 1981) has been achieved .

The symptoms of the BBSD in highbush blueberries become apparent in early summer and include downward leaf cupping and puckering, accompanied by a reduction in leaf size. Leaves on affected plants are often chlorotic, especially along leaf margins and between lateral veins. Midribs and lateral veins retain their normal color. Stem internodes are shortened and the bush looks stunted overall, which gives rise to the name of the disease (Figure 1).

The BBSD can be experimentally transmitted by dodder and grafting, but its major mechanism of transmission in nature is by insects. Tomlinson et al. (1950) determined that Scaphytopius magdalensis (Prov.) (Figure 2) and S. verecundus (Van D.) were vectors of the disease. Maramorosch (1955) and Hutchinson (1955) reported that only S. magadalensis was able to transmit the disease.

Present control strategies for BBSD include field inspections, roguing of infected plants and insecticidal spray programs to control the vector. Researchers are looking for other alternatives to control BBSD such as finding blueberry cultivars resistant to S. magdalensis (Meyer and Ballington, 1990) and methods to rid infected plants of the causal MLO, using high carbon dioxide thermotherapy which entails growing plants at 38ºC in 1200 ppm of CO_2 (Converse and George, 1987).

These strategies need some additional development to be

more effective. Some examples are: a) Development of a method to detect specifically, rapidly and easily, infected bushes that do not show conspicuous symptoms, b) Develop biological information on the vector according to the transmission of the disease, in order to properly time insecticidal sprays, and c) Determination if other Scaphytopius spp., commonly found in blueberry plantings, are also involved in the transmission of the disease.

Figure 1. Symptoms of Blueberry Stunt Disease in highbush blueberry

Figure 2. External view of adults of Scaphytopius magdalensis (male, left and female right)

Methods of detection of mycoplasma-like organisms (MLO) in insects and plants

The MLOs are cell wall-less prokaryotes which are believed to cause many yellows-type diseases of economically important plants worldwide (Davis and Whitcomb, 1971; Maramorosch, 1973; Lovisolo, 1973; Maramorosch and Raychaudhuri, 1981; Ploaie, 1981).

Since the initial observation of MLOs by Doi et al. (1967) using the transmission electron microscope to examine the phloem of infected plants, several other research approaches have been carried out to detect them in plants and insects.

Electron microscopy has been used extensively, alone or in combination with immunological techniques. Pleomorphic bodies have been reported to be in plants infected by "yellows-type diseases" e.g. little peach, X-disease (Jones et al., 1974; Nasu et al., 1974a), blueberry witches broom (Blattný and Vana, 1974), mulberry dwarf (Yamada et al., 1978), blueberry stunt disease (Chen, 1971; Hartman et al., 1972; Dale and Moore, 1978; Gocio, 1982) and others. MLOs were also observed in vector insects (Sinha and Paliwal, 1970; Nasu et al., 1974b; Stanarius et al., 1976; Sinha et al., 1986; Lherminier et al., 1990) by electron microscopy.

Even though several attempts have been made to culture MLOs *in vitro*, up to the moment none has been successful (Jacoli, 1981; Lee and Davis, 1986). Efforts are being made to study the phloem composition of host plants in order to devise

a suitable culture medium (Lee et al., 1983; Kollar and Seemüller, 1990). Frozen insects and leaf tip cultures have been proposed as an alternative method to maintain a supply of infective MLOs for research purposes (Smith et al., 1981; Chiykowski, 1983; Sears and Klomparens, 1989).

MLOs have been extracted from infected tissues and vectors (Nasu et al., 1974a; Nasu et al., 1974b; Nasu et al., 1974c; Sinha, 1974; Sinha, 1979; Sinha and Chiykowski, 1984). Insects were reported as a better source for extracting MLOs and their extracts as more infective than those from plants (Kirkpatrick, 1986; Kirkpatrick et al., 1987). This can be explained by several factors. One factor is the deleterious effects of plant sap on MLO viability. A second factor is the higher titers of MLO cells in infected insect vectors than in infected plants. A third factor is the lower mechanical forces needed to triturate insect tissues compared to plant phloem (Kirkpatrick, 1989).

These MLO extracts were used to develop antigens for serological detection e.g. standard double antibody sandwich plate ELISA (Sinha and Chiykowski, 1984; Boudon-Padieu et al., 1989; Clark et al., 1989), dot-ELISA (Boudon-Padieu et al., 1989; Lin et al., 1990) and immunosorbent electron microscopy (Lherminier et al., 1989; Sinha and Chiykowski, 1986) in either vectors or plants.

Monoclonal antibodies have been obtained for aster yellows (Lin and Chen, 1985; Lin and Chen, 1986), flavescence dorée (Schwartz et al., 1989), maize bushy stunt

(Chen and Jiang, 1988), primula yellows (Clark et al., 1989) and peach eastern X-disease (Jiang et al., 1989) and have been recommended as very sensitive and specific with few cross-reactions occurring with healthy host antigens.

Fluorescent light microscopy has been extensively used for MLO detection in infected plants (Hiruki et al., 1974; Seemüller, 1976; Cazelles, 1978; Deeley et al., 1979; Marwitz and Petzold, 1980; Petzold and Marwitz, 1980; Douglas, 1986; Rocha et al., 1986; Hiruki and Rocha, 1986). This method involves the staining of cryosections or free-hand sections with fluorescent dyes. Aniline blue was traditionally used as an indirect method of detection, staining the excess of callose formed in the sieve tubes as a response to MLO infection. Most recently a DNA-specific fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI) has been successfully used in MLO detection in plant tissues. The DAPI binds to DNA and forms a highly fluorescent compound.It is not clear yet if the binding is intercalatively or not (Hiruki and Rocha, 1986). According to Schapper (1985) MLO stunt DNA can be readily distinguished by its brightness from blueberry phloem nuclei DNA and mithocondrial DNA, which are possible sources of error. Roots are the most suitable tissues in which to look for stunt MLO, specially in symptomless samples. Petiooes can be used but it is possible to fail to detect the blueberry stunt agent if it is not at high concentrations (Schapper, 1985).

Matteoni and Sinclair (1983) reported that stomatal

closure can be also useful to differentiate MLO infected plants (American and red elms, white ash and chokecherry) from healthy ones, since that phenomenon, when produced independently of water deficit in leaves, can be a symptom of MLO pathogenesis.

The detection of plant pathogenic MLOs based on electron microscopy or fluorescent microscopy has limited usefulness because neither method can differentiate among different MLOs that cause different diseases. Serological techniques have made some progress but there is cross reaction with antigens of healthy plants. Even though this can be solved by using monoclonal antibodies, their contribution is limited because they are based on a single epitope and strains from different geographical locations could differ in that epitope (Kollar et al., 1990).

With the development of molecular biology techniques, MLO DNAs, chromosomal and extrachromosomal DNAs have been isolated and cloned. Several disease-specific DNA probes, both radioactive and non-radioactive (biotinylated), have been constructed and used in MLO detection (Kirkpatrick et al., 1987; Davis et al., 1988; Lee et al., 1988; Davis et al., 1990; Bertaccini et al., 1990; Lee et al., 1990; Kruske et al., 1991). Short ribosomal RNAs (rRNAs), oligonucleotide probes with sequences specific to MLOs have been proposed for detection also (Kuske and Kirkpatrick, 1989; Kirkpatrick et al., 1990). These DNA and RNA probes not only are useful in detection due to their sensitivity, but also have contributed

to the study of the relatedness among MLOs from various sources (Davis et al., 1988; Lee et al., 1988).

The pAY22 probe was developed from extrachromosomal DNA of Aster Yellows MLO. It is able to detect many MLOs including BBSD-MLO. Among those MLO it detects hybridizing at 42ºC are: Ash Yellows, Aster Yellows, Tomato big bud, Connecticut periwinkle little leaf, Potato witches' broom, Canada X-disease, Clover proliferation, Clover phylody, Western X-disease and Vinca virescence. It does not detect Elm Yellows. (I.M. Lee unpublished data).

MLO Transmission by Leafhoppers

Many leafhopper species have been reported as vectors of MLO-caused diseases of economically important plants, most of which belong to the Homoptera-Auchenorhyncha : Cicadellidae (Chiykowski, 1962; Taboada and Burger, 1967; Rosenberger and Jones, 1978; Chiykowski, 1979; Purcell, 1979; Shaw et al., 1990; Chiykowski, 1981; Suslow and Purcell, 1982).

The mode of MLO transmission by leafhoppers is generally circulative, propagative or persistent (Chiykowski, 1981; Daniels and Markham, 1982). Most leafhoppers feed in phloem, having short test feedings in many plants until finding the preferred one where they carry out long feedings, thereby transmitting the disease.

Leafhoppers have a well developed sensory system that enables them to reject the most unsuitable host plants without

sampling sap. As the vector penetrates plant tissue, two types of saliva are produced, one solidifies and produces the flange and salivary sheath, and the other is watery and may contain enzymes to help with tissue penetration and ingestion (Daniels and Markham, 1982).

The leafhopper vector can acquire the pathogen after feeding on infected plants for several hours or days. It cannot transmit the MLO immediately but instead, requires an incubation period of about 10 to 45 days. The length of acquisition and incubation periods depend on the MLO agent, the vector, light and temperature regimes (Gold and Silvester, 1982; Suslow and Purcell, 1982; Chiykowski and Sinha, 1988) and the plant itself (Purcell et al., 1981). Different durations were reported by various authors working in different diseases (Chiykowski, 1962; Rosenberger and Jones, 1978; Chiykowski, 1979; Purcell, 1979; Sinha and Chiykowski, 1980; Smith et al., 1981; Gold and Sylvester, 1982; Suslow and Purcell, 1982; Boudon-Padieu et al., 1989).

During the incubation period, the MLO multiplies and distributes throughout the insect. The acquired infection agent passes through the gut wall into the blood or hemolymph which circulates throughout the body (Daniels and Markham, 1982). Aster Yellows MLOs (AY-MLOs) have been reported in the alimentary canal, hemolymph, salivary glands, and ovaries but not in the malpighian tubules, mycetomas, fat body, testes or brain of Macrosteles fascifrons(Stal). Athysanus argentatus (Metc) Fab. showed a similar content pattern but, MLOs were

detected in the ovaries and fat body also (Chiykowski, 1979). Nasu et al. (1974c) reported multiplication of western-X MLO in the brain of Colladonus montanus (Van D.). Boudon-Padieu et al. (1989) detected flavescence-dorée in salivary glands of Scaphoideus titanus (Ball) and Lherminier et al. (1990) in Euscelidius variegatus (Kbm).

In some cases MLO multiplication causes damage to the vector, e.g. aberrant spermatogenesis in Macrosteles laevis (Rib.) produced by Aster Yellows, (Raatikainen et al., 1976) or premature death in C. montanus by Western-X MLOs (Jensen, 1959; Nasu, 1974).

After the incubation period, the leafhopper remains a vector for life, but for transmission tests it is recommended to use acquisition access feeding periods of 1-7 days, because it takes some time for the insect to get accustomed to a new plant (Daniels and Markham, 1982).

Several transmission tests have been performed for many MLO diseases. These tests allowed the determination of the approximate length of the periods in the transmission process, the determination of new vectors, and elucidation of the relative efficiencies of vectors of the same disease (Chiykowski, 1962; Rosenberger and Jones, 1978; Chiykowski, 1979; Purcell, 1979; Sinha and Chiykowski, 1980) and to show some specificity of leafhopper species to certain host plants (Chiykowski, 1962; Rosenberger and Jones, 1978). Even though most of the MLO transmission studies were performed with laboratory-reared insects (Rosenberger and Jones, 1978;

Chiykowsky, 1979; Purcell, 1979; Sinha and Chiykowski, 1980; Suslow and Purcell, 1982), some of them have been carried out using field trapped adults (Rosenberger and Jones, 1978).

Not all of the insects used in transmission tests become infective after the acquisition period. Chiykowski and Sinha (1988) found that a high proportion of insects in a population of Paraphlesius irroratus (Say.) were unable to transmit Peach Eastern X-MLO (48-94%). However 82% of the insects of the same population became inoculative after being injected with the inoculum. They suggested that the gut of some leafhoppers could be a barrier to the initial infection by the ingested pathogen since the injection directly to the haemocoel gave higher number of inoculative insects.

Kirkpatrick et al. (1990) detected Western X-MLO DNA in 41 of 50 experimentally infected Colladonus montanus and Fieberiella florii leafhoppers 2 weeks after feeding them on celery plants for 1 week. This ratio changed to 35 of 50 and 22 of 50 if the leafhoppers were left to hang in yellow sticky traps under field conditions for two and three weeks, respectively, indicating that the infectivity of the captured insects would be underestimated by approximately 15% if the insects used in MLO-DNA detection came from yellow sticky traps replaced every 2 weeks from the field. When several species of leafhoppers captured in fruit crops were tested for MLOs, using probes for Western X-disease MLO and Aster Yellows, an average of 2.5% of them tested positively.

The Sharpnosed Leafhoppers (Scaphytopius spp.) in blueberry

The sharpnosed leafhoppers, classified within the Tribe Scaphytopiini, genus Scaphytopius Ball (Tumeus DeL., Hebenarius DeL.) subgenus Cloanthanus Ball., can be recognized by the long, pointed vertex (top part of the head between the eyes) and by the genae (part of the head on each side of the eyes) being visible from above. Ten species have been reported in North America, separated mainly by characters of the internal male genitalia : S. diabolus, S. frontalis, S. acutus, S. sarisus, S. cinnamoneus, S. magdalensis, S. angustatus, S. oregonensis, S. latus and S. argutus (Beirne, 1956).

Several Scaphytopius spp. have been reported to transmit MLO-caused diseases. S. acutus is one of the vectors of Eastern-X Disease of peach and cherry (Hildebrand 1953; Gilmer et al., 1966; Taboada et al., 1975; Rosenberger and Jones, 1978); aster yellows (Chiykowski, 1962); alfalfa witches' broom (Menzies 1944; Glover and Mc Allister, 1960); and clover phyllody (Chiykowski, 1962). Scaphytopius nitridus was mentioned as an inefficient vector of Western-X disease (Purcell, 1979) and of Citrus Stubborn. Scaphytopius magdalensis has been confirmed to be a vector of blueberry stunt disease (Tomlimson et al., 1950).

Scaphytopius acutus, S. latus and S. frontalis were reported to be trapped from grasses, Prunus serotina and Rubus spp. (Hoffman and Taboada, 1960; Taboada, 1965; and Taboada

and Hoffman, 1965) and S. magdalensis from blueberries (Taboada and Burger, 1967) in Michigan.

The life cycles of S. magdalensis and S. acutus have been studied. Scaphytopius magdalensis is found in low, moist locations which are also the natural habitat of their ericaceous host plants (Tomlimson, 1950). Feeding is most commonly observed on leaves or in leaf-bearing twigs in the lower shaded portions of the blueberry bush. Eggs are laid between epidermal surfaces of the leaves of the host plant; the leaves chosen usually being on the lower portions of the bush (Tomlimson, 1950). They winter in the egg stage in fallen blueberry leaves. Eggs start hatching in late April or early May in New Jersey. First generation adults appear in early June; the population reaches a peak in late June and early July. Nymphs of the second generation are present in the field from early July until late September. Whereas, adults of the second generation appear about mid-August and their population builds up to a peak in late September and early October (Tomlimson, 1950; Hutchinson, 1955).

Five nymphal instars, with a nymphal period lasting about 1 month, were reported for S. magdalensis and S. verecundus by Hutchinson (1955) in New Jersey.

Meyer (1984) studied the life cycle of S. magdalensis in North Carolina, where a third generation occurs. Egg hatch for the first generation occurs soon after blueberry leaf buds open in early March. Nymphs of the first generation mature and adult males emerge in 9 to 11 wk (average 10 wk). For males,

the period from 1st to 2nd generation adults (including incubation of the eggs) averages 9 wk (range 8 to 10), and the period from 2nd to 3rd generation adults averages 13.5 wk (range 11 to 15). Female populations peak 1 to 2 wk after those of males. The longer development time for 3rd generation nymphs probably reflects lower average temperatures late in the season and perhaps a deterioration in nutrient quality of the host plant (Meyer, 1984). Populations of S. frontalis followed a phenology similar to that of S. magdalensis but S. cinereus and S. acutus seemed to develop more slowly and reached peak adult populations 1 to 2 wk later than S. magdalensis. Males of all four species were found on the sticky traps in greater numbers than females, while sweep-net collected insects were found in nearly equal proportions by sex over a course of a year (McClure, 1980; Meyer, 1984). Hutchinson (1955) reported males of S. magdalensis appearing earlier than females, and the females being more numerous in late autumn.

Palmiter et al. (1960) determined the duration of the stages in the life cycle of S. acutus reared in the laboratory at 70 to 75ΩF. Eggs hatched in 11 to 14 days. Adults were available 20 to 25 days after hatching and remained alive for 20 to 30 days. Five molts of nymphs were recorded.

Population dynamics of Scaphytopius spp. have been studied in the field using yellow sticky traps (Hutchinson, 1955; Palmiter et al., 1960; Hoffman and Taboada, 1960; Taboada et al., 1975; Meyer, 1984) or vacuum aspiration (Burger, 1966;

Meyer, 1984). In the Northern United States, Scaphytopius spp. have a bivoltine distribution showing two generations a year. The periods of major adult activity for S. acutus were in early July and mid-September (Palmiter et al., 1960; Mc Clure, 1980); June-July and September-October (Taboada et al., 1975); the last week of June or first 2 weeks of July and the second in mid September-early October (Rosenberger and Jones, 1978). For S. magdalensis, the periods of late June-early July and late September-early October (Tomlimson, 1950); early June and late August (Hutchinson, 1955) were the times of major adult activity. Meyer (1984) reported three periods of adult activity of S. magdalensis for the Southern states of the United States. The first period was recorded in blueberry fields during May and June, the second on July-August and the third on October-November. Scaphytopius frontalis showed a similar phenology to S. magdalensis, but S. acutus seemed to develop more slowly and reach peak adult populations 1 to 2 weeks later than S. magdalensis.

Epidemiological Studies on MLO Diseases

Most of the research on the epidemiology of MLO-caused diseases of woody plants was performed on Eastern X-Disease of peach. Gilmer et al. (1954) and Rosenberger and Jones (1977b) using graft transmission from infected peach and chokecherry trees to peach, found that peach buds were not infective

during winter (mainly infective during July-August) while chokecherry buds were infective all year round, showing that in peach, Eastern X-Disease MLOs may overwinter in roots, while in chokecherry, it overwinters in both roots and buds. Rosenberger and Jones (1977a) stressed the role of the vector insect as a reservoir of MLO prevalence in peach orchards in Michigan. Vectors sheltered from insecticidal applications in perennial grasses would be as important as chokecherry as an MLO source for the orchard.

Suslow and Purcell (1982) studied the efficiency of transmission of Western X-disease MLO by Colladonus montanus from peach and cherry during the season, to celery, finding that the highest percentage of transmission took place from cherry in August-September, even though there was transmission all during the season, except during 8-15 April. They did not find transmission from peach.

Rosenberger and Jones (1978) exposed peach and chokecherry indicator plants in an Eastern X-diseased orchard to measure the efficiency of transmission during two seasons and found that 37% of the transmissions took place during July-August, 21% in the middle of June and 17% at the end of August.

The role of weeds in the epidemiology of MLO diseases has been also determined; they are not only a source of MLOs for crops but also as shelter of vectors (Mc Clure, 1980; Mowry and Whalon, 1984; Larsen and Whalon, 1988; Vicchi and Belardi, 1988).

Paraphlepsius irroratus, a vector of Eastern X-Disease of

peach, has been reported to spend daytime hours in the orchard ground cover and to feed on fruit trees after twilight (Gilmer et al., 1966). This insect was preferentially captured in box light traps, 0.5 hrs before to 2 hrs after sunset (Larsen and Whalon, 1988). Mc Clure (1980) found that the capture of S. acutus was positively correlated with the number trapped in wild host plants [sumac (Rhus spp.), strawberry (Fragaria spp.), cherry (Prunus spp.), rose (Rosa spp.), and blackberry (Rubus spp.)]. The population peaks were earlier than or at the same time as those in peach, suggesting that this vector invades peach orchards from wild hosts.

Mowry and Whalon (1984) performed vacuum aspiration of leafhoppers with a D-vac machine (D-Vac Corp. Riverside, Ca.) in the ground cover of sprayed and unsprayed peach orchards in Michigan [orchard grass (Dactylis glomerata), red clover (Trifolium pratense), and many other herbaceous weeds]. They found that most leafhoppers prefer herbaceous hosts for feeding and oviposition, being non-resident vector species which move in and out of the orchard.

Weeds and wild vegetation are also important in the life cycle of Scaphytopius spp. in Michigan where many species were first reported from grasses, blackcherry (Prunus serotina) and Rubus spp. (Hoffman and Taboada, 1960).

METHODS AND MATERIALS

Leafhopper Population studies

A blueberry planting (cultivar Jersey) with a high incidence of stunt disease was selected for a study of the population of *Scaphytopius* spp. relative to the incidence of stunt disease during the 1989, 1990 and 1991 growing seasons. The field was located near Imlay City, Michigan. No insecticides were sprayed during these studies.

Yellow sticky traps of approximately 15.3 x 20.3 cm (6 x 8 in.) were hung in or near the crop (Figure 3). A total of ten traps were used in 1989, when two shapes, flat and tent, were compared. Twenty traps were used in 1990, seven to monitor the leafhopper population in blueberries, six in wild cherries located near the crop [two of each in: pincherry (*Prunus pennsylvanica*), blackcherry (*P.serotina*), and chokecherry (*P.virginiana*)], and seven to provide leafhoppers to test for MLO content during the growing season.

In 1991 only seven traps were hung, all of which were to provide leafhoppers to be tested for the presence of MLOs.

The traps were changed weekly for the periods of 3 May to

20 October in 1989 and from 29 April to 22 October in 1990. In 1991 the traps were changed every 2 wk from 3 May to 3 October.

Leafhoppers belonging to the genus Scaphytopius were counted and identified to the species level. Each insect was removed from the trap, the sticky material dissolved using Histo-Clear™ (National Diagnostics, Manville, NJ 08835) and kept in 70% ethanol until identification was done. Species determination was performed by observing male genitalia using the following method developed by Dr. O. Taboada: The abdomen of male specimens was separated and treated with 10% potassium hydroxide on a warm hot plate to soften the exoskeleton for 10-15 min. Then, under a dissecting stereomicroscope, male genitalia were separated, observed and compared with those described by Beirne (1956). Since the identification of female insects was not possible, their species were assumed to be in the same proportion as the males identified from the same trap.

The individuals from 1990 and 1991 to be tested as MLO carriers were only identified to the genus level and saved in 70% ethanol, in a refrigerator or -20ΩC freezer respectively, until analyzed.

The number of Scaphytopius spp. in the crop and adjacent vegetation during the daytime was estimated weekly in 1990 by performing 5-min vacuuming periods using a D-Vac® suction sampler (D-Vac Corp. Riverside, Ca.) on blueberry bushes (Figure 4), wild cherries (Figures 5-7), bushes belonging to

the Rubus pensilvanicus complex (Figure 8) and the ground cover between the rows of the crop. The predominant plant species in the ground cover were: bristly sarsaparilla (Auralia hispida) (Figure 9), sheep red sorrel (Rumex acetidiella) (Figure 10) and a Rubus sp. belonging to the R.setosus complex (Figure 11). Vacuuming was performed weekly on the same dates when yellow sticky traps were changed. The insects collected were also identified at the species level. The identification of the wild cherries and weeds was performed by M. Case from the Beal-Darlington Herbarium at Michigan State University.

Figure 3. Flat-type yellow traps hung in blueberry bushes

Figure 4. Vacuuming of insects from blueberry bushes using the D-vac® aspirator machine

Figure 5. Pincherry (Prunus pennsylvanica)

Figure 6. Blackcherry (Prunus serotina)

Figure 7. Chokecherry (Prunus virginiana)

Figure 8. Plants belonging to the Rubus pensilvanicus complex

Figure 9. Bristly sarsaparilla (Auralia hispida).

Figure 10. Sheep red sorrel (Rumex acetidiela).

Figure 11. Plants belonging to the Rubus setosus complex.

Determination of the period of transmission during the growing season.

Sets of 21 2-yr-old potted nursery plants, cultivar Jersey, provided by Mr. J. Nelson, Director of Research for the Michigan Blueberry Growers Association, known to be stunt-free, were placed in three groups of seven each under diseased bushes and replaced every 14 days with new plants (Figure 12). Trap plants were set out from 3 May to 20 October in 1989 and from 29 April to 29 October in 1990. Exposed plants were stored in a greenhouse and transferred to a nethouse in summer months before they were tested for MLOs. They were kept for 2 mo. in a cold room at 4ΩC in order to satisfy dormancy requirements when needed.

Figure 12. Sets of blueberry trap plants field-exposed every two weeks during the growing seasons of 1989 and 1990.

Blueberry Stunt Transmission tests

Twenty transmission tests were carried out during 1990. Sets of Scaphytopius spp. were collected using the D-Vac® aspiration machine in two locations that provided different species: 1) in State lands in Southwestern Michigan, near the town of Pullman, from wild blueberries and 2) near Imlay City, on bushes of the Rubus pensilvanicus complex, within the same property used for the other studies but relatively far (one half mile) from the blueberry crop. An average of forty insects were collected at each visit. They were transported in plastic boxes with adequate aeration, containing some wild blueberry or Rubus sp. leaves as a food source, depending on the location. Boxes were placed in an ice chest during the trip to keep insect activity to a minimum. Upon arrival at the laboratory they were transferred to plastic lantern-type cages 15.24 cm long and 5.08 cm of diameter (6 in. x 2 in.) attached to stunt diseased and healthy bushes by using panty hose cloth taped to the base of the cage (Figure 13). They were allowed to feed for an acquisition access period of 2 days and then moved individually to healthy plants of the cv. Bluecrop prepared by P. Callow by micropropagation. Cages were opened in plastic bags and individual insects were collected using a mouth aspirator, cushioned with cotton to prevent leafhopper damage during the procedure. Then, they were transferred inside cages of the same kind to the healthy small, tissue culture-grown cv. Bluecrop blueberry plants. That operation

was carried out inside a plastic bag to prevent the insects from escaping. White styrofoam circles were used at the base of the cages to separate the leafhoppers from the wet soil and to facilitate their localization (Figure 14).

For tests numbered from 1 to 11, the insects fed for 2-4 days on the healthy plants, and for tests 12 to 20, insects fed for approximately 3 weeks. Since 2 days was considered too short for transmission, and the amount of tissue-cultured plants for the season was fixed, some of the plants already used in tests 1-11 were used again for tests 12-20, keeping separated those that were exposed to leafhoppers which fed on healthy or infected plants.

Once the test ended, the insects were collected and identified to the species level and the plants were stored to be tested later for MLO infection.

A total of 71 plants were used in control transmission tests made at the same time using healthy plants as an acquisition source instead of diseased plants in the acquisition period.

A third source of Scaphytopius spp., from blueberries and raspberries from an isolated location (Onondaga, MI) was found. It was further from wild BBSD sources than the previous two, and leafhoppers were used in 1991 for an additional healthy control transmission test. This leafhopper population was isolated from any source of BBSD MLOs, to complement the results obtained in 1990. The procedure was identical to that used in 1990 for the healthy controls.

Figures 13. BBSD transmission tests. Acquisition access period in stunt diseased bushes.

Figure 14. Materials used in the BBSD transmission tests.

Detection of MLO in non-blueberry plants within or adjacent to the blueberry planting

Leaf samples were taken for MLO-tests from plants other than blueberry in or near the diseased plantation, as follows: ten from pin cherries, five from black cherries, four from chokecherries, ten from bristly sarsaparilla, ten from sheep red sorrel, ten from bushes belonging to the Rubus setosus complex and ten from R. pensilvanicus complex.

Fifteen samples from wild blueberries, composed of five from each of three different places in Southwestern Michigan were also tested for MLOs.

Methods of MLO detection in plants and insects.

DNA HYBRIDIZATION TECHNIQUES.

DNA was extracted from the following sources: i) those blueberry plants exposed in the field during 1989 and 1990; ii) all of the tissue culture-grown blueberry plants used in the transmission tests; iii) the Scaphytopius spp. trapped in the seven traps designated for insect-MLO detection in 1990 and 1991; iv) plants other than blueberry in or near the planting; and v) some wild blueberry leaf samples collected in South West Michigan.

Two separate methods of DNA extraction were used for

plants and insects since one of them did not work well for blueberry samples under our conditions.

For the first method (Davis et al. unpublished data) used with plant samples (Figure 15), approximately 2 g of leaf tissue were ground in liquid nitrogen in pre-cooled mortars. The powder was allowed to warm up slightly and then 7 ml of grinding buffer was added (21.7 g $K_2HPO_4.3H_2O$ + 4.1 g KH_2PO_4 + 100 g sucrose + 1.5 g bovine serum albumin (BSA) + 20 g polyvinylpirrolydone MW 10,000 in a final volume of 1 L of glass distilled water) followed by filtration through Miracloth® (Calbiochem Corp., La Jolla, CA). This process was repeated a second time with an additional 7 ml of grinding buffer and then dispensed into 15 ml Corex tubes. The tubes were kept in ice until centrifuged at 20,000 x g for 20 min at 4ΩC in an IEC No. 870 rotor (IEC Co., Needham Heights, MA 02194). The pellet was resuspended in 4 ml of extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl pH 8.0). Then, 80 µl of a 5 mg/ml solution of proteinase K (Sigma Chemical Co., St. Louis, MO 63178) and 440 µl of Sarkosyl 10% (N-Lauroyl-sarcosine, sodium salt, Sigma Chemical Co., St. Louis, MO 63178) were added to each tube and incubated for 2 hr at 55ΩC. The solution was centrifuged at 4400 x g for 10 min at 4ΩC in an IEC No. 870 rotor. The supernatant was saved and mixed in a corex tube with 0.6 vol of cold isopropanol and incubated at -20ΩC for 30 min. The mixture was centrifuged at 7,500 x g for 15 min at 4ΩC in an IEC No. 870 rotor. The pellet was resuspended in 3 ml of TE buffer (10 mM Tris-HCl,

1 mM EDTA, pH 8). Sodium dodecyl sulphate [SDS, 75 µl of 20% (w/v)] and 60 µl of a 5 mg/ml solution of proteinase K were added to each tube and incubated 1 hour at 37ΩC. Later, 525 µl of 5 M NaCl and 420 µl of CTAB/NaCl solution (10% hexadecyl trimethylammonium bromide in 0.7M sodium chloride) were added to each tube and incubated at 65ΩC for 10 min. The samples were then extracted twice in an equal volume of chloroform/isoamyl alcohol mixture (24:1 v/v) and centrifuged at 4400 x g for 5 min in an IEC No. 870 rotor. The aqueous phase was saved, extracted with an equal volume of a mixture of chloroform/isoamyl alcohol (24:1 v/v) and TE-saturated phenol (1:1 v/v) and centrifuged at 4400 x g for 5 min in an IEC No. 870 rotor. Isopropanol (0.6 vol) was added to the aqueous phase and kept overnight at -20ΩC. It was then centrifuged at 4400 x g for 10 min, and the pellet washed in 70 % (v/v) ethanol by centrifugation for 10 min at 4400 x g in an IEC No. 870 rotor. The final DNA pellet was resuspended in 100 µl of TE buffer and stored in a -20ΩC freezer for later use for hybridization tests.

In the second method used for insects (Lee and Davis, 1988; Lee et al. 1988) (Figure 16), single individuals were macerated in eppendorf tubes with plastic mini-pestles after adding 400 µl of extraction buffer (100 mM Tris, 50 mM EDTA, 500 mM NaCl, pH 8.0), 2 µl of 2-mercaptoethanol and 20 µl of 20% (w/v) SDS in distilled water. The solution was further centrifuged at 2000 rpm for 10 min in an Eppendorf Microfuge, model 5415C (Brinkmann Instruments, Westbury, NJ 11590). The

supernatant was saved and the pellet was further centrifuged at 8000 rpm for 10 min. The second supernatant was added to the first and incubated 5 min at 65ΩC and cooled on ice. The solution was centrifuged again at 14000 rpm for 10 min and extracted twice in a 1:1 (v/v) mixture of chloroform/isoamyl alcohol (24:1 v/v) and TE-saturated phenol by centrifuging at 4400 rpm for 5 min. The aqueous phase was saved and 2.5 vol of absolute ethanol was added and kept overnight at -20ΩC. On the following day the solutions were centrifuged at 14000 rpm for 15 min and the DNA pellet was resuspended in 100 µl of 6X SSC, pH 7 (0.9 M NaCl and 0.09 M sodium citrate).

The procedure used for blotting, hybridizing, blocking and detecting was the same for the DNA extracted by both methods. Six µl of 2N NaOH were added to the DNA extracted from each sample and boiled for 10 min. To restore the pH of the previous solution, 6 µl of 2M Tris buffer pH 7.0 and 2 µl of 1.5 M sodium acetate were added to each tube and 50 µl were blotted on nitrocellulose membranes (BioRad Laboratories, Richmond, CA 94804) pre-wetted in 2X SSC using a slot blot apparatus (designed and made in Dr. James Hancock's laboratory, Michigan State University, East Lansing). The membranes were baked for 2 hr at 80ΩC in a vacuum oven (NapCo Model No. 5831, Tualatin, OR 97062). A pre-hybridization procedure was performed for 2-4 hours at 42ΩC in a seal-a-meal^{R} bag (Dazey Corp., Industrial Airport, KS 66031) using 20-100 µl of solution/cm^{2} of filter. The solution was composed of 5 ml of formamide, 2.5 ml of 20X SSC, 1 ml of 50X

Denhardt's solution (5 g of ficoll, 5 g of polyvinyl-pyrrolidone and 5 g of BSA, Fraction V in 500 ml of autoclaved distilled water), 1 ml of 250 mM sodium phosphate pH 6.5 and 0.5 ml of denatured salmon sperm DNA.

Filters were hybridized for 12-16 hr at 42ºC in a seal-a-meal® bag, using 20-100 µl of probe-solution/cm^2 membrane. The solution consisted of 5% (w/v) dextran sulfate, 45% formamide, 5X SSC, 1X Denhardt's solution, 20 mM sodium phosphate pH 6.5, 0.2 mg/ml denatured salmon sperm DNA and pAY22 DNA probe at 0.1-0.2 mg/ml. The pAY22 probe was kindly provided by Dr. R.E. Davis of USDA/ARS, Beltsville, MD 20705. The pAY22 probe was from a cloned portion of the genome of the Aster Yellows MLO, but showed better results for BBSD in preliminary tests than did other three probes also provided by Dr. Davis [CN40, CN41 from "orchard one MLO" (from a peach tree in Connecticut) and BB115 from tomato big bud MLO].

The probe solution was saved after hybridization and re-used several times. Membranes were washed with : a) 50 ml 20X SSC (3 M NaCl, 0.3M sodium citrate) and 10 ml 5% SDS (w/v) in 500 ml of autoclaved distilled water, at room temperature for 3 min; b) 5 ml 20X SSC and 10 ml 5% SDS in 500 ml of autoclaved distilled water, at room temperature for 3 min and, c) 4 ml 20X SSC and 10 ml 5% (w/v) SDS in 500 ml of autoclaved distilled water, at 50ºC for 15 min. Every wash was performed twice using 250 ml each time. These solutions and all the ones used after this step were filter-sterilized using a 0.45 µm pore size Nalgene® Disposable filter (Nalgene Co., Rochester,

NY 14602). The amount of liquids used here and in future steps was calculated for approximately 100 cm^2 membrane area.

After these washings, the filter was blocked using 200 ml of blocking solution [0.1 M Tris HCl, 0.15 M NaCl, containing 3% BSA Fraction V (w/v), pH 7.5] for 1 hr at 64ΩC. It was then washed for 1 min in buffer No.1 (0.1 M Tris HCl, 0.15 M NaCl, pH 7.5) before conjugation.

For conjugation 10 µl of streptavidin alkaline phosphatase (SAAP) conjugate in 10 ml of buffer No.1 were used, agitating gently for 10 min. Membranes were washed twice for 10 min with buffer No.1, using 20-40 times the volume used in conjugation, and once with 25 ml of buffer No.3 (0.1 M Tris HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5).

The results were visualized using 10 ml of buffer No.3 containing 44 µl Nitroblue Tetrazolium (NBT) and 33 µl of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The membranes were kept in the dark, slowly agitating the solution. The color was allowed to develop for 30 min to 3 hr.

When the color of the reaction was considered appropriate, the filters were washed with termination buffer (20 mM Tris, 0.5 mM EDTA, pH 7.5) and dried in a vacuum oven at 80ΩC for 3 min.

Positive and negative control samples were included on every filter. Stunt-diseased blueberry bushes collected from the field and kept in the greenhouse were used as positive controls. Blueberry bushes from the same source as those exposed in the field as trap plants were used as negative

controls. In some tests, "orchard one"-MLO-infected periwinkle (Catharantus roseus) leaves provided by Dr. R. Davis were used as a positive control and healthy periwinkle samples as the negative controls. In insect analyses, the negative controls were MLO-free Macrosteles fascifrons insects laboratory-reared and kindly provided by Dr. K. Klomparens. The positive controls were "orchard one"-MLO-infected periwinkle leaves. A sample was considered positive when it was darker than or as dark as the positive control.

The SAAP, BCIP, NBT used in this work were part of the BluGENE^{R} Non Radioactive Detection System (Bethesda Research Laboratories Life Technologies Inc., Gaithersburg, MD 20877).

Figure 15. Procedure used for DNA extraction from plant samples.(Davis et al. unpublished data)

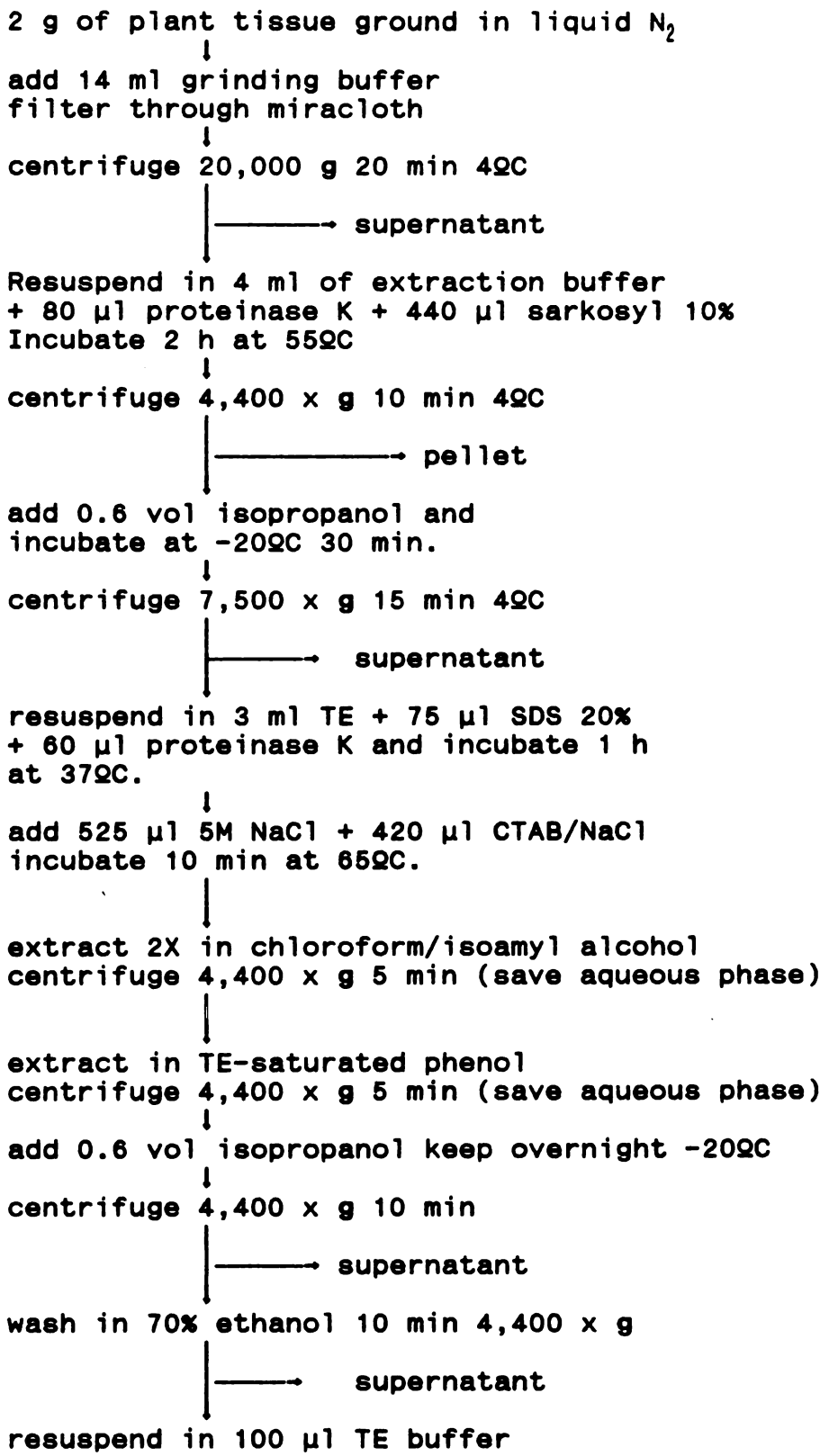
2 g of plant tissue ground in liquid N_2
add 14 ml grinding buffer
filter through miracloth
centrifuge 20,000 g 20 min 4ºC
supernatant
Resuspend in 4 ml of extraction buffer
+ 80 µl proteinase K + 440 µl sarkosyl 10%
Incubate 2 h at 55ºC
centrifuge 4,400 x g 10 min 4ºC
pellet
add 0.6 vol isopropanol and
incubate at -20ºC 30 min.
centrifuge 7,500 x g 15 min 4ºC
supernatant
resuspend in 3 ml TE + 75 µl SDS 20%
+ 60 µl proteinase K and incubate 1 h
at 37ºC.
add 525 µl 5M NaCl + 420 µl CTAB/NaCl
incubate 10 min at 65ºC.
extract 2X in chloroform/isoamyl alcohol
centrifuge 4,400 x g 5 min (save aqueous phase)
extract in TE-saturated phenol
centrifuge 4,400 x g 5 min (save aqueous phase)
add 0.6 vol isopropanol keep overnight -20ºC
centrifuge 4,400 x g 10 min
supernatant
wash in 70% ethanol 10 min 4,400 x g
supernatant
resuspend in 100 µl TE buffer

Figure 15

Figure 16. Procedure used for DNA extraction from insect samples (Lee and Davis, 1988; Lee et al. 1988).

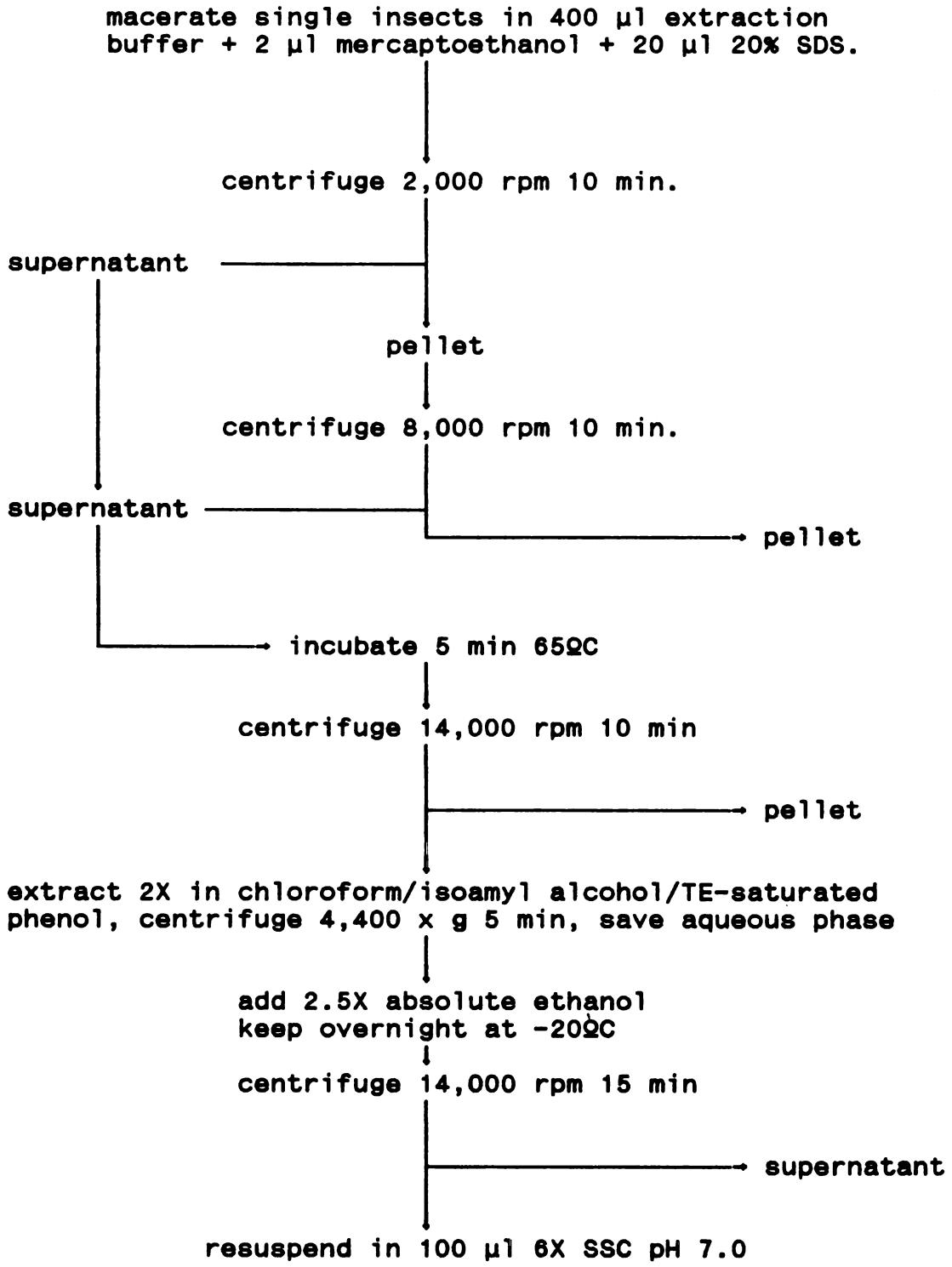
macerate single insects in 400 µl extraction buffer + 2 µl mercaptoethanol + 20 µl 20% SDS.
centrifuge 2,000 rpm 10 min.
supernatant
pellet
centrifuge 8,000 rpm 10 min.
supernatant
pellet
incubate 5 min 65ΩC
centrifuge 14,000 rpm 10 min
pellet
extract 2X in chloroform/isoamyl alcohol/TE-saturated phenol, centrifuge 4,400 x g 5 min, save aqueous phase
add 2.5X absolute ethanol
keep overnight at -20ΩC
centrifuge 14,000 rpm 15 min
supernatant
resuspend in 100 µl 6X SSC pH 7.0

Figure 16

DAPI STAINING

Fluorescent light microscopy was used as an alternative method to corroborate the results of the DNA hybridization method only for approximately 10% of the blueberry plants exposed in the field in 1989 and 1990. Two tissues were used to look for MLOs in the phloem; leaf petioles and roots. Ten percent of the plants were selected at random among those that yielded positive and negative results for each date of exposure to the field. Samples of four petioles and four roots were collected from each plant. The tissue was washed, cut in pieces of about 2 mm^2 and fixed overnight in 5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer pH 7.0 at 4ΩC. The samples were then washed three times with 0.1 M phosphate buffer pH 7.0 for 3 min each time and then sectioned. The sectioning was performed longitudinally with a cryostat microtome model CTD (IEC, Needham Heights, MA 02194.) with the thickness control set in 8 µm. Sections were placed on cover slides coated with a solution of 0.5 g gelatin and 0.05 g chromium potassium sulfate, dodecahydrate in 100 ml of water. They were stained for 20 min with a 1 µg/ml solution of the DNA-specific compound DAPI (4',6-diamidino-2-phenylindole) (Sigma Chemical Co., St. Louis, MO 63178) in 0.1 M phosphate buffer pH 7, washed three times again with 0.1 M phosphate buffer pH 7 and then mounted on slides with a cover slip ringed with fingernail polish.

Fluorescence was observed with a Reichert Microstar IV

light microscope (Cambridge Instruments Inc., Buffalo, NY 14215) equipped for epifluorescence with an HBO 100 W/2 mercury source, and a Reichert-Jung filter set, including an exciter filter at 365 nm, a dichromatic beam splitter at 395 nm and a barrier filter at 420 nm. Twenty sections were examined per sample and tissue type, and the number of them in which objects that resembled MLOs appeared, was recorded. The method of evaluation was very conservative trying to minimize the effects of artifacts in the sections. A code was used so that the source of the samples observed was unknown by the operator.

Positive and negative controls consisting of blueberry plants were also observed for comparison.

RESULTS

Leafhopper Population studies

Three different Scaphytopius spp. were identified among the insects collected during this research. The genitalia used in the identification of these species are shown in Figure 17.

S. acutus, S. frontalis and S. magdalesis were trapped from blueberry bushes in 1989 at Imlay City, MI using yellow sticky traps. The flat shaped trap was more efficient in capturing Scaphytopius spp. than the tent shape, for the same trapping area. The numbers of insects trapped on each date are shown in Figures 18 and 19. According to these graphs, two periods of major adult activity were exhibited; one from mid-June to mid- July and a second and bigger one from mid-August to the end of October. These two periods of activity correspond to two phenological stages of the blueberry bushes. These were respectively, from petal fall to full sized fruit and from harvest until leaf drop. They also correspond to the two main periods of active vegetative growth of blueberries.

Two Scaphytopius spp. were recorded during 1990: S. frontalis and S. acutus . The number of leafhoppers trapped in the seven sticky traps from blueberry bushes appears in Figure

20. Two main periods of adult activity were also observed. The first was from the beginning of June to end of July, and the second and larger from mid-August to the beginning of October. These periods corresponded also to the same two phenological periods as previously mentioned. S. frontalis was the most frequently trapped species.

The capture in the six traps hung in wild cherries was similar, but S. acutus was more prevalent than S. frontalis (Figures 21 to 24). Among the traps located in wild cherries chokecherries contributed the greatest numbers of trapped Scaphytopius spp. These were mainly S. acutus (Figure 23).

The number of insects vacuumed with the D-Vac® aspirator from blueberry bushes, wild cherry trees, bushes of the Rubus pensilvanicus complex and from the ground cover are shown in Figures 25-28. It is evident that the number of leafhoppers captured in blueberry bushes or in wild cherries was smaller than from the other plants. The first adult activity peak was almost imperceptible, and was composed mainly of S. acutus. In the vacuuming from the R. pensilvanicus complex plants and ground cover, the number of leafhoppers was dramatically higher; both species were more balanced even though S. acutus continued being more frequently trapped. The periods when peak numbers of Scaphytopius spp. were vacuumed were approximately 2 wk earlier from bushes of the Rubus pensilvanicus complex and from the ground cover than in either blueberries or wild cherries.

Table 1 shows the ratio of males:females among

Scaphytopius spp. sticky-trapped and vacuumed during 1990. Considering the whole season, the ratio of males:females was lower when the activity of the adults was measured by the number of individuals trapped by vacuuming compared to trapping by yellow sticky traps. The male:female ratio during the season was irregular, but most of the values from yellow sticky traps remained > 1 and those from vacuuming < 1, especially at the beginning of the season.

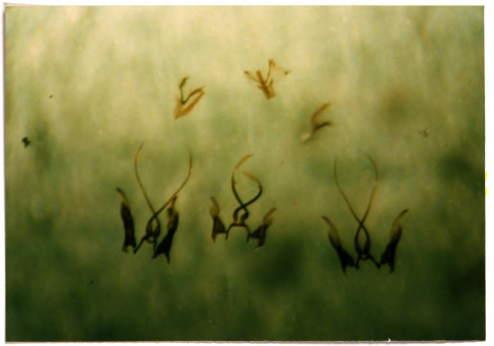

Figure 17. Detail of the genitalia used to identify Scaphytopius spp. (Left= S. acutus, middle= S. magdalensis, right= S. frontalis).

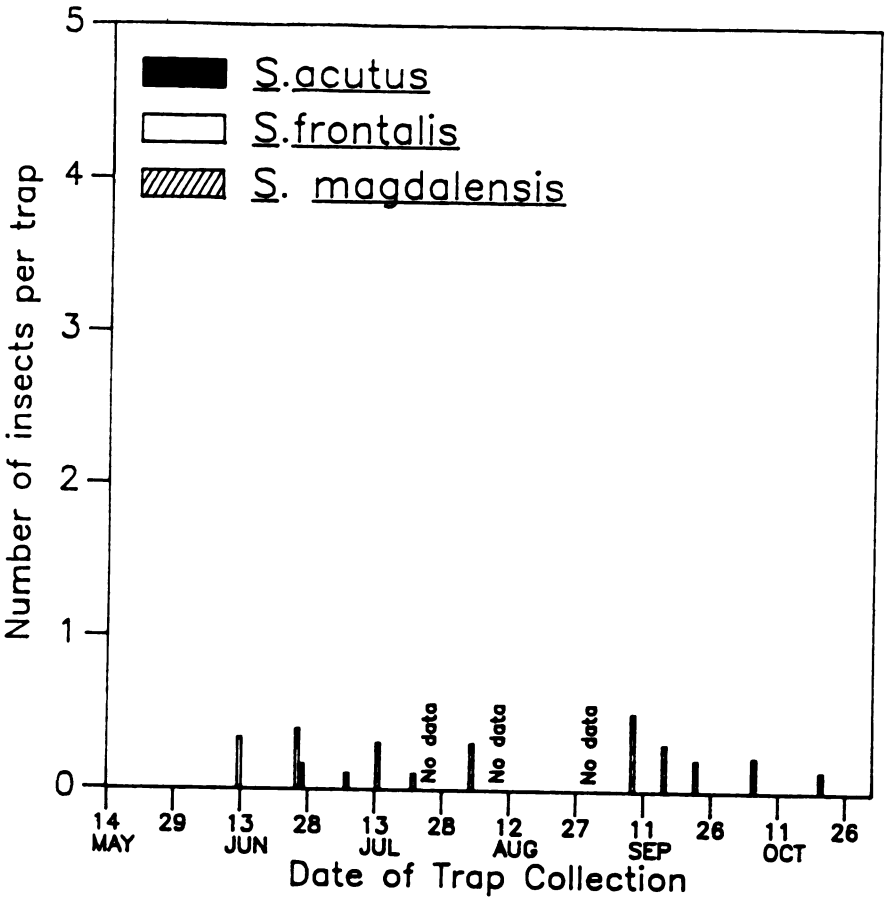

Figure 18. Scaphytopius spp. trapped in blueberry bushes with tent yellow sticky traps during 1989.

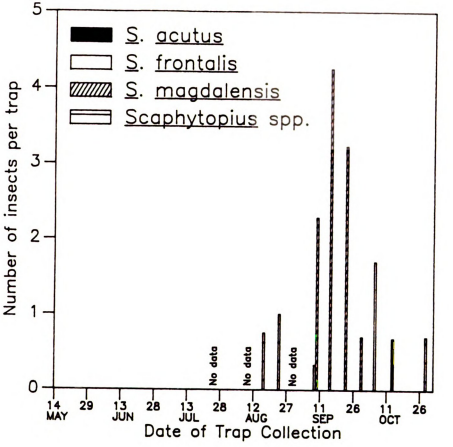

Figure 19. Scaphytopius spp. trapped in blueberry bushes with flat yellow sticky traps during 1989.

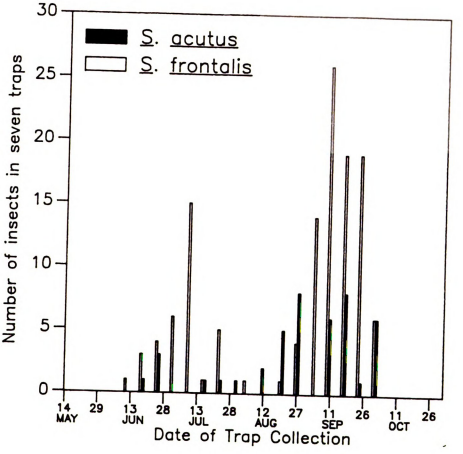

Figure 20. Scaphytopius spp. trapped in blueberry bushes with flat yellow sticky traps during 1990.

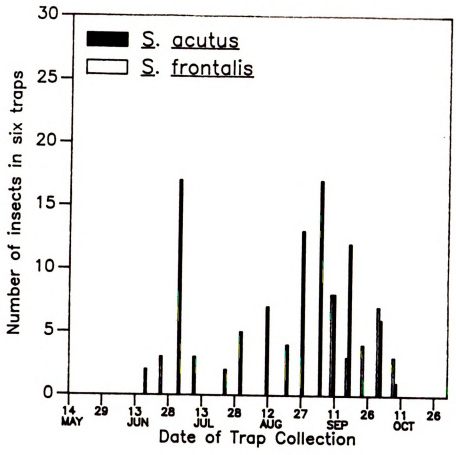

Figure 21. Scaphytopius spp. trapped in wild cherry trees with flat yellow sticky traps during 1990.

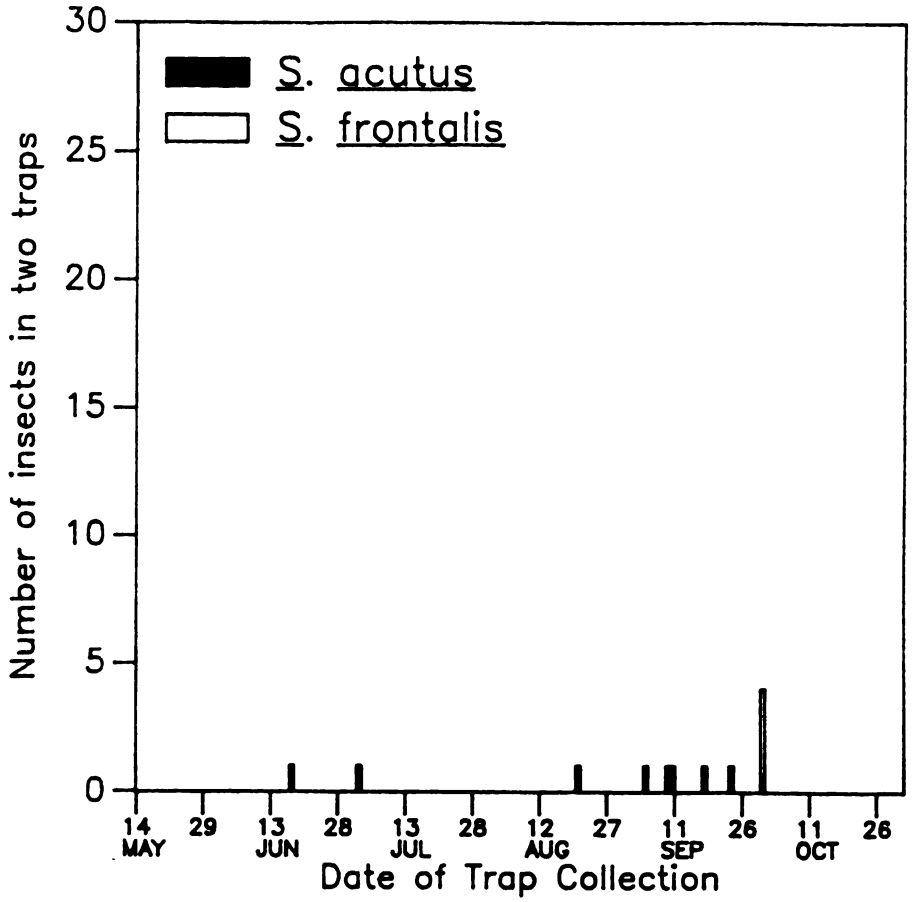

Figure 22. Scaphytopius spp. trapped in black cherry trees with flat yellow sticky traps during 1990.

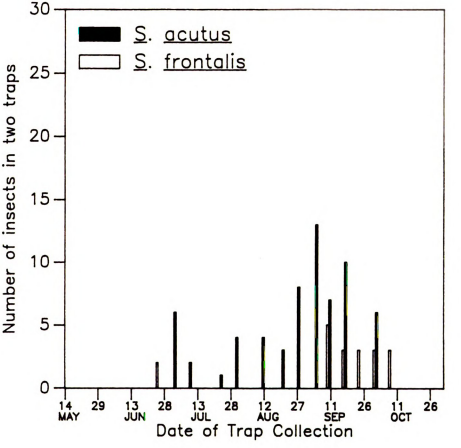

Figure 23. Scaphytopius spp. trapped in chokecherry trees with flat yellow sticky traps during 1990.

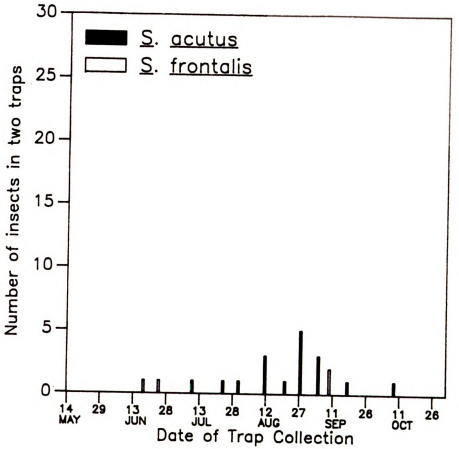

Figure 24. Scaphytopius spp. trapped in pincherry trees with flat yellow sticky traps during 1990.

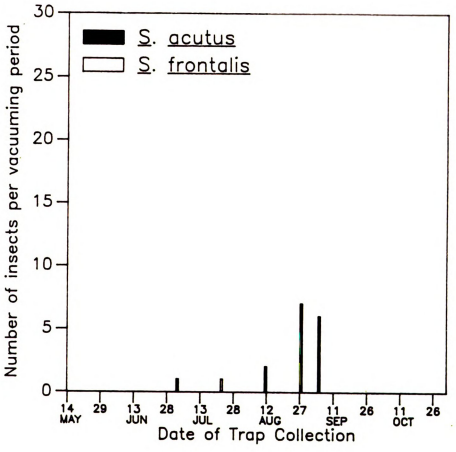

Figure 25. Scaphytopius spp. vacuum aspirated from blueberry bushes during 1990.

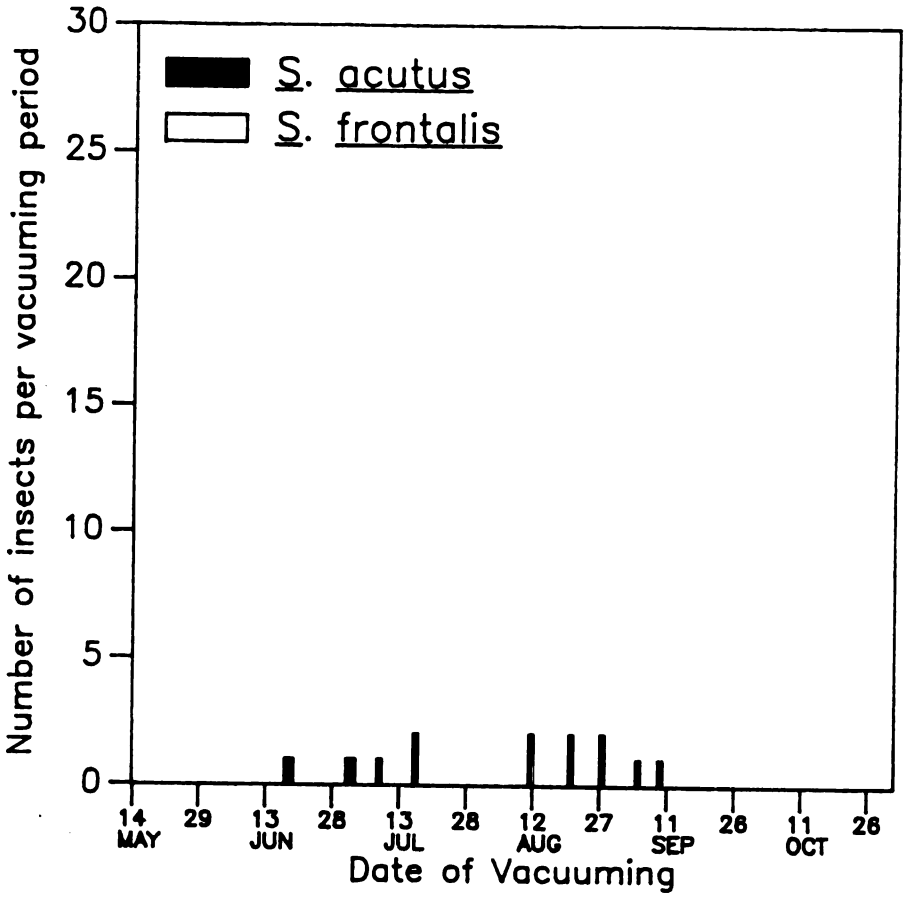

Figure 26. Scaphytopius spp. vacuum aspirated from wild cherry trees during 1990.

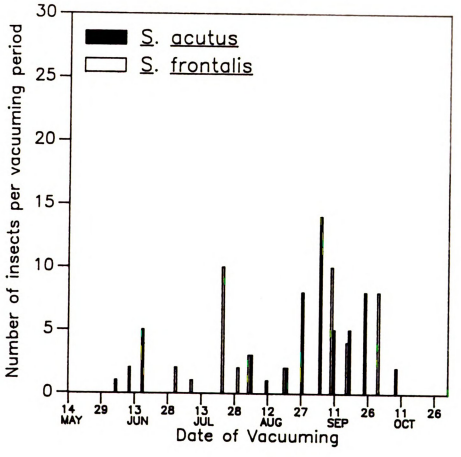

Figure 27. Scaphytopius spp. vacuum aspirated from bushes belonging to the Rubus pensilvanicus complex during 1990.

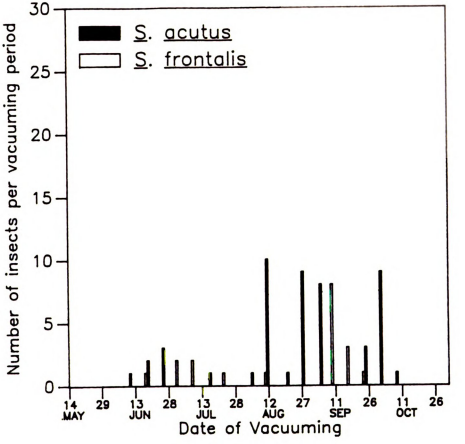

Figure 28. Scaphytopius spp. vacuum aspirated from the ground cover during 1990.

Table 1. Number of males:females of Scaphytopius spp. trapped during 1990.

Duration sticky traps were in the field and dates of vacuuming[a]	Yellow traps hung in: blue-berries	wild cherries	Numbers collected by vacuuming (total)
5/28-6/4	0/0	3/0	0/1
6/4-6/11	1/0	0/0	2/1
6/11-6/18	2/2	2/0	1/4
6/18-6/25	2/5	3/0	2/1
6/25-7/2	5/4	7/0	3/3
7/2-7/9	7/8	1/3	1/3
7/9-7/16	1/1	0/0	1/7
7/16-7/23	2/4	2/0	1/11
7/23-7/30	1/0	5/0	0/2
7/30-8/4	0/1	0/0	1/3
8/4-8/11	1/1	5/2	14/2
8/11-8/20	2/3	1/4	4/5
8/20-8/27	6/6	2/11	6/18
8/27-9/4	10/4	8/9	19/10
9/4-9/10	16/16	10/7	9/10
9/10-9/17	16/11	6/9	6/6
9/17-9/24	10/10	0/5	7/6
9/24-10/1	8/4	8/4	4/13
10/1-10/8	0/0	3/1	1/2
10/8-10/15	0/0	0/0	0/0
10/15-10/22	0/0	0/0	0/0
Totals	90/80	66/55	82/108

[a] Vacuuming was done on the first date of each pair of dates shown.

Determination of periods of transmission during the growing season.

MLO detection in *Scaphytopius* spp.

Figures 29 and 30 show the number of *Scaphytopius* spp. trapped respectively, during 1990 and 1991, for testing by DNA hybridization in order to determine the percentage of them that carried MLOs. Both figures display the same bimodal tendency found in the population studies with *Scaphytopius* spp. in the previous section. For 1990, the periods of major adult activity were between mid-June to the end of July and end of August to mid-October. In 1991, the population dynamics were very similar.

Fifteen percent of the insects trapped on sticky traps tested MLO-positive during 1990. The number of MLO-positives tended to follow the distribution of the total number of leafhoppers trapped for testing, being higher in the peak periods. The highest number of MLO-positive *Scaphytopius* spp. was trapped from the end of August to mid-October.

Only three insects out of 101 tested MLO-positive in 1991. One of them was trapped in the middle of July (30% fruit ripe) and the remaining two at the beginning of September (two weeks after harvest was completed). Perhaps allowing the traps to hang in the field for 2 wk may have led to degradation of MLOs inside the insects.

Not all of the insects trapped for MLO detection in 1990

were sexed as they were in 1991. Tables 2 and 3 show the sex ratio in the insects trapped to be tested and among those that tested MLO-positive in 1990 and 1991.

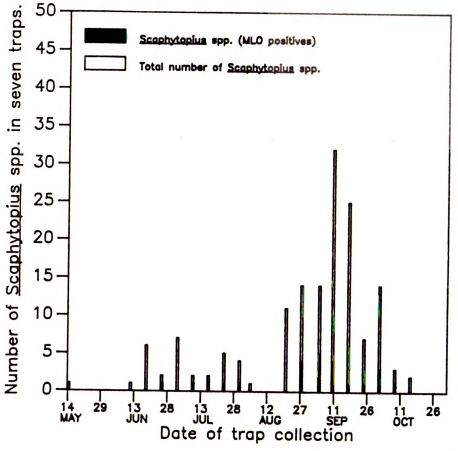

Figure 29. Scaphytopius spp. trapped in 1990 and tested for MLOs using DNA-hybridization.

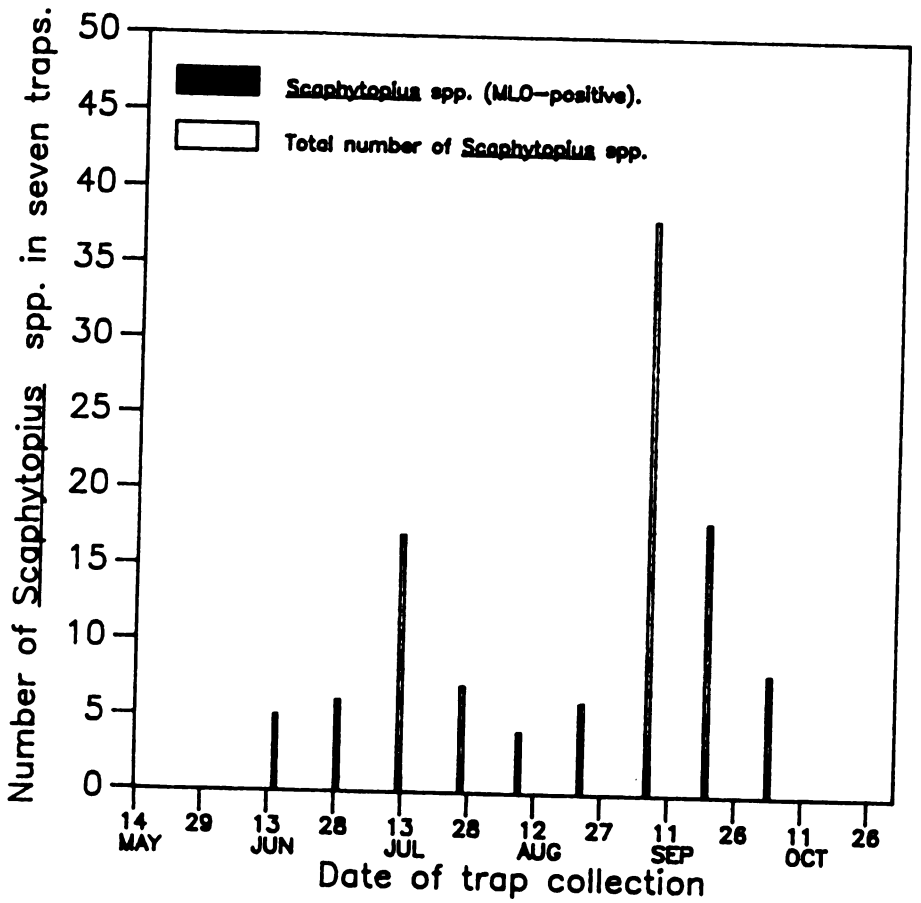

Figure 30. Scaphytopius spp. trapped in 1991 and tested for MLOs using DNA-hybridization.

Table 2. MLO detection in Scaphytopius spp. and distribution according to sex during 1990.

Date trapped	Number of Scaphytopius spp. trapped[a]			Number of Scaphytopius spp. MLO-positive		
	Females	Males	Total	Females	Males	Total
6/4-6/11	1	0	1	0	0	0
6/11-6/18	7	3	10	0	0	0
6/18-6/25	2	1	3	1	0	1
6/25-7/2			11			0
7/2-7/9			3			1
7/9-7/16	1	1	2	0	1	1
7/16-7/23			5			1
7/23-7/30			5			1
7/30-8/4	0	1	1	0	0	0
8/4-8/11	0	0	0	0	0	0
8/11-8/20	6	5	11	0	0	0
8/20-8/27	8	6	14	1	1	2
8/27-9/4	1	13	14	0	4	4
9/4-9/10			31			5
9/10-9/17			26			1
9/17-9/24			7			1
9/24-10/1			11			3
10/1-10/8			3			1
10/8-10/15			2			0

[a] An empty space means no sex determination was performed for that date.

Table 3. MLO detection in insects and distribution according to sex during 1991.

	Number of *Scaphytopius* spp. trapped			Number of *Scaphytopius* spp. MLO-positive		
Date	Females	Males	Total	Females	Males	Total
5/31-6/14	0	5	5	0	0	0
6/14-6/28	2	4	6	0	0	0
6/28-7/12	14	3	17	1	0	1
7/12-7/26	4	3	7	0	0	0
7/26-8/8	2	2	4	0	0	0
8/8-8/22	6	0	6	0	0	0
8/22-9/6	17	21	38	1	1	2
9/6-9/19	14	4	18	0	0	0
9/19-10/3	4	4	8	0	0	0
Totals	63	46	109	2	1	3

MLO detection in field-exposed blueberry trap plants

A total of 223 and 267 blueberry trap plants exposed in the field for two weeks during 1989 and 1990 respectively, were tested using the pAY22 DNA probe. The percentage of these plants that tested as MLO-positive among the 21 exposed during each period is shown in Figures 31 and 32. Figure 33 shows some of the MLO-positive plants as detected on nitrocellulose blots.

Forty seven percent of the blueberry plants exposed in the field during 1989 tested MLO-positive by DNA hybridization. There were two periods when the percentage of MLO-positive plants was higher. These were the beginning of June to mid-July and the beginning of August to mid-October.

Forty four percent of the blueberry plants exposed in the field during 1990 tested MLO-positive by DNA hybridization. There were also two periods in 1990 when more exposed plants tested as MLO-positive. These were the beginning of June to the beginning of August and the beginning of September to the end of October.

The results obtained by checking 10% of the plants exposed during 1989 and 1990 with DAPI stain are shown in Tables 4 and 5. Figures 34 and 35 show the petiole and root sections used in the test. "MLO-like" bodies were not abundant in the sections in which they were observed. The number of sections that showed such cells in the phloem was low compared to the total number observed. There was little or no correlation

between the results obtained with DNA hybridization and those by DAPI staining. When samples of known, symptomatic blueberry stunt-diseased and known healthy were observed previously, they could be separated easily as MLO-positive or negative by DAPI, in both petiole and root sections. Eight out of 18 and 5 out of 11 root and petiole sections of stunt diseased blueberry contained MLO-resembling bodies in their phloem. Only one section with MLO cells was found in leaf petiole sections of blueberry samples used as negative controls. No bodies were found in the root sections of the negative controls.

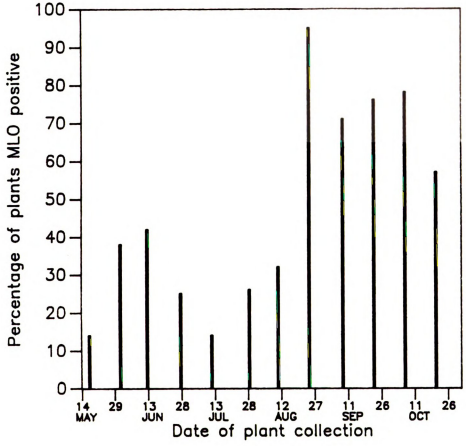

Figure 31. Percentage of field-exposed blueberry plants that tested MLO-positive using DNA-hybridization (1989).

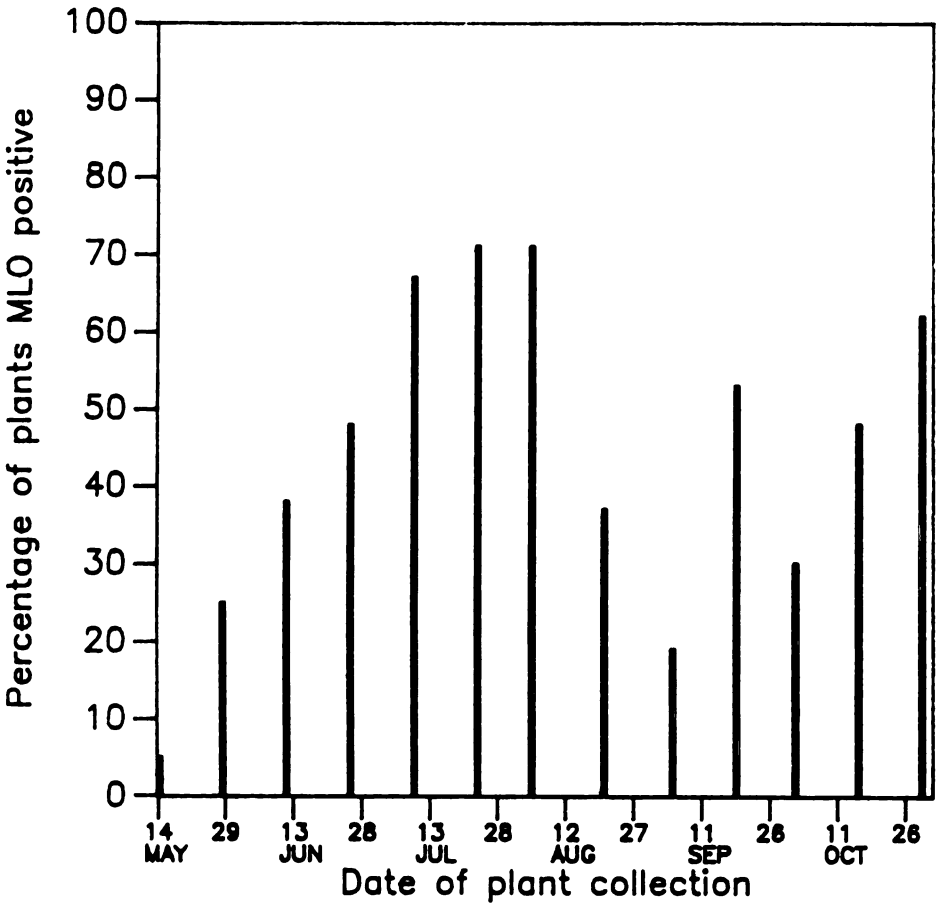

Figure 32. Percentage of field-exposed blueberry plants that tested MLO-positive using DNA-hybridization (1990).

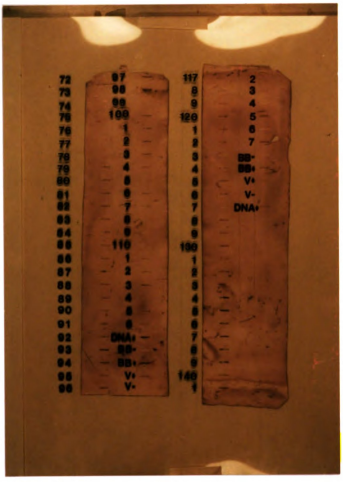

Figure 33. Detection of MLO-DNA in blueberry samples by DNA-hybridization on Nitrocellulose membranes. (Samples 79-96, 100-104, 106, 111-113, 120-122, 125-126, 128-130, 132, 134-141, 145, 147, BB+, V+ and DNA+, were recorded as MLO positive).

Table 4. Results of MLO detection in 10 % of the blueberry trap plants field-exposed in 1989, using DAPI stain, compared with those using DNA hybridization for detection.

Table 4

Dates trap plants were field-exposed	Petiole sections[a] Total Number of samples	Number of MLO positive (DAPI)	Percent positive	Root sections[a] Total Number of samples	Number of MLO positive (DAPI)	Percent positive	DNA hybridization Results[b]
5/3 - 5/17	34	7	21	22	0	0	+
5/3 - 5/17	10	0	0	22	0	0	-
5/17- 5/31	16	1	6	26	0	0	+
5/17- 5/31	13	0	0	27	1	4	-
5/31- 6/12	12	0	0	25	1	4	+
5/31- 6/12	20	6	30	21	2	10	-
6/12- 6/27	21	4	19	21	1	5	+
6/12- 6/27	15	0	0	15	0	0	-
6/27- 7/14	12	1	8	20	2	10	+
6/27- 7/14	17	0	0	20	1	5	-
7/14- 7/28	19	2	11	21	7	33	+
7/14- 7/28	11	0	0	22	2	9	-
7/28- 8/10	22	12	55	20	1	5	+
7/28- 8/10	30	0	0	19	2	11	-
8/10- 8/24	11	6	55	20	4	20	+
8/10- 8/24	14	2	14	12	0	0	-
8/24- 9/8	25	8	32	27	0	0	+
8/24- 9/8	11	0	0	21	2	10	-
9/8 - 9/22	15	2	13	12	0	0	+
9/8 - 9/22	26	5	19	24	2	8	-
9/22- 10/6	21	1	5	22	0	0	+
9/22- 10/6	15	2	13	18	0	0	-
10/6-10/20	25	7	28	18	1	6	+
10/6-10/20	30	2	7	21	4	19	-
Totals	223	51	23	254	17	7	+
Totals	212	17	8	242	16	7	-

Table 4 (cont'd).

[a] Sections were taken from individual plants previously tested by DNA-hybridization.
[b] Results from individual plants randomly selected.Ten percent of the DNA-hybridization positive and 10% of the negative plants were tested.

Table 5. Results of MLO detection in 10 % of the blueberry trap plants field-exposed in 1990, using DAPI stain, compared with those using DNA hybridization for detection.

Table 5

Dates trap plants were field exposed	Petiole sections[a] Total number of samples	Number of MLO positive (DAPI)	Percent positive (%)	Root sections[a] Total number of samples	Number of MLO positive (DAPI)	Percent positive (%)	DNA hybridization Results[b]
4/30 - 5/14	13	1	8	24	0	0	+
4/30 - 5/14	20	0	0	31	3	10	-
5/14 - 5/28	25	1	4	25	0	0	+
5/14 - 5/28	25	4	16	25	2	8	-
5/28 - 6/11	14	1	7	21	0	0	+
5/28 - 6/11	17	1	6	22	0	0	-
6/11 - 6/25	18	0	0	22	0	0	+
6/11 - 6/25	18	1	6	23	4	17	-
6/25 - 7/9	12	1	8	17	1	6	+
6/25 - 7/9	20	3	15	26	2	8	-
7/9 - 7/23	12	0	0	22	0	0	+
7/9 - 7/23	20	3	15	27	0	0	-
7/23 - 8/4	19	1	5	23	2	9	+
7/23 - 8/4	14	0	0	23	0	0	-
8/4 - 8/20	25	3	12	23	1	4	+
8/4 - 8/20	20	1	5	24	1	4	-
8/20 - 9/4	20	2	10	21	0	0	+
8/20 - 9/4	17	1	6	25	4	16	-
9/4 - 9/17	25	5	20	23	0	0	+
9/4 - 9/17	21	1	5	20	1	5	-
9/17 - 10/1	24	2	8	28	1	4	+
9/17 - 10/1	17	2	12	25	1	4	-
10/1- 10/15	21	4	19	23	0	0	+
10/1 -10/15	20	0	0	26	0	0	-
10/15-10/29	20	1	5	24	2	8	+
10/15-10/29	16	0	0	21	1	5	-

Table 5. (cont'd).

Dates trap plants were field exposed	Petiole sections[a] Total number of samples	Number of MLO positive (DAPI)	Percent positive (%)	Root sections[a] Total number of samples	Number of MLO positive (DAPI)	Percent positive (%)	DNA hybridization Results[b]
Totals	248	22	9	296	7	2	+
Totals	245	17	7	318	19	6	-

[a] Sections were taken from individual plants previously tested by DNA-hybridization.

[b] Results from individual plants randomly selected. Ten percent of the DNA hybridization positive and 10% of the negative plants were tested.

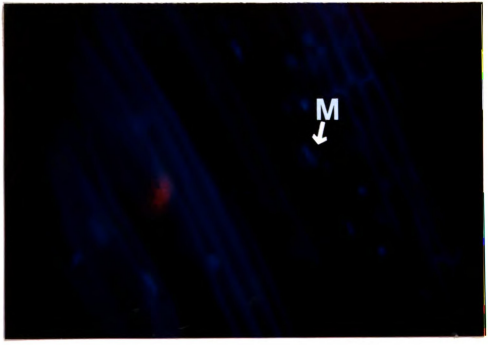

Figure 34. DAPI stained sections of blueberry leaf petioles.
M = Objects resembling MLOs. (630 X)

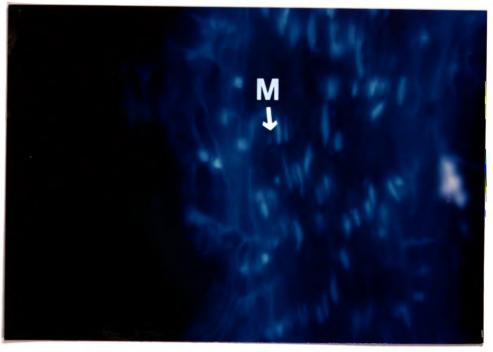

Figure 35. DAPI stained sections of blueberry roots.
M = Objects resembling MLOs. (630 X)

Blueberry stunt transmission tests

Twenty transmission tests were performed between 29 June and 11 October 1990. Tables 6 and 7 show the details and results achieved in the transmission tests.

The highest mortality of Scaphytopius spp. in the transmission tests was recorded during the acquisition access period (AAP). Among those that were transferred to the test plants for an inoculation feeding, only a few survived the 3 wk period scheduled for inoculation. There were not apparent differences in survival between Scaphytopius spp. and locations from where they were collected.

The three Scaphytopius species used in the tests, transmitted MLOs according to DNA hybridization results (Table 7). Thirty percent of the plants tested were MLO-positive following inoculation feeding by S. magdalensis and 28% when S. acutus was used. Scaphytopius frontalis showed the highest percentage of transmission (61%). If only the transmission by male insects was considered, (species identification was carried out in males only) S. acutus and S. magdalensis showed the highest values of transmission (Table 8).

Some of the plants in the test, which were inoculated by insects that had their AAP on healthy blueberry plants, tested as MLO-positive. When the "healthy" plants used in the AAPs were tested, some of them were also MLO-positive (tests 4, 5, 7-11, 12-18).

According to the identification of genitalia, the male

insects collected at Onondaga, MI, used in 1991 for the additional controls of the transmission tests were all S. acutus. Among them only 2 out of the 14 survived the three-week transmission period. None of the plants used in this test were detected as MLO-positive by DNA hybridization tests (Table 9).

Table 6. Details of the blueberry stunt transmission tests performed with different species of Scaphytopius during 1990.

Test No.	Place and date of collection[a]	Length of Acquisition Access Period (AAP) (days)	Percentage of mortality during AAP[b]	Length of inoculation feeding on test plants (days)	Percentage of mortality on test plants during inoculation feeding[b] 7d[d]	14d	21d
1	SW-MI, 6/29	2	N.D.[c]	4	100		
2	SW-MI, 7/3	2	50	4	20		
3	SW-MI, 7/13	2	50	4	13		
4	SW-MI, 7/19	2	40	5	18		
5	SW-MI, 7/27	2	7	4	28		
6	SW-MI, 8/10	2	52	4	5		
7	Imlay, 8/17	2	18	2	3		
8	Imlay, 8/20	2	45	2	8		
9	Imlay, 8/24	2	63	3	13		
10	Imlay, 8/27	2	30	3	13		
11	Imlay, 9/4	2	88	3	3		
12	Imlay, 9/10	2	63	22		23	14
13	SW-MI, 9/11	2	43	21		30	27
14	Imlay, 9/17	2	37	21	53	8	2
15	SW-MI, 9/20	2	15	18	28	18	5
16	Imlay, 9/24	2	75	21	13	5	7
17	SW-MI, 9/27	2	62	18	5	12	8
18	Imlay, 10/1	2	77	21	12	8	3
19	Imlay, 10/8	2	90	14	10		
20	SW-MI,10/11	2	80	11	0	20	

[a] - The places of insect collection were: SW-MI= South West Michigan, State land near Pullman, MI; Imlay = Imlay City,Michigan.

[b] - Calculated over an average of 40 insects trapped on each field trip.

[c] - No data available.

[d] - d = Days on healthy tissue culture-grown blueberry plants.

Table 7. Scaphytopius spp. used in transmission tests and results of MLO detection in blueberry test plants by DNA hybridization.

Table 7

Test No.	Species of Scaphytopius	Number of plants Used as: Test plants[a]	Healthy checks[b]	DNA tested Test plants	Healthy checks	MLO-positive Test plants No.	(%)	Healthy checks No.	(%)
1	S.magdalensis	8	4	1	0	0	0	--	--
2	S.magdalensis	15	5	0	0	--	--	--	--
3	S.magdalensis	14	6	4	5	2	50	2	40
4	S.magdalensis	19	5	10	4	6	60	2	50
5	S.magdalensis	30	4	27	4	6	22	2	50
6	S.magdalensis	14	5	14	5	2	14	1	17
7	S.acutus	4	3	0	0	--	--	--	--
8	S.acutus	14	7	1	6	1	100	2	33
	S.frontalis	0	1	0	1	--	--	1	100
9	S.acutus	7	8	0	3	--	--	1	33
10	S.acutus	8	4	1	3	1	100	0	0
11	S.frontalis	4	1	0	1	--	--	1	100
12	S.acutus	12	2	12	2	5	42	0	0
	S.frontalis	1	0	1	0	1	100	--	--
13	S.magdalensis	20	2	18	2	5	28	0	0
	S.frontalis	1	1	1	1	1	100	0	0
14	S.acutus	19	4	19	4	2	11	2	50
	S.frontalis	2	0	2	0	--	--	--	--
15	S.magdalensis	30	4	29	3	3	10	1	33
16	S.frontalis	10	1	10	1	6	60	1	100
17	S.magdalensis	14	1	14	1	9	64	1	100
18	S.acutus	6	3	6	3	2	67	1	33
19	S.acutus	1	0	1	0	1	100	--	--
20	S.magdalensis	1	0	1	0	0	0	--	--
Totals	S.magdalensis	165	36	118	24	33	28	9	38
	S.frontalis	18	4	14	4	8	56	3	75
	S.acutus	71	31	40	21	12	30	5	24

[a] - The insects placed on these test plants spent their AAP (2 days) on a stunt diseased bush.

[b] - The insects placed on these plants spent their AAP (2 days) on a healthy bush.

Table 8. Results of MLO detection in blueberry plants used in transmission tests with male leafhoppers identified to species.

Table 8

Test No.	Leafhopper species	DNA Tested		Number of plants MLO-positive			
		Test Plants No.[a]	Healthy Checks No.[b]	Test Plant No.	Test Plant %	Healthy checks No.	Healthy checks %
1	S.magdalensis	0	1	0	0	0	0
3	S.magdalensis	3	3	1	33	2	67
4	S.magdalensis	2	2	0	0	1	50
5	S.magdalensis	1	1	0	0	0	0
6	S.magdalensis	2	1	1	50	0	0
8	S.acutus	1	2	1	100	1	50
	S.frontalis	0	1	0	0	1	100
9	S.acutus	0	1	0	0	0	0
10	S.acutus	1	1	1	100	0	0
12	S.acutus	5	1	3	60	0	0
	S.frontalis	1	0	0	0	0	0
13	S.magdalensis	5	1	1	20	0	0
	S.frontalis	1	1	1	100	0	0
14	S.acutus	1	1	1	100	1	100
	S.frontalis	2	0	0	0	0	0
15	S.magdalensis	7	1	0	0	0	0
16	S.frontalis	1	0	0	0	0	0
17	S.magdalensis	1	0	1	100	0	0
18	S.acutus	1	0	1	0	0	0
20	S.magdalensis	1	0	1	0	0	0
Total	S.magdalensis	22	10	5	23	3	30
	S.frontalis	7	2	1	14	1	50
	S.acutus	9	6	7	78	2	33

[a] - The insects placed on these plants spent their AAP (2 days) on a stunt diseased bush.

[b] - The insects placed on these plants spent their AAP (2 days) on a healthy bush.

Table 9. Results of the transmission test performed as an additional control, using insects captured from cultivated highbush blueberry free from stunt disease, at Onondaga, Michigan, 1991.

Plant	Insect species	Sex	Days on healthy plant before death	DNA hybridization results
1	*Scaphytopius* spp.	female	7	-
2	*Scaphytopius* spp.	female	14	-
3	*Scaphytopius* spp.	female	7	-
4	*Scaphytopius* spp.	female	14	-
5	*Scaphytopius* spp.	female	21	-
6	*Scaphytopius* spp.	female	7	-
7	*Scaphytopius* spp.	female	7	-
8	*S. acutus*	male	14	-
9	*S. acutus*	male	14	-
10	*S. acutus*	male	7	-
11	*Scaphytopius* spp.	female	7	-
12	*S. acutus*	male	21	-
13	*Scaphytopius* spp.	female	7	-
14	*S. acutus*	male	7	-

Detection of MLOs in non-blueberry plants within or adjacent to the blueberry plantation.

The results of testing plants other than blueberry in or adjacent to the plantation at Imlay City, for MLO and from testing wild blueberries are shown in Table 10.

MLO-positive samples were found in all the plant species tested even though no apparent symptoms of disease were observed (there was no proliferation of shoots or phyllody as is commonly reported for Aster yellows disease).

Table 10. MLO detection by DNA-hybridization in non-blueberry plants in or adjacent to the blueberry plantation at Imlay City,MI and in wild blueberry plants from Ottawa Co.,MI.

Plant type	Number of samples tested	Number of samples MLO-positive	Percentage (%)
Wild blueberries[a]			
location 1.	5	1	20
location 2.	5	1	20
location 3.	5	3	60
Rubus pensilvanicus complex	10	3	30
R. setosus complex	10	4	40
Pincherry (Prunus pennsylvanica)	10	4	40
Blackcherry (P.serotina)	5	3	60
Chokecherry (P.virginiana)	4	2	50
Sheep red sorrel (Rumex acetidiella)	10	2	20
Bristly sarsaparilla (Auralia hispida)	10	6	60

[a] All wild blueberries were highbush type (Vaccinium corymbosum L.). These were sampled in Ottawa Co., MI, near commercial highbush plantations.

DISCUSSION

Three different species of Scaphytopius were trapped during studies at the commercial plantation near Imlay City, MI. These were S. magdalensis, S. acutus and S. frontalis. This agrees with the results of previous leafhopper surveys in Michigan (Hoffman and Taboada, 1960; Taboada, 1965; Taboada and Hoffman, 1965; and Taboada and Burger, 1967).

Scaphytopius magdalensis was not trapped during 1990 in the yellow traps or by vacuuming plants at Imlay City. The reason for this is unknown, since it was an important component of the Scaphytopius spp. trapped in 1989.

From the results obtained in 1989 using yellow sticky traps, it is evident that the shape of the trap strongly affects the number of Scaphytopius spp. trapped, keeping the area of the trap constant. The flat-shaped trap caught more Scaphytopius spp. than the tent-shaped yellow sticky trap and for that reason it was selected for the further studies. Several reasons could account for these results. It is possible that Scaphytopius spp. do not like dark places e.g. the inner part of the tent traps or, the attractive properties of the yellow color could be diminished by the shadowing in

the inner part of the tent, reducing the effective trapping area of the yellow trap by a half. The most probable reason for the flat sticky vertical traps being most effective is that more surface area was exposed to the leafhoppers.

The bimodal nature of the Scaphytopius spp. population dynamics found in most of our trapping, regardless of the method used (sticky trap or vacuuming), host (blueberry, wild cherries, Rubus spp. etc.) or season (1989,1990 or 1991) is consistent with the life cycles of the species reported for the Northern states of the United States where two generations a year of Scaphytopius spp. have been detected (Tomlimson, 1950; Hutchinson, 1955; Palmiter et al., 1960; Taboada et al., 1975; Rosenberger and Jones, 1978; Mc Clure, 1980).

Two periods of major adult activity were found using yellow sticky traps in blueberry and wild cherries. The dates for those periods were relatively consistent over the three seasons in both hosts. These periods are generally from mid-June until the end of July and a second and larger one from mid-August to the beginning of October. In 1989 and 1990 these periods corresponded to petal fall to full sized fruit, and harvest time to leaf drop in the blueberry crop and also to the two major preiods of vegetative growth of the plants. While in 1991, when the growing season was earlier, these periods corresponded to fruit set to almost full sized fruit and from harvest time to leaf fall. The ratio of Scaphytopius spp. trapped in 1990 was different between the traps located in blueberry bushes and those located in wild cherries.

Scaphytopius acutus was more commonly trapped in wild cherries while the proportion of species was more balanced in blueberry bushes indicating some host preference. The traps hung in chokecherry trees yielded more leafhoppers than those in the other wild cherries. Most of them were S. acutus. This is probably due to the fact that chokecherry is an important host of Eastern-X disease MLO and S. acutus is one of the vectors of that disease (Hildenbrand, 1953; Gilmer et al., 1966; Taboada et al., 1975; Rosenberger and Jones, 1978).

Even though the results from the population dynamics of Scaphytopius spp. as measured by collection with the D-Vac^{R} aspirator in 1990, were generaly similar to those obtained from yellow sticky traps, some differences were observed. The amount of insects vacuumed from bushes of the Rubus pensilvanicus complex and the ground cover, was larger than that from blueberry bushes and wild cherry trees. It appears that S. acutus and particularly S. frontalis prefer to live in those plants, at least during the day time, and probably feed in blueberry and cherry after twilight, when they are trapped in the yellow sticky traps. This behavior was already reported for Paraphlepsius irroratus, a vector of Eastern X-Disease of peach (Gilmer et al., 1966). The fact that the periods when more Scaphytopius spp. were vacuumed, were earlier in the season in bushes from the Rubus pensilvanicus complex and the ground cover than in blueberry and wild cherries also supports this idea. A similar effect was found by Mc Clure (1980) for S. acutus, suggesting that this vector invades orchards from

wild hosts.

The sex ratio of Scaphytopius spp. trapped during 1990 was slightly different for yellow sticky traps and the D-Vac® aspirator. Slightly more females than males were captured when vacuum aspiration was used. That was also found by Meyer (1984) with S. magdalensis. This phenomenon may occur because males tend to be more mobile and disperse to find mates, while females are more sedentary, spending a greater proportion of their time feeding and laying eggs.

The number of MLO-positive Scaphytopius spp. observed over time tended to follow the same pattern found for the total number of Scaphytopius spp. trapped during the season. This was especially true in 1990. The highest number of MLO-positive Scaphytopius spp., was trapped in 1990 from August to mid-October when the highest number of insects was also recorded. However, the percentage of MLO-positive Scaphytopius spp. was not uniform with respect to the overall growing season. Few MLO-positive leafhoppers were trapped at the beginning of the growing season. This could be due to the fact they require an incubation period to build up detectable MLO levels in their bodies after they acquire MLOs. The overall percentage of MLO-positive Scaphytopius spp. was 15% in 1990 and 3% in 1991. These results can be compared to the 2.5% obtained by Kirkpatrick et al. (1990) using DNA probes for Western X-Disease and Aster Yellows MLOs to test several species of leafhoppers trapped in fruit orchards. The higher percentages showed by the Scaphytopius spp. trapped in the

blueberry planting in 1990, could be due to a higher sensitivity of the DNA probe used, a lower specificity (pAY22 can detect several MLOs), or the characteristics of the MLOs involved in the system (their multiplication inside Scaphytopius spp.) and the high incidence of BBSD observed. The lower detection of MLOs in Scaphytopius spp. during 1991 could have been the result of replacing the yellow sticky traps every 2 wk instead of every week (as was done in 1990), exposing the trapped leafhoppers to weathering over a longer period. A similar effect was reported by Kirkpatrick et al. (1990), for experimentally infected Colladonus montanus and Fieberiella flori with Western X-Disease MLO.

MLO detection in the blueberry trap plants field exposed during 1989 and 1990, showed that the periods of infection tended to closely follow the bimodal distribution of the Scaphytopius spp. populations. Higher infection values of trap plants occurred when the leafhoppers were at higher population levels. These results can be compared to those of Rosenberger and Jones (1978) for Eastern X-Disease in a peach orchard where peach and chokecherry were the exposed indicator plants. They found transmission of the disease after the middle of June through the end of August. Suslow and Purcell (1982) using reared leafhoppers fed at different intervals during the season in infected cherry, demonstrated an increased percentage of transmission to celery from the end of May to the end of September.

Almost 50% of the total number of trap plants exposed in

both seasons became infected by MLOs. Those percentages are higher when compared to those found by Rosenberger and Jones (1978) for Eastern X-disease or by Suslow and Purcell (1982) for Western X-disease. That could be due to the higher sensitivity of the DNA hybridization tests compared to symptom expression or other methods of MLO detection used by those authors. In addition, the MLOs involved in our studies could have been able to infect blueberry more readily than X-disease MLO infects its hosts.

The results achieved in the detection of MLOs in blueberry plants using DNA hybridization could not be confirmed using DAPI staining of either leaf petiole or root sections. Correlations between DNA hybridization positives for MLO and DAPI positives for MLOs were almost non-existent for 1989 and 1990 trap plants. The higher sensitivity of the DNA hybridization techniques enabled the detection of very small amounts of MLOs in samples and without the risk of interfering artifacts encountered with DAPI. A very important factor which could influence the results obtained with DAPI, are threshold levels used to determine when a plant is positive or negative, since MLO-resembling objects were found in almost all of the samples, whether taken from petioles or roots. There are no references in the literature concerning this problem. Most of the publications on DAPI report on the comparison of results with plant samples whose status of MLO infection is known, or versus results obtained with other techniques (electron microscopy or other fluorescence techniques). Usually there is

no reference as to the number of sections per sample analyzed or the amount of them required to establish that a sample was indeed positive. In our observations, even though objects resembling MLOs were found in almost all the samples, their number per section and the percentage of positive sections per sample was small (less than 25% on average). Root sections did not show more MLO resembling bodies than leaf petiole sections as was reported by Schaper (1985) for BBSD-MLO. This inconsistency in our results could be due to the fact that all of the blueberry plants tested were symptomless. DAPI stain was reported to be not very efficient in detecting latent MLO infections because of the low number of organisms in the phloem and their irregular distribution (Douglas, 1986).

Even though the D-Vac aspiration machine, provided an abundant source of Scaphytopius spp. to perform BBSD transmission tests, high insect mortality was found during the acquisition access period. Several things could have lead to this problem. Too many insects placed together in a small cage could cause their decline. Scaphytopius spp. may need a combination of plant hosts for their diet in order to survive. Insect damage during vacuuming, the trip from the field to campus, and other manipulations could have occurred. Very few insects survived the 3 wk period of inoculation feeding on the micropropagated healthy blueberry test plants as a result of poor insect longevity. Palmiter et al. (1960) reported that adults of S. acutus lived only 20 to 30 days. Perhaps many of the leafhoppers were near the end of their life when collected

in the field. Excessive heating in the greenhouse, insects hit by water drops during watering could be other causes of the mortality observed. Ideally, for future transmission tests, reared insects should be used. However, earlier attempts at rearing Scaphytopius spp. in the greenhouse on caged blueberry were unsuccessful (Ramsdell, unpublished data). In future tests, larger cages should be used. Even with the relatively high mortality experienced, nearly 30% of all the plants from the transmission tests were MLO-positive according to DNA-hybridization tests. Some of the test plants used as "healthy" controls, upon which insects that spent their AAP on healthy bushes were placed, also were MLO-positive. The percentage of detection was the same from test plants upon which the insects fed for 1 or 3 wk. Since the test plants used in the transmission experiments were MLO-free before the test, and the need of an incubation period in the leafhoppers is a extensively reported phenomenon, one can surmise that the insects trapped in the field were already carrying MLOs and ready to transmit them. Perhaps the locations selected to vacuum the control insects for the transmission tests were too near the commercial blueberry planting with BBSD. Another factor that contributes to this supposition is that some of the "healthy" plants used in the AAP as controls were found to be MLO-positive when tested after the transmission tests. It appears that the "control" leafhoppers from the field inoculated the plants during the "control" AAP. When transmission tests were performed in 1991 with insects trapped

at a place far from BBSD sources (Onondaga, MI) and using only healthy plants, none of these plants tested MLO-positive. This finding has strengthened our confidence that cross-contamination of plants in the greenhouse by stray leafhoppers was not responsible for MLO-positives in the control test plants.

All three Scaphytopius spp. used in the test transmitted MLOs according to the results of the DNA hybridization with probe pAY22. The analysis of these results (Table 7) shows that the total number of plants that tested MLO-positive in the test after S. magdalensis feeding was the highest (33+9) but if the percentage of positive plants over the total used is considered, S. frontalis had the highest values of transmission. One factor that could influence these results, is the fact that we assumed that females belonged to the same species as did the males which were identified in the same capture. Females cannot be identified to species, since male genitalia are used. If only males were considered, the relative importance of the species in MLO transmission would change. Scaphytopius acutus would be the most efficient, followed by S. magdalensis. Since S. acutus and S. frontalis were always trapped together at the same locations, our extrapolation in the identification of species could have altered the actual species composition in our samples. Scaphytopius frontalis or S. acutus specimens are sometimes hard to separate by male genitalia since there is a high variation of shapes in nature according to the patterns given

in the key for species determination. S. frontalis has not been reported as an MLO vector yet, and according to our results it should be considered in future MLO transmission tests.

Many of the non-blueberry plants in or near the blueberry planting studied, tested MLO-positive. This indicates that weeds and plants other than blueberry could be sources of MLOs (pathogenic or not) for insect vectors, playing an important role in the epidemiology of diseases caused by MLOs. It should be reiterated at this point, that since the detection probe pAY22 came from an aster yellows type MLO, the MLOs detected in the plants other than blueberry may not all be BBSD MLOs. This phenomenon of other hosts near the commercial crops, carrying MLOs has been reported for chokecherry and Eastern X-disease MLO. Since Rubus spp. and wild blueberry samples tested as MLO-positive, it helps to explain why the insects collected for the transmission tests could have been carrying BBSD MLOs or other MLOs despite the relative distance from BBSD sources.

The DNA hybridization technique using the pAY22 probe was very useful for MLO detection in our studies. It had some limitations, i.e. the ability of pAY22 to detect not only BBSD-MLO but also certain other MLOs. We cannot say absolutely that the MLOs we detected were those that cause BBSD until the plants show symptoms of the disease. All of the plants used in this study are being kept until symptoms are expressed to confirm the results obtained. In order to avoid this problem

of specificity, other detection techniques must be investigated. Two alternatives are to construct a probe specific to BBSD-MLO or to prepare a highly specific monoclonal antibody to it. These specific detection methods could help to detect symptomless, mild infections. Such plants could be "silent" carriers of the BBSD-MLO.

If methods are to be used in mass-detection of samples, they must be easy to perform and not extremely time consuming. The method of DNA-extraction used by us, even though successful for avoidance of interfering natural blueberry-compounds in detection, was excessively time consuming. It would not be advisable for use in a certification program, since only sixteen samples a day could be processed, which involved 9 hr of work. DAPI is not a suitable method because of its high degree of subjectivity and the need for expertise required to determine if a particle is an MLO. It does not allow one to determine if the particle being seen is BBSD-MLO or not.

In summary, the population dynamics of Scaphytopius spp. were determined in a commercial blueberry field containing a high percentage of bushes with BBSD, and its surrounding area. Two periods of maximum adult activity were shown during the growing season. Non blueberry plants in or near the crop were shown to be effective shelters for Scaphytopius spp., as aided by use of a D-vac® aspirator. These leafhoppers could readily invade the blueberry crop. These Scaphytopius spp. were detected as MLO-carriers in a proportion that tends to follow

the population dynamics of the insect during the season. There was MLO transmission through the season in 1989 and 1990 but the percentage of it tended to correspond with the amount of Scaphytopius spp. trapped in the area. Scaphytopius magdalensis, S. acutus and S. frontalis transmitted MLOs to blueberry plants in our transmission tests. The fact that transmission also took place in healthy controls showed that the insects came infected from the field. Our last set of controls remained healthy, because that set of control leafhoppers was from an area well isolated from BBSD. Future transmission tests should be done with laboratory reared insects to avoid these problems and those derived from sex extrapolations in the species identification. Since S. frontalis was never reported as an MLO vector and it is very frequently trapped in Michigan, additional tests must be done to confirm its MLO-vector nature. All the plant species near the crop tested as MLO-positive. This finding indicates that growers must be aware not only of the control of vectors but also to eliminate additional sources of MLOs by keeping their plantings clean from plants that shelter leafhoppers and could be sources of MLOs.

Finally, some recommendations to the growers could be made according to our results in order to improve the strategies currently used for the control of BBSD. Since Scaphytopius spp. are present and transmitting MLOs during the entire season from May-June to leaf drop, insecticide sprays to control them should not be discontinued when harvest starts

(of course, keeping in mind the obligatory insecticide-free periods to avoid fruit contamination) and followed until leaf drop in autumn, when there is no risk of transmission. Roguing of stunt-diseased blueberry plants, as well as keeping the planting free of wild blueberry plants and weeds that could be sources of MLOs, will help to keep transmission levels low, since chemical control of vectors is never fully effective.

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