A STUDY OF THE RELATIONSHIP OF PRUNUS NECROTIC RINGSPOT VIRUS, PRUNE DWARF VIRUS AND TOMATO RINGSPOT VIRUS TO SOUR CHERRY DECLINE IN SOUTHWEST MICHIGAN

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ABSTRACT

A STUDY OF THE RELATIONSHIP OF PRUNUS NECROTIC RINGSPOT VIRUS, PRUNE DWARF VIRUS AND TOMATO RINGSPOT VIRUS TO SOUR CHERRY DECLINE IN SOUTHWEST MICHIGAN

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The symptoms of stone fruit decline (SFD) in sour cherry include reduced life expectancy and reduced fruit production. The relationship of Prunus necrotic ringspot virus (PNRSV), prune dwarf virus (PDV), and tomato ringspot virus (TmRSV) to SFD was investigated in twelve commercial cherry orchards in southwestern Michigan. At each orchard, leaf and cambial tissue was tested for PNRSV, PDV and TmRSV by enzyme-linked immunosorbent assay. Levels of infection by all three viruses were significantly higher in the decline orchards than in the non-decline orchards. Both TmRSV and PNRSV were associated with poor tree vigor, trunk damage and high orchard levels of tree death and replanting. Distribution of TmRSV within sour cherry tissues was irregular; the cambial tissues at or below the graft union and the roots were the most reliable source of virus. Infection by TmRSV was not well associated with pitting symptoms, vector nematodes or weed hosts.

To the cherry trees and cherry tree growers of Michigan,
whose cooperation made this research possible

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INTRODUCTION

Sour or tart cherry (*Prunus cerasus* L.) is an important commerical fruit crop in Michigan. Michigan has long been the national leader in sour cherry production, with approximately 75 percent of the nations' sour cherry crop produced in the state. Nationally, the 300 million sour cherry trees in production brought 60 million dollars to growers in 1986. The 33,000 acres of sour cherry in Michigan produced a 170 million pound crop in 1986, valued at 38 million dollars (Mich.Ag.Statistics, 1987). Other important sour cherry producing states are New York, Pennsylvania, Wisconsin and Utah.

In southwest Michigan the sour cherry growing region is centered in Van Buren and Berrien counties. In west-central Michigan and northwest Michigan, the production centers are in Oceana and Grand Traverse/Leelanau Counties respectively (Figure 1.1).

Ninety percent of all the sour cherry planted consists of a 'Montmorency' cultivar budded onto a 'Mazzard' (*P.avium* L.) or 'Mahaleb'(*P. mahaleb L.)* rootstock. Typically, trees will begin to bear a crop six years after planting and be productive for a minimum of 20 years, with some plantings producing well into their 3rd decade.

In the past 20 to 30 years growers and extension agents

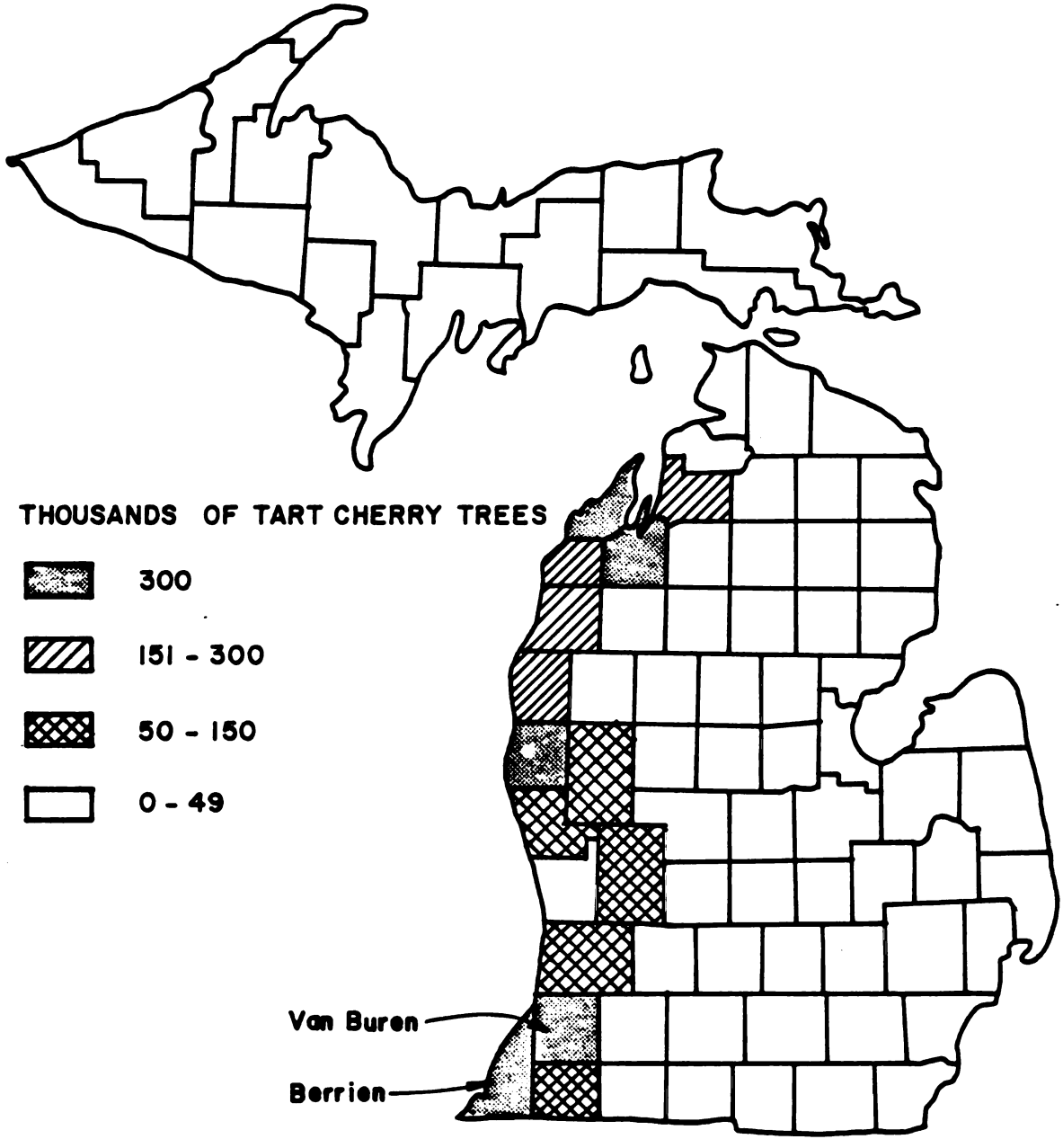

Figure 1.1 Location of major tart cherry growing regions in Michigan (Michigan Agricultural Statistics, 1987)

in all cherry growing regions have reported a field and production decline of mature cherry orchards that has steadily worsened. Trees which produced for up to 35 years in the past die or decline in yield and vigor in 20-25 years or even less. In addition to decreased life expectancy, growers report declining or inconsistent yields and decreased tree vigor beginning at only 8 years of age. Trees apparently healthy one year may suddenly decline in yield or vigor and may die in the early spring of the following year. Trees may break easily at the graft union, or develop bare, thin vegetative growth. Trees usually begin to show signs of decline as they come into their bearing years, and the resulting decline in or loss of productivity can seriously affect the growers ability to turn a profit on an often substantial intial investment.

The diverse symptoms reported for decline suggest a complex, multi-faceted cause. Researchers in Michigan have approached the investigation of the problem in a multi-disciplinary fashion. Present research in Michigan includes investigation of a number of factors which may be limiting tree growth and production. As possible causal agents to the decline syndrome many biological factors are being investigated. These include nematodes, bacterial canker, mycoplasma caused X-disease, tree borers and the soil pathogens *Phytophthora, Pythium* and *Armillaria*. Cultural and environmental factors such as soil structure, harvesting equipment damage, and frost and winter injury are also being

studied. Long term research includes the investigation of genetic resistance to decline and development of accelerated production strategies for sour cherry. Research is also in progress on tree decline in peach and plum, which includes varietal studies, biocontrol of *Cytospora* canker and investigations of various cultural practices.

This research was undertaken as part of the multi-disciplinary program. Its purpose was to investigate the role and the relationship of several plant pathogenic viruses to the declining sour cherry orchards in southwest Michigan.

Several viruses are known to infect and adversely affect sour cherry. Two are classified in the Ilarvirus group. These are Prunus necrotic ringspot (PNRSV) and prune dwarf virus (PDV). They are pollen borne and have been reported in Wisconsin, New York, Oregon, Washington and Ontario (Keitt and Clayton, 1943; Mink, 1980). All have been shown to cause biological stresses on sour cherry trees resulting in reduced tree vigor and lowered fruit production.

Tomato ringspot virus (TmRSV), a nepovirus, has been associated with poor production in a number of stone and pome fruits. Prunus stem pitting (PSP) in peach and nectarine, apple union necrosis and decline (AUND) in apple, and the Prunus brownline (PBL) disorders of plum and prune have all been found to be caused by TmRSV infection.

The purpose of this work was to determine the extent to which PDV, PNRSV, and TmRSV exist in southwest Michigan plantings of sour cherry; to investigate nurseries as the

source of these viruses; and evaluate the contribution of these three viruses to sour cherry decline in Michigan.

I. LITERATURE REVIEW

Prunus Necrotic Ringspot Virus and Prune Dwarf Virus

Prunus necrotic ringspot virus (PNRSV), and prune dwarf virus (PDV), are both included in the category known as 'ilar' (icose-hedral labile ring spotting) viruses. In the present study, these two viruses were considered as possible influencing agents in stone fruit decline. The effects that PDV and PNRSV infection produce in sour cherry include yield reduction, yield fluctuation and reduced tree vigor.

Serologically unrelated, PNRSV and PDV may occur together in sour cherry and other *Prunus* species. Prunus necrotic ringspot virus causes a necrotic leaf ringspot disease in many species of *Prunus*. In cherry, infection by PDV causes sour cherry yellows (SCY), the most economically damaging disease of sour cherry in the Great Lakes fruit belt (Jones, 1982).

Often PDV infection is accompanied by PNRSV infection. This relationship, a crossover in some host symptomatology between the two viruses, and the fact that both viruses may be latent in cherry has caused some confusion in the literature (Willison et al., 1957; Fulton, 1958 & 1960; Berkeley, 1948; Pine, 1964). Prior to antibody-specific testing the two viruses were sometimes inaccurately and inefficiently identified.

Prunus necrotic ringspot virus virions are quasi-spherical and range in diameter from 22 to 27 nm. The protein coat consists of subunits with a molecular weight of approximately 2.5×10^4 daltons (Barnett and Fulton, 1969).

Prunus necrotic ringspot virus is an RNA virus with the nucleic acid accounting for about 16 percent of the total particle weight. Loesch and Fulton (1975) found three species of particles that have sedimentation coefficients of 72S, 90S and 95S. Alone, the bottom particles are slightly infectious while the middle and top particles are noninfective. Mixing the bottom and middle particles markedly increases infectivity. Particle molecular weights range from 5.2 to 7.3 x 10^6 daltons. The A260/280nm ratio for PNRSV is 1.56. The virus is unstable in crude, undiluted sap; infectivity is lost within a few minutes at room temperature. In diluted sap maximum longevity is 9 to 18 hours. When the virus is in a stabilizing buffer, the thermal inactivation point (10 minute exposure) ranges from 55 to 62^{o} C, depending on the isolate of the virus (Waterworth and Fulton, 1964; Fulton, 1970b).

Prune dwarf virions are also quasi-spherical, with a diameter of approximately 19 to 35 nm. Halk and Fulton (1978) found that PDV sedimented in sucrose density gradients as five closely spaced zones of about 75, 81, 85, 98, 113S. The composition of the virus protein coat has not been determined. The virus contains 14 percent RNA. Only the faster sedimenting particles are infective. The A260/A280nm ratio is 1.56. The virus is unstable and loses one half its infectivity within 30 seconds in undiluted cucumber sap. The virus is stable in EDTA and salt solutions of 0.03 to 0.3 M. Infectivity is lost quickly in solutions of divalent cations. The thermal inactivation point of PDV (after 10 minutes

exposure with infectivity stabilized) is 45 to 54° C depending on the isolate (Fulton, 1970a).

Prunus species other than *P. cerasus* are susceptible to PDV and PNRSV. Some of the more important species are; *P. fasiculata* (Torr.) Gray, *P. dulcis* (Mill) D.A.Webb, *P. persica* L. (Batsch), *P. mahaleb* L., *P. pumila* L., *P. virginiana* L., *P. americana* Marsh, *P. bessyi* Bailey, *P. cerasifera* Ehrh., *P. fruticosa* Pall., *P. serotina* Ehrh., *P. pennsylvania* L. and *P. salicina* L. Genera other than *Prunus* that are susceptible to these two viruses are as follows: *Malus, Humulus, Rosa,* and *Rubus* (Fridlund, 1970; Cochran and Hutchins, 1941; Keitt et al., 1951; Kirkpatrick et al., 1962; Bock, 1966).

Transmission of PDV and PNRSV

Both PDV and PNRSV are pollen-borne in cherry. Gilmer and Way (1960) presented conclusive evidence for pollen transmission when a healthy parent cherry inoculated with diseased pollen produced diseased seeds. Extensive pollination studies involving caged trees, bud removal, and bee and hand pollination established pollen transmission of PNRSV and PDV from tree to tree (George and Davidson, 1964; Davidson, 1976). Prune dwarf virus was transmissible at lower rates than PNRSV.

Cole et al., (1982) have found that PNRSV is carried on the exterior of the pollen grain and they proposed that pollen germination may not be required for virus transmission. Other researchers demonstrated that mechanical

transmission of PDV and PNRSV does occur, both through foliage contact and from pollen to the host plant during fertilization (Hamilton et al., 1984; Swenson and Carpenter, 1961; Das and Milbrath, 1961).

Megahed and Moore (1967) have reported that up to 70 percent of the seed from *Prunus* species infected with PNRSV will carry the virus, and 70 to 80 percent of the seed from PDV infected trees will carry PDV. 'Mazzard' and 'Maheleb' seed and seedlings were found to have both viruses at appreciable levels and may have been the initial source of infection in some orchards (Davidson and George, 1965; Gilmer and Kamalsky, 1962). Mink and Aichele, (1984) tested the levels of both viruses in the seed of eight *Prunus* species. High levels of PDV and PNRSV were found in 'Mazzard' and 'Maheleb' seed, yet much lower levels were found in seedings from the same seed lot. This suggested that seed infected with these viruses often did not germinate or did not produce acceptable seedlings.

Prune dwarf virus and PNRSV have no known insect vector. This fact was established after extensive insect tests involving 130,293 individuals including 30 aphid, 18 leafhopper and 12 other insects species (Swenson and Milbrath, 1964). Nematode transmission has been reported (Fulton, 1970a and 1970b).

Host Symptoms of PNRSV and PDV

Researchers in North America and Ontario began to report on a ringspot-causing disease in cherry and other hosts around 1940. (Cochran and Hutchins, 1941; Keitt and Clayton, 1939; Hildebrand, 1943; Willison et al.,1948). Typical symptoms of ringspot on sour cherry include delayed foliation and blooming and small (1mm) dark necrotic rings on emerging leaves. The necrotic rings may fall out leaving a shot hole or shredded leaf appearance. The affected leaves are small but remain normal in color and are not dropped from the tree. Symptoms are most evident 1 - 3 weeks post bloom and can affect a few leaves, a branch or the entire tree. Affected branches may show stunted shoot growth and terminal necrosis. There is a wide range of symptom severity with milder symptoms even disappearing in midsummer. Symptoms on an individual tree may vary greatly year to year. The first year is typically the most severe, and is considered the "shock" or acute stage (Lewis, 1947; Berkeley and Willison, 1948). Lack of symptoms after the initial stage often masks the incidence of the virus and the seriousness of the losses it can produce (Berkeley & Willison, 1948; Cochran et al., 1950). More ringspot disease is found with increased tree age.

Early studies noted that similar ringspot symptoms were occurring in widely separated locations and in related species. A peach ringspot strain was transferred to almond, myrobalan plum, and 'Mahaleb' and 'Mazzard' cherry, producing typical ringspot leaf symptoms (Cochran and Hutchins, 1941).

Parker and Cochran (1951) reported on transmission of ringspot inducing virus strains from New York, California and Oregon as well as a strain from peach to 'Montmorency' and 'Mazzard' cherry. It was determined that all the strains were actually one virus, eventually termed Prunus necrotic ringspot virus (Berkeley et al., 1951).

In 1919, Stewart described the symptoms of a chlorosis problem in sour cherry. Twenty years later Keitt and Clayton (1939) reported a yellows condition occurring throughout cherry orchards in Wisconsin. Other researchers had noted a similar problem in New York and Oregon (Rasmussen, 1938; Gloyer and Glasgow, 1928). Keitt and Clayton (1943), established the viral nature of this yellows condition and ruled out fungal or bacterial causes. Citing the conspicuous yellow leaves produced, they suggested the name sour cherry yellows.

Yellows symptoms develop in sour cherry 3 to 4 weeks after petal fall, and progress from older to younger leaves. Typical leaf symptoms include conspicuous chlorotic areas of lighter than normal green, variable in size and shape. Leaves are relatively large in size and either drop off or change from paler green to bright yellow. Little or no necrosis occurs. Major defoliation (as high as 50 percent) occurs in late June or early July (Keitt and Clayton, 1939, 1943). The spur system of the tree may be reduced due to an initial overproduction of flower buds, leaving a willowy type of growth associated with long bare spaces on twigs which is sometimes termed "blindwood" (Lewis, 1947). Sour cherry

yellows may also produce "shock" symptoms similar to, yet milder than, those produced by PNRSV (Fulton, 1970). Overall, however, the disease tends to occur gradually and does not "kill the trees outright but...impairs their health and fruitfulness and probably tends to shorten their lives" (Keitt and Clayton, 1943).

Hildebrand (1943), after a nine year study, noted that at least one strain of the SCY virus was in fact PDV, first described as a disease of prune in 1936 (Thomas and Hildebrand, 1936). Later researchers confirmed that PDV was the SCY causal agent (Fulton, 1960; Gilmer and Brase, 1963; George and Davidson, 1964).

Berkley (1948) and Cameron and Moore (1956) both noted the high incidence of PNRSV infection on PDV infected trees, although work by Fulton and Hamilton (1960) and Tremaine et al. (1964) clearly establishes the two viruses as serologically unrelated. Fulton (1970) and Willison et al. (1957) using serological and electron microscopy techniques found no evidence that SCY requires more than one virus for expression.

Symptom expression of sour cherry yellows and ringspot was found to be particularly temperature dependent. Mills (1946) concluded that the chief factor in determining SCY symptom severity is temperature during the 30 day period following petal fall. Keitt and Moore (1943) found from greenhouse experiments that yellows symptoms developed freely on potted 'Montmorency' that were in greenhouses which fluctuated from 12 to 16^{o} C night temperatures and 24 to 28 ^{o}

C day temperatures, with the best symptoms developing at 16° C. At constant temperatures of 20° C or greater no symptoms developed on yellows infected 'Montmorency'. Moore (1946) observed that ringspot symptoms were expressed over the entire 16 to 28° C temperature range, with more rapid symptom development and necrosis occurring at the higher temperatures. Best leaf symptom expression occurred at 20 to 24° C.

Effects of PDV and PNRSV on Growth and Yield

The effects of both PDV and PNRSV on cherry growth and yield are well documented. Keitt and Clayton considered yellows an "unfruitful condition" as early as 1939 with their experiments showing a range of 16.7 to 41.6 percent yield reduction due to virus. Rassmussen and Cation (1942) showed SCY diseased trees had only 47 percent of the production of healthy trees while a 1946 report (Moore) noted yield reduction due to sour cherry yellows could be as high as 62 percent. The largest yield reductions occurred several years after infection (Lewis, 1951; Keitt and Clayton, 1943). Fruit produced on yellows infected trees had the same sugar content as fruit from healthy trees yet tended to be larger in size (Klos and Parker, 1960; Keitt and Clayton, 1943).

Lewis (1951) indicated that ringspot infection can cause up to 35 percent yield reduction, although it was noted that in subsequent years after initial infection, the bearing capacity of ringspot infected trees can approach that of healthy trees. Klos and Parker (1960) obtained results in

agreement with Lewis. The yield reduction caused by ringspot was considered important even at low levels, however, due to the sheer prevalence of the disease (Cochran et al., 1950).

Cain and Parker (1951) and Way and Gilmer (1963) noted that it was the decreased fruit set, possibly due to decreased pollen viability, that accounted for decrease in production for both yellows and ringspot infected trees. Nyeki and Vertesy (1974) showed morphological and physiological effects on pollen to be responsible for the lower rates of fertilization and fruit set. Infected pollen grains were smaller in size, variable in shape and had decreased viability and incomplete pollen tube formation.

In a study of ringspot effects on growth, Milbrath (1950) noted that virus-free trees were taller and sturdier than those with ringspot infection. Parker et al.(1959) also reported growth reduction for ringspot infected trees. Spur production was reduced for trees infected with ringspot (Keitt and Clayton, 1943) and yellows (Cain and Parker, 1951) where infection was associated with terminal extension (Klos and Parker, 1960). Cain contrasted this with a healthy condition:

> " The type of growth resulting when terminal extension continues with a reduced formation of spurs and lateral branches is commonly associated with trees infected with cherry yellows. The lateral buds on these shoots are largely flower buds which set a few fruits the second year and are completely barren thereafter. In contrast, on vigorous healthy shoots one or more of the basal buds are vegetative, forming spurs the second year and a fruit cluster the third year. These spurs then remain productive for several years."

Young tree growth was also affected by ringspot. A comparison of ringspot-inoculated young trees and virus-free trees showed that ringspot induced serious growth reduction. The largest difference in growth was in numbers of branches formed; the tree heights were approximately the same (Millikan, 1955). Davidson and George (1965) inoculated young trees with PDV, PNRSV, or both. Inoculations of trees at 1,2,4, and 6, years of age retarded growth. The younger the tree was at the time of inoculation the more severe was the growth reduction. Prune dwarf virus inoculation caused up to 60 percent growth reduction, with effects especially severe 2 to 5 years after inoculation. Prunus necrotic ringspot virus infected trees had less reduction in growth but yield reduction was substantial, as high as 56 percent less than healthy. Trees infected with both viruses had reductions in yield and growth similar to those only inoculated with PDV.

Prunus necrotic ringspot virus and PDV also affect the growth and yield of peach trees. A study in California showed up to 51 percent reductions in growth and up to 32 percent reductions in yield on peach trees infected by PNRSV and PDV (Pine, 1964). Work done in Bulgaria from 1975 to 1981 showed a decrease in leaf size, limb circumference, fruit set and fruit size in peach trees infected with PNRSV and PDV (Topchiiska, 1982). In addition, yields were reduced from 37-99 percent and high rates of tree death were seen in the infected trees. Estimates of the economic damage PNRSV causes to peach crops in California suggest that an intial

infection level of four percent could, after fourteen years, cost up $2200.00 per hectare (Heaton et al.,1981). Current research in Pennsylvania, Georgia and West Virginia show PNRSV to be prevalent in peach orchards and nurseries, and possibly related to slow decline in peach (Jaffee et al., 1986; Wells et al., 1986; Barrat and Otto, 1985).

Detection of PDV and PNRSV

Originally detection methods for PDV and PNRSV exploited the characteristic reactions of different *Prunus* species upon graft inoculation with infected bud tissue (Berkeley, 1948; Moore and Keitt, 1949; Berkeley et al., 1951; Hampton et al., 1966). For example, the necrotic reaction of 'Shirofugen' flowering cherry (*P.serrulata* Lindl.) when inoculated with tissue infected with PDV and/or PNRSV can be used to determine the presence of and sometimes distinguish between these viruses. Such bud inoculation is an inefficent methodology, however, especially in detecting early PNRSV or PDV infections. Hampton (1966) for example, showed that there was up to a 63 percent chance of failing to detect PDV using this method.

Later, more rapid virus detection was achieved by mechanical inoculation of virus infected tissue to different herbaceous hosts (Moore and Keitt, 1941; Fulton, 1957; Gilmer, 1961). Host reactions could distinguish virus presence in 10 to 14 days. In 1964, Waterworth and Fulton developed a PDV and PNRSV detection protocol using a combination of serology (microprecipitin or gel diffusion

tests), host range, thermal inactivation point and symptoms on selected hosts.

With the advent of enzyme linked immunosorbent assay (ELISA) PNRSV and PDV were detected and discriminated more rapidly and conveniently than possible with host range, grafting, or gel diffusion (Voller et al., 1976; Clark and Adams, 1977). ELISA was more discriminating than precipitin tests in distinguishing between serologically related strains of these viruses (Barbara et al., 1978).

Experiments by Torrence and Dolby (1984) report that detection of PNRSV and PDV by ELISA is possible throughout the year, yet the ELISA test is most sensitive early in the season. No evidence was found for uneven virus distribution in trees, yet it was recommended that multiple samples should be taken from any given tree to increase the chances of detecting recent infections.

Tomato Ringspot Virus

Tomato ringspot virus (TmRSV) has often been associated with systemic disease in perennial plants and has been present as a limiting factor in stone fruit longevity and production in particular. Tomato ringspot virus has been shown to cause Prunus stem pitting (PSP), apple union necrosis and decline (AUND), and Prunus brownline. All of these disorders display some symptoms that are similar to those noted in declining cherry orchards and this suggested TmRSV as a part of the cause of stone fruit decline.

Tomato ringspot virus was described by Price in 1936. Tomato ringspot virus is an RNA-containing virus with isometric particles about 28 nm in diameter. A purified preparation of TmRSV has three major classes of particles, designated top (T), middle (M), and bottom (B). The particles have sedimentation rates of 53S, 119S and 126S, respectively. The T particles consist of protein subunits and lack RNA. M and B particles contain the infective nucleoprotein. Forty-one percent of the particle weight of M and 44 percent of the particle weight of B is RNA (Stace-Smith, 1984).

In tobacco sap, the virus loses infectivity after 10 minutes at 58^{o}C, 2 days at 20^{o}C, 3 weeks at 4^{o}C, or several months at -20^{o}C. Dilution end point of sap from inoculated tobacco leaves or cucumber cotyledons is 10^{-3}, that of sap from systemically infected tobacco is less than 10^{-1} (Stace-Smith, 1970; 1984).

Tomato ringspot virus is both seed transmitted (Kahn, 1956; Mellor and Stace-Smith, 1963) and transmitted by adults and three larval stages of the dagger nematode *Xiphinema americanum* Cobb,1913. The nematodes can acquire the virus within 1 hour and transmit it into healthy plants within 1 hour. Single nematodes can transmit the virus (Teliz et al., 1967). Tomato ringspot virus is also transmitted by *X. rivesi* and *X. californicum* (Forer and Stouffer, 1982; Forer et al., 1984; Bonsi et al., 1984; Hoy et al., 1984). Insect transmission has not been reported.

Cadman and Lister (1961) found through plant protection and serological tests that peach yellow bud mosaic (PYBMV), reported to cause serious diseases in almond, nectrine, plum and sweet cherry, (Schlocker and Traylor, 1976) was antigenically no different than TmRSV. Civerolo and Miricetich (1972) found the stem pitting isolate of TmRSV was related to, but distinct from PYBMV.

Tomato ringspot virus is similar in size, shape, physical properties, host reaction, and geographic distribution to other nematode-borne viruses. Tomato ringspot virus is especially similar to tobacco ringspot (TRSV), yet the two viruses are serologically unrelated (Tall et al., 1949).

Tomato ringspot virus has been reported in a wide variety of plants, although mostly in ornamental and woody or semiwoody species. Affected species include *Vitus vinifera* and french hybrid grape (in combination with TRSV), geranium, cymbidum orchid, strawberries, raspberries, blueberries, passion fruit, gladious, and tobacco. (Uyemoto, 1975;

Christensen and Paludan, 1978; Goff and Corbett, 1977; Convese, 1981; Stace-Smith, 1962; Johnson, 1972; Koening and Fribourg, 1986; and Stace-Smith, 1970).

TmRSV and Prunus Stem Pitting

Wood or stem pitting was first observed in 1956 (Guengerich and Millikan) as being associated with a disorder of virginia crab apple. The disorder was found to be graft transmittable to healthy tissues which suggested a viral cause. The first description of pitting in peaches was by Barrat in 1964. He noted "the pitting was of the rough, ridged type with fissures up to 1/8" deep, with occasional wood enations." The first report of stem pitting in cherry was by Lott in 1967, which he termed a "xylem abberation." Stouffer et al. (1969) gave a fuller description of pitting in several species of *Prunus* including pitting on the 'Montmorency' cultivar on 'Mahaleb' rootstock. He described a "prominent union, invaginated, with pockets of brown necrotic tissues embedded. This suggests union incompatibility and weak root systems, possibly inducing winter injury."

Other researchers described pitting on other species of cherry, peach, wild peach, apricot, nectarine, several plum varieties, Italian prune and pear (Mircetich and Fogle, 1969; Stouffer et al., 1969; Aldwinkle and Arneson, 1972; Dowler et al., 1968; Mircetich, et al., 1968; Cameron, 1968; Millecan et al., 1964). Pitting was noted in eastern Europe, Greece, and in many areas of the United States (Zagaja and Millikan,

1972; Gavalas, 1970; Agrios, 1971). Early reports listed TmRSV in the following states: New York, Delaware, Maryland, Pennsylvania, West Virginia, New Jersey, North Carolina, South Carolina and later, in states further west - Illinois, Ohio, Michigan, and California (Aldwinkle and Arneson, 1972; Miricetich and Fogle, 1976; Mircetich et al., 1968; Dowler et al., 1968; Thornberry, 1970; Jones and Janeson, 1969; Smith and Traylor, 1969). Thornberry (1970) suggested movement was from the east by vector movement in wet soil.

Barrat et al. (1968) first named this stem pitting disorder in peach as stem pitting of peach. Later, the disorder was renamed to Prunus stem pitting (PSP). Barrat described the complex of symptoms on peach. The foliar symptoms were listed as girdling, brachytic terminal growth, leaf curl, droop, chlorosis, and early leaf abscission. Also mentioned were early fruit ripening and drop. The lower trunk showed enlargement at or below ground level, with extremely thickened bark (2 to 4 times thicker than normal) and wood pitting. Some trees broke off at or below ground level. Disorganized tissues were found in the woody cylinder, and xylem elements were abnormal. The trees did not appear to recover and it was noted there were no signs of incompatibility since the union was normal.

In 1968, Lewis et al., documented the decline problem on peach in Pennsylvania and Maryland. The foliar and pitting symptoms were similar to those described by Barrat et al.(1968). Pitting was found to extend to the roots and up to 12 inches above the union on the scion, although sometimes

the pitting was limited to the rootstock. Lewis et al. found pitting on over 90 percent of the declining peach trees but not on vigorously growing trees. The symptoms were found in trees as early as 3 months post-budding, but 3 to 5 years post-budding was most common. Symptomatology led the researchers to suggest that a combination of virus, winter injury and drought was causing the problem. Also in 1968, Mircetich et al., described a pitting disorder on peach, apricot and nectarine in east coast regions. A detailed description of the pitted wood showed that the secondary tissues produced by the affected cambium were highly disorganized. Phloem tissue was thickened and spongy, with enlarged rays. The woody cylinder was disorganized, and inclusions of large wide xylem rays and phloem islands were noted. Lignification was incomplete, resulting in a weak woody cylinder. The pitting was noted as affecting trees from one to 20 years in age, and the point was made that some of the trees were affected well after maturity had been reached (10 to 15 yrs). This indicated that the causative factor must be there or needed to have certain environmental factors present for expression. Nematode involvement was suggested. Mircetich and Fogle (1969), after noting symptoms on several fruit species supported the theory that PSP was more than just a graft union incompatibility problem.

In transmission studies in the early 1970's, Mircetich et al.(1970) found the disorder to be graft-transmissible and noted that the causal agent may be present before "macroscopic" stem pitting develops. This was supported by

the fact that some healthy trees inoculated with symptomless tree roots developed pitting. No correlation was found between leaf symptoms and stem pitting. In further work on the peach stem pitting problem in Pennsylvania, Stouffer and Lewis, (1969) reported that there seemed to be no association between the pitting and PNRSV infection. This was later verified by Mircetich et al.(1971).

Research in 1971 (Mircetich et al.) and 1972 (Mircetich and Civerolo) showed that the PSP agent was graft transmissible across *Prunus* species, indicating the same agent was involved in all pitted species. Tomato ringspot virus was recovered from plum, cherry and apricot seedlings inoculated with PSP root chips from naturally infected peach and apricot trees. Root chips of affected trees grafted into healthy trees were found to be the most effective tranmission method. Symptoms were reproduced in five months, including pitting and grooving in the woody cylinder, cambial and bark necrosis, trunk enlargement and disorganization of xylem. Researchers suggested at that time that the stem pitting agent was not uniformly distributed, citing graft transmittable viruses of fruit trees (green ring mottle and apple mosaic virus) that were known to have localized distribution within the plant. Soulen et al., (1972) also found the PSP agent to be graft transmissible, and found high rates of graft transmission were possible.

Mircetich showed in 1972 that the spread of the disorder was from tree to tree in an apricot to sour cherry transmission study. A soil agent was suspected to be

involved and a virus was recovered that was related to TmRSV and peach yellow bud mosaic virus (PYBMV). Stouffer et al. (1972) soon after proved PSP was transmitted through the soil.

Other workers in the same year (Civerolo and Miricetich, 1972) serologically compared the stem pitting (SP) isolate and PYBMV isolate and found them to be related, yet distinct. No correlation was found between the virus associated with the pitting symptom or the experimentally infested trees, yet seedlings planted in infected peach sites were showing symptoms of stem pitting within 5 months. They attributed the lack of association between TmRSV and stem pitting, to the virus not being present in all trees, or to inefficient detection methods.

In 1970, Smith and Stouffer had used cucumber baiting to show that tomato and tobacco ringspot viruses were present in the soil around pitted trees, including sour and sweet (*Prunus avium* L.) cherry. In 1973, Smith et al., mechanically inoculated TmRSV into peach and reproduced PSP symptoms - thick bark, elongated pits and grooves, reduced terminal growth and chlorotic, or red/purple leaves. No pitting was found to be associated with TRSV or PNRSV. This was the first concrete experimental evidence positively linking TmRSV and the PSP problem.

Other workers associated stem pitting and grooving with the presence of the dagger nematode, *Xiphinema* spp., known to be a virus vector (Bloom et al., 1972). Stouffer, working with chemical (fumigant) control, experimentally confirmed

the soil/nematode nature of PSP spread and showed that control of *Xiphinema* reduced stem pitting and increased yields (Stouffer et al., 1975; Stouffer and Mowery, 1980).

By 1977, Mircetich et al., working in California, found a consistent association of stem pitting and decline in several *Prunus* species. Variations were found in the nature and severity of rootstock pitting, with 'Mazzard' and 'Napoleon' showing little or no pitting. 'Mahaleb' rootstock exhibited short and relatively shallow pits without cambial necrosis and 'Bing' in contrast, showed extensive, severe pitting. No stem pitting was found on trees with severe yellow bud mosaic, suggesting that only specific viral strains cause the stem pitting phenomenon.

Extensive work involving over 22,000 sweet cherry trees in Washington state revealed that reduced vigor was associated with severe pitting only for trees with 'Montmorency' interstocks (Mink and Howell, 1980). Although it was noted that sweet cherry orchards planted with certified virus-tested trees showed less stem pitting, there was little consistent overall association of thick bark and unthrifty tree growth with pitting. Stem pitting was noticed on trees that were otherwise normal. In some but not all cases, the symptomology recorded for sweet cherry was similar to stem pitting recorded in peach. Graft transmission of the sweet cherry pitting agent was not successful.

Other work with sweet cherry in Washington involving an attempt to transmit a stem pitting agent, was unsuccessful as well (AlMusa et al., 1980). There were *Xiphinema* spp.

present, yet TmRSV could not be isolated from trees, bait plants, or weeds. No pitting developed on peach or cherry seedlings that were grown in infested soil or experimentally inoculated. Again, although it was noted that pitting was present, it was not associated with an overall decline; normal growth was observed.

Lister et al., (1980), in work with detection of TmRSV in apple and peach found that the virus was somewhat localized at the base of the trees, yet led to impairment of translocation resulting in dwarfing and death of the canopy.

In 1984 Barrat et al., noted that PSP remains a serious problem in peach orchards in West Virginia and Pennsylvania. Surveys of peach trees and dandelion showed that TmRSV is widespread throughout these areas.

Prunus stem pitting was found to be caused by TmRSV infection via transmission by *Xiphinema* spp., yet the strengh of the connection between symptomatology, virus, and nematode varies with *Prunus* species. Even though programs have been implemented to control virus dissemination through infected stock, reservoir plants and nematodes have established the disease in some areas. Under these conditions complete control is not feasible, and hope for the future in eradicating PSP lies in TmRSV resistant germ plasm or cross protection with mild strains of TmRSV.

TmRSV and Apple Union Necrosis & Decline

In 1976, Stouffer and Uyemoto reported that girdling-type symptoms had been occurring in northeastern United States

apple orchards. Symptoms included leaves that were sparse, small and chlorotic. Terminal growth was reduced and twigs exhibited a bunchy type growth. Fruits were small and highly colored. Many trees had cracked or broken off at the graft union. Tissue at the graft union was abnormally thick and spongy and a distinct scion/stock necrotic line was often present. The 'Delicious' trees on Malling-Merton 106 rootstocks were found to be the most severely affected. Initially, the disease was reported from Pennsylvania and Virginia, but later, reports came from all apple growing regions of the eastern United States.

Researchers isolated TmRSV from apple rootstocks from Pennsylvania, Virginia and New York (Stouffer and Uyemoto, 1976; Stouffer et al., 1977). A common etiology for PSP and the apple disorder was shown when 100 percent of 37 peach trees inoculated with wood chips from the affected apple trees developed PSP (Stouffer and Powell, 1979). Later reports showed TmRSV had been isolated from trees with the apple disorder in the western United States as well (Parish and Converse, 1981).

Further work in the eastern United States (Rosenberger et al., 1983) found the disease (now termed AUND) was prevalent in orchards in the Hudson river valley of New York. Although the virus was found to be unevenly distributed in the trees, in one experiment, TmRSV was found in 89 percent of trees with graft union symptoms. It was theorized that the rootstocks were susceptible or tolerant to TmRSV and the scions were sensitive or hypersensitive, resulting in the

graft union symptoms. Rosenberger et al., (1983) observed that the stressed trees (heavily fruited, waterlogged, bark injured) were more susceptible to TmRSV infection and /or developed AUND symptoms quicker than non-stressed trees. It was noted that the combination of high levels of *Xiphinema* spp. and high numbers of TmRSV infected dandelions was favorable for the spread of the virus from dandelions to trees.

The researchers theorized that initially the virus entered the orchard via dandelion seed. If the nursery had been the primary source of TmRSV, a higher incidence of the problem should have been seen then, not in the eastern Hudson Valley, but in western New York where more of the susceptible MM106 rootstocks had been planted. Reasons suggested for the differences noted in eastern and western New York included the fact that there were different strains of virus/vector, different percentages of infected weeds, and differences in environmental conditions.

As observed with other TmRSV fruit tree infections, it was noted that apple tree infection seemed to depend on more than just susceptible plants, virus presence and nematode vectors. For example, very little PSP was found in New York and often AUND symptoms were found next to an unaffected peach. Yet there was Stanley constriction (also caused by TmRSV) of plum in New York, in areas where there was little or no AUND and PSP.

Histological investigation showed that the disturbed union was not a hypersensitive reaction in apple, but was

associated with increased production of ray and axial parenchyma and decreased vessel and fiber cells. Apparently the union disorder occurs after the plant switches from vegetative to reproductive phase with a loss in ability of cambium to differentiate normally (Tuttle & Gottleib, 1982).

In 1985 Rosenberger et al., continued research on AUND in the Hudson river valley of New York. The AUND syndrome was noted on seven scion cultivars propagated on MM106 rootstocks although different symptoms were noted for the different cultivars. In four orchards, dandelions and trees were tested for TmRSV and the soil was tested for *Xiphinema* spp. In the trees there was a high correlation of AUND symptoms with virus infection. The combined tree, dandelion and nematode results supported the theory that the TmRSV came in on dandelion seed and was spread by nematodes from dandelion to tree.

Studies reported in 1985 (Rosenberger et al.) experimentally provided evidence that AUND can be reproduced by inoculating trees with TmRSV-infected bark, although it was indicated that particular strains of TmRSV may not be capable of causing AUND.

Control of AUND has been partially achieved through removal of MM106 rootstocks and removal of weed hosts. It has been postulated, however, that the totality of the AUND problem has yet to be realized. In the years 1965-1975, 40 percent of the apples planted in New York were on susceptible MM106 rootstocks. It has been speculated that it is only a matter of time before many of these trees become infected

with TmRSV and develop AUND, but the numbers of trees involved preclude wholesale tree removal as a preventative measure (Bitterlin et al., 1984).

TmRSV and Prunus Brownline Disease

In the late 1950's, researchers noted a constriction problem in 'Stanley' plum (Brase and Parker, 1955; Kirkpatrick et al., 1958). Symptomatology led them to suspect virus and/or winter injury as the cause. Later work by Parker and Gilmer (1976) supported the winter injury theory, suggesting that a mild union incompatibility predisposed the trees to injury in the first place.

A similar hypersensitive necrotic reaction at the union of prune and peach was noted by Mircetich and Hoy (1981) who named the problem prune brownline disease. They detected TmRSV in rootstocks of both species and noted that detection was high from all tissues early in the onset of the disease and lower when the trees were dormant or dying. Serological tests indicated that the TmRSV strain was related, but not identical to, the strain causing PSP, yet was the same as the TmRSV-PYBMV strain.

Comparative studies by Hoy et al.(1984), showed that inoculation of prune with similar isolates - PYBMV, PSP, cherry leaf mottle and California PSP - did lead to union infection. Hoy et al. also noted that *Xiphinema californicum* could transmit the virus and that one plum rootstock, 'Marianna' 2624, did not become infected with TmRSV.

Noting the similarity of 'Stanley' constriction and

decline and prune brownline, Cummins and Gonsalves (1986) suggested the use of the name Prunus Brownline (PBL). As with the AUND syndrome, the researchers thought that PBL was caused by interaction of TmRSV infection, presence of an efficient vector, and use of a susceptible but tolerant rootstock with a resistant scion cultivar. They presented a scenario of original nursery infection leading to infected trees becomming widely dispersed in the orchard industry.

Association of TmRSV with Nematodes and Weed Hosts

The PYBMV strain of TmRSV, was first associated with *Xiphinema* spp. in 1959 (Breece and Hart). Nearly ten years later PYBMV was transmitted experimentally to apple and peach by *Xiphinema americanum* (Teliz et al., 1967). This was an important finding, because *Xiphinema* has worldwide distribution and virus vector capability (Lownsberry and Maggenti, 1963).

Braun et al.(1966), considered nematode populations in relation to apple vigor. They found a positive relationship of *Xiphinema* and another nematode, *Pratylenchus*, to tree vigor, although there was no investigation of virus on the site. *Pratylenchus* had been previously associated with serious damage to cherry and other tree fruits in New York and Michigan (Parker and Mai, 1956; Knierim, 1964; Davidson, 1957; Mai and Parker, 1967). Control of *Pratylenchus* resulted in a decrease in a sour cherry decline associated with 'Mazzard' and 'Mahaleb' rootstock (Edgerton and Parker, 1958).

Two *Xiphinema* species, *Xiphinema rivesi* in Pennsylvania and *Xiphinema californicum* in California, were also shown to transmit TmRSV to stone and pome fruit trees with the same efficiency as *X. americanum* (Forer and Stouffer, 1981; Forer et al., 1984; Bonsi, 1984; Hoy *et.al.*, 1984).

In 1981, Powell et al., noted that dandelion was associated with persistence of TmRSV-caused diseases. Later, seed transmission of TmRSV was established in dandelion, proving that dandelions can be a source of TmRSV inoculum and a major factor in orchard and inter-orchard movement of the virus (Murant and Lister, 1967; Lister and Murant, 1967; Mountain et al., 1983). This is especially true since the nematode movement is slow and nematodes can only retain TmRSV for a short time. Weed seeds are one initial source of virus and infected weeds are the source for nematodes reacquiring the virus (Murant and Lister, 1967). This can explain why some viruses like TmRSV are widely distributed in a scattered and localized way even though nematodes can not move over much distance.

Research has shown that dandelion, chickweed, henbit, creeping sorrel, common plaintain, strawberry, sorrel and red clover, can all be infected with TmRSV (Miller,1980; Powell et al., 1982). The most frequently infected, ubiquitous weed is dandelion. Work by Powell et al. (1984a) established criteria for a weed to be an important virus reservoir and showed that only dandelion fulfills these criteria.

Powell et al. (1984b) compared the population of *Xiphinema* and the presence of dandelions, with the incidence

of TmRSV and PSP. More dandelions infected with TmRSV were found in orchards with PSP than orchards without PSP or non-orchard sites. This work supported the hypothesis that TmRSV originates in the nursery or in dandelion seed and is often well established in weed hosts.

Current data indicate, however, that dandelion density and dagger nematode density are not the limiting factors to TmRSV spread or tree infection. Tomato ringspot virus initially comes in on nursery stock, then low tillage facilitates virus movement. As weed infection increases, the probability of tree infection also increases, on both an orchard and on a regional level.

Detection of TmRSV

Initally, researchers detected TmRSV in plant tissues by combining a number of detection methods, including host range and serology. In 1953, Yarwood first mechanically transmitted the PYBMV strain of TmRSV from peach tissue to an herbaceous host plant. In the years following, various herbacous hosts were tested and the symptoms that TmRSV induced were recorded. Typically, tissue from infected trees was sap inoculated to selected herbaceous hosts, and then this infected tissue was tested for presence of virus using serological methods such as microprecipitin and gel diffusion (VanSlogteren, 1955; Crowle, 1961). Slower methods of detection were also used, such as inoculations of infected fruit tree tissue into a healthy fruit tree, which was then "read" for symptoms after 3 to 4 months (Soulen et al.,

1972). As previously noted, Mircetich et al.(1971) found root chips were the most efficient tissue to use for this type of test.

In the 1970's the use of enzyme linked immunosorbent assay (ELISA), originally developed for use in the medical field, was applied to plant virus detection (Engvall and Perlman, 1971; Voller et al., 1976; Clark and Adams, 1977). ELISA has since been valued as the method of choice for efficiently detecting plant viruses, often at below the concentrations detected by many other standard procedures. For detection of TmRSV in woody hosts in particular, ELISA simplified the investigation of etiology and epidemiology of TmRSV induced diseases like PSP and AUND (Lister et al., 1980).

Converse (1978), working with naturally infected raspberry tissue, first used ELISA to detect TmRSV. He found that ELISA detected partially purified TmRSV at concentrations as low as 41 ng/ml. In 1981, Hoy et al., compared the abilities of ELISA, radio-immunosorbent assay (RISA) and mechanical transmission (MT) to detect TmRSV in *Prunus* species. When *Prunus* species were inoculated with a stem pitting strain of TmRSV, the virus was detected in 95 percent of the trees by ELISA and only in 13 percent by MT. Tests of naturally infected prune found MT detected TmRSV in 12 percent of the trees tested, while ELISA detected the virus in 51 percent of the trees, and RISA detected 97 percent TmRSV infection.

ELISA can be used to detect and assay both the virus

(direct method) and the antibody prepared against the virus (indirect method) (Voller, 1977). Both direct and indirect ELISA techniques can detect TmRSV, with indirect techniques slightly more sensitive (Powell, 1984). Indirect ELISA has less viral strain specificity (Van Regemortel and Burckard, 1980; Koenig, 1981) and the benefit of using one enzyme conjugate for detection of more than one virus (Lommel et al., 1982).

A commonly used direct ELISA is the double antibody sandwich (DAS) form of ELISA which uses immunoglobulin purified from an antiserum prepared in an animal to trap viral antigen. The antigen is then detected though use of an enzyme-labeled immunoglobulin which attaches to the the bound antigen and is then reacted with a substrate. The substrate initiates a color reaction upon degradation of the enzyme and this reaction is measued spectrophotometrically.

$F'(ab)_2$-based indirect ELISA uses pepsin derived $F'(ab)_2$ fragments of immunoglobulin from specific antiserum to trap virus. Trapped virus is then reacted with intact immunoglobulin. Enzyme-labeled Protein A that reacts specifically with the F_c portion of the intact immunoglobulin is added and then reacted with a substrate to give a characteristic measurable color reaction (Clark, 1981; Barbara and Clark, 1982).

Another indirect ELISA, heterologous antibody double antibody sandwich (HADAS) uses immunoglobulin prepared in two animals for virus detection. The second immunoglobulin in

the 'sandwich' is from a different animal species than the species used to prepare the first immunoglobulin. This second immunoglobulin is then reacted with an enzyme labeled anti-immunoglobulin and the color reaction is measured spectrophotometrically upon the addition of substrate (Bar-Joesph, 1982).

Dot-Blot immunoassay or dot-ELISA is similar to the DAS form of ELISA except this system uses a nitrocellulose membrane (NCM) to bind either the antigens or the virus specific antibodies. The NCM has a better binding capacity than the polystyrene binding surface used in the other forms of ELISA (Banttari and Smith, 1984; Banttari and Goodwin, 1985).

It is a concern in the use of ELISA that not all antisera can detect all isolates of a virus. For TmRSV in particular, serological differences among isolates could complicate diagnosis (Bitterlin and Gonsalves, 1988; Lister et al., 1980). Bitterlin and Gonsalves tested 30 isolates of TmRSV and found they fell into four serogroups. Antiserum to one isolate, PYBMV, detected 30/33 isolates of TmRSV when using direct ELISA, and 32/33 when an indirect ELISA technique using partially purified antigen was employed. Antisera to three other TmRSV strains were also tested. Results indicate that proper antisera must be used to assure efficient detection of isolates which belong to various serogroups. Bitterlin and Gonsalves suggest that antibodies to both the PYBMV and the 'Chickadee' isolate be used to assure TmRSV detection if the direct DAS method of ELISA is

used.

Though simplifying work with detecting TmRSV, ELISA has not eliminated the fact that TmRSV is often irregularly distributed in woody tissues. Various researchers have tested different tissues in attempts to locate the most reliable and efficient detection methods.

Lister et al.(1980) found that TmRSV is not systemic in apple, and that root pieces (2 to 4mm long) were the best tissue sources for detection. In another study, however, apple roots were an erratic source of TmRSV, while leaves (when a representative sample was taken), and bark tissues were more consistent (Bitterlin et al., 1984).

Barrat et al.(1984), found that sampling the the root/trunk transition zone in peach was an inefficient detection methodology, since only 48 percent of the peach trees with PSP symptoms were TmRSV positive when tested in this manner.

Recent studies, however, contradict Barrat's finding. Bitterlin et al. (1988) found that TmRSV and pitting symptoms were irregularly distributed within peach trees but mostly concentrated at and below soil line. Their experiments investigated distribution of TmRSV and pitting symptoms in 7-year-old peach trees and in 'Halford' (a peach rootstock) seedlings. Both orchard trees and seedlings were extensively sampled at roots, stem, leaves and branches. The ELISA test results indicated that the cambium on the trunk below the soil line was the most reliable sampling location for detection of TmRSV. Root tissues and the cambial tissues

above the soil line were less efficient for detection and no virus was detected in leaves or cambial tissues from the tree branches. To evaluate the use of pitting symptoms as a detection method, the results of TmRSV detection by ELISA were also compared to the pitting symptoms present in the orchard trees and seedlings. Pitting symptoms were similarly distributed in most trees, with most pitting occurring at the stem below the soil line. All symptomatic (pitted) tissues were ELISA positive for TmRSV, yet some tissues were positive that were not symptomatic.

Cummins and Gonsalves (1986) performed similar work in plum to determine the appropriate sampling methods for field surveys of TmRSV infection. Trees were sampled around the trunk circumference at the graft union and brownline symptoms were noted. Symptoms and virus distribution were found to be non-uniform. It was determined that detection of TmRSV through symptoms was possible visually if readings were taken from at least four locations around the graft union. Reliable detection of TmRSV by ELISA was possible if at least three locations at the graft union were sampled. Sampling was most efficient in July and August, when the bark was 'slipping' and was easily separated from the TmRSV-infected vascular tissues.

The contradictions in sampling techniques between species most likely are due to irregular distribution of TmRSV in individual trees. Bitterlin et al.(1988), noted that the age of the trees, the mode of inoculation, particular sampling techniques and environmental conditions could also have

affected results. In addition, they noted that particular isolates of TmRSV differ in their abilities to infect tissues, with PYBMV the most likely strain to systemically infect fruit trees. The possible effects of the mode of inoculation were noted by Bitterlin et al. (1984) in one study where TmRSV was detected in the roots in 62 percent of nematode inoculated trees, yet in no bud inoculated trees. Bitterlin et al. (1988) when testing cambial tissues noted that positive and negative samples were sometimes only 1 cm apart. In their studies they used larger and less diluted samples than Lister et al.(1980) and Barrat et al.(1984), who by using smaller and fewer samples may have missed the "pockets" of virus infection.

Cummins and Gonsalves (1982), noted that transmission, maintenance and reproduction of TmRSV within plant tissues, as well as the recovery of TmRSV from plant tissues, appears to be influenced by a number of factors. Upon initial grafting of tissues between stock and scion there may be variations in the establishment of new vascular tissue. Particular cultivars could differ in the capacity to support reproduction of TmRSV, or to destroy TmRSV upon infection. The physical status of host plants within a given clone may also produce different responses. Plant defense mechanisms, in response to the relatively slow influx of TmRSV may temporarily or permanently inhibit the movement of the virus. Whatever the cause or influencing factors, the irregular distribution of TmRSV, especially in woody tissues, must be considered in all field sampling for TmRSV and in

implementing any control measures, such as cross protection.

II. PRUNUS NECROTIC RINGSPOT VIRUS AND PRUNE DWARF VIRUS

MATERIALS AND METHODS

Field and Nursery Survey for PNRSV and PDV

Plot Design. During the 1983 and 1984 growing seasons, twelve sour cherry orchards in Van Buren and Berrien counties of southwest Michigan were indexed for prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV). Six decline orchards and six non-decline orchards were selected. The decline-affected or decline-free status was agreed upon after conferring with growers and extension agents. All trees consisted of a 'Montmorency' cultivar budded onto 'Mazzard' or 'Mahaleb' rootstocks. Trees ranged from 6 to 12 years of age and the average tree age was 8.5 years. Within each of the twelve orchards a rectangular plot of 200 trees was randomly selected for the study. Plots were mapped and individual tree ages and orchard cultural characteristics were noted.

For each of the orchards studied, the nursery source of the trees was determined and samples were then taken at these nurseries for virus testing. Three of the four nurseries studied (nurseries A,B, and C) were located in southwest Michigan and sampling was done at the nursery site. One nursery (D) was located outside of Michigan. This nursery was tested for virus by testing recently purchased, field-planted trees. At nursery A, 120 'Montmorency' / 'Mazzard' and 120 'Montmorency' / 'Mahaleb' 1-year-old

"liners" were tested. Sixty 'Mazzard' and 60 'Mahaleb' rootstocks were also tested. At nursery B, 160-IR2 project and 300-Michigan Department of Agriculture (MDA) certified virus-free bud wood trees were tested. In addition, 100 'Mazzard', 100 'Mahaleb' and 30 'Turkish Mahaleb' rootstocks and 50 'Montmorency'/'Mahaleb' 1-year-old "liners" were tested. Two hundred 1-year-old 'Montmorency'/'Mahaleb' trees were tested at nursery C. The fourth nursery (D), was tested by sampling 46 2-year-old 'Montmorency'/'Mazzard' trees received from the Sierra Gold nursery of Yuba City, California within the 10 days prior to testing. In all cases, representative samples of the field selection were taken.

Sampling. At all twelve orchard sites and the four nurseries, the trees were tested for PNRSV and PDV by enzyme linked immunosorbent assay (ELISA). Spring buds, flowers, or young leaf tissue samples were taken from each tree. For the orchard sites and the nursery budwood trees, samples consisted of tissue from eight locations on each tree, equally distributed around the canopy. The eight subsamples were combined to make one sample per tree. In the nurseries, two to four subsamples were taken from each 1-2 year old tree tested. Samples were placed in plastic bags and stored at 5° C until assayed for virus. Samples were always processed within 10 days.

ELISA Procedures. Virus assay was performed following the double antibody sandwich ELISA procedures of Clark and Adams (1977). Samples of 0.5 g to 1.0 g per tree were

diluted 1:10 (v/w) in cold extraction or PBS-Tween /PVP buffer (phosphate buffered saline, pH 7.4, containing .05 % Tween 20, 2 % polyvinylpyrrolidone MW 40,000, 0.2 % ovalbumin and .02 % sodium azide). The composition of PBS per liter was 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCL, 0.2 g NaN_3. Samples were ground with a Tekmar Tissumizer (SDT-182EN shaft, Tekmar Co., Cincinnati, OH 45237) for 30 to 60 seconds at approximately 60 percent of maximum speed (20,000 rpm) and then strained through 1 to 2 layers cheesecloth. The filtrates were stored at 5° C for no more than 4 hours.

Rabbit antiserum prepared against the G isolate of PNRSV and antisera to PDV was obtained from American Type Culture Collection, (ATCC, 2301 Parklawn Dr., Rockville, Maryland 20852). Anti-virus IgG (coating antibody) was purified from the antiserum by ammonium sulfate precipitation and by passing through a DEAE-22 cellulose (Sigma, St Louis, Mo 63178) column (Clark and Adams, 1977).

Flat-bottomed polystyrene Microelisa® substrate plates (Immulon® I, Dynatech Lab Inc., Alexandria, VA 22305) with 96 wells per plate were used for the ELISA evaluations. For each virus the procedure consisted of the following steps: **1**) 200 µl of 1.0 µg/ml purified IgG (PNRSV or PDV) in sodium carbonate buffer, pH 9.6 was placed in each of the 96 wells. The plates were then incubated in plastic bags for 4 hours at 37° C. **2**) Plates were emptied and rinsed with phosphate buffered saline solution, pH 7.4 with 0.5 % (v/v)

polyoxyethylene sorbitan monolaurate (Tween 20). This rinse procedure was repeated at least three times with at least 3 minutes between rinses. **3**) A 200 µl sample of tissue extract was placed in each plate well. Each plate included five to 10 healthy controls wells, two to four diseased control wells and one to two wells containing extraction buffer. The remaining wells were filled with test samples, which were each replicated twice on the same Microelisa® plate. Filled plates containing sample extract were stored in plastic bags at 5° C overnight. **4**) After rinsing as per step 2, 200 µl of a solution containing alkaline phosphatase-conjugated-IgG (anti- PNRSV or PDV) diluted 1:800 in extraction buffer was added to each well. Plastic covered plates were then incubated for 3 to 6 hours at 37° C. **5**) After rinsing as per step 2, 200 µl of a solution containing p-nitrophenyl phosphate(Sigma no. 104, Sigma Chemical Co, St Louis, MO 63178), freshly prepared at a concentration of 1.0 mg/ml in substrate buffer [10 % diethanolamine (v/v), pH 9.8, in distilled water containing .02 % sodium azide (w/v)] and was added to each well. Plates were incubated at room temperature for 15 to 30 minutes for PNRSV, and 30 to 60 minutes for PDV. The wells were rated visually for color change and absorbance was measured at 410 nm with a Microelisa® minireader (Dynatech Lab Inc., Alexandria, VA 22305). Plates were zeroed on an empty well and at least two readings were made of each plate. For visual readings, a distinct yellow color was considered ELISA-positive, and a colorless well, ELISA negative. Photometrically, samples

were considered ELISA-positive if the mean absorbance at 410 nm of each of the duplicate sample wells was greater than the mean A_{410} nm of the healthy control samples plus three standard deviations.

Tree Evaluation

All the trees in the six decline and the six non-decline plots were evaluated for various characteristics in 1985. The presence of natural wounds, gummosis, borer injury, blindwood and stunting was noted for each tree. Mechanical harvesting damage to the trunk portion of the tree, if present, was noted as mild or severe. Trees were also rated for overall vigor. A rating of 1 was given to a non-vigorous, stunted or sparsely foliated or fruited tree, often with excessive bare spaces on the branches. A rating of 5 was given to an extremely vigorous tree with a even ratio of shoot growth to fruit spur formation. In all twelve orchards the incidence of replanted and dead trees within the plots was also noted.

RESULTS

Field Survey for PNRSV and PDV

Prunus Necrotic Ringspot Virus. The 1983 survey revealed widespread infection of commercial sour cherry plantings in Van Buren and Berrien counties by PNRSV. Prunus necrotic ringspot virus was detected by ELISA in all six decline and all six non-decline orchards in the study. Infection levels per orchard ranged from 1.02 percent to 99.4 percent of the trees tested, with eight of the twelve orchards having over 25 percent of the trees infected (Table 2.1). Within the selected 200-tree plots, the six decline orchards had an average of 62.48 percent of the trees testing ELISA-positive for PNRSV while the non-decline orchards had an average of 23.05 percent of the trees testing ELISA-positive for PNRSV. Chi-square analysis found the incidence of trees testing ELISA-positive for PNRSV in the decline and non-decline orchards to differ significantly (P=.001) from the expected 1:1 ratio (Table 2.2). Figure 2.1 shows the percentage of the individual trees infected with PNRSV in each orchard and the overall mean percentage of trees that were detected as ELISA-positive for PNRSV in the six decline and six non-decline orchards.

Prune Dwarf Virus. Table 2.3 shows the levels of trees ELISA-positive for PDV for each of the twelve orchards tested. Prune dwarf virus was present in seven of the twelve

Table 2.1 Incidence of trees ELISA-positive for Prunus necrotic ringspot virus in six declining and six non-declining sour cherry orchards in southwest Michigan.

Orchard Number	Orchard Status	# of Trees PNRSV positive / # of Trees Sampled	Incidence of Virus (%)
1	Decline	167/187	89.30
2	Decline	48/186	25.81
3	Decline	135/182	74.18
4	Decline	79/200	39.50
5	Decline	172/194	88.66
6	Decline	115/197	58.38
7	Non Decline	3/200	1.50
8	Non Decline	197/198	99.49
9	Non Decline	2/196	1.02
10	Non Decline	12/200	6.00
11	Non Decline	5/200	2.50
12	Non Decline	56/199	28.14

Table 2.2 Chi-square analysis of PNRSV incidence between decline and non-decline orchards.

Treatment Comparison	Observed[a]	Expected[b]	X^2	P
	Decline: Non Decline[c]			
PNRSV positive	716:275			
PNRSV negative	430:918	1:1	370.48[d]	<.001

[a]Observed values are for ELISA positives (+) and ELISA negatives (-) for the compared treatments summed over six decline plots and the six non decline plots.

[b]Null hypothesis: The number of diseased and healthy trees are independent of the decline and non-decline status.

[c]Decline treatments consisted of 1146 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six declining orchards. Decline treatments consisted of 1193 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six non-declining orchards.

[d]A significant X^2 indicated that PNRSV incidence is not independent of decline and non-decline status.

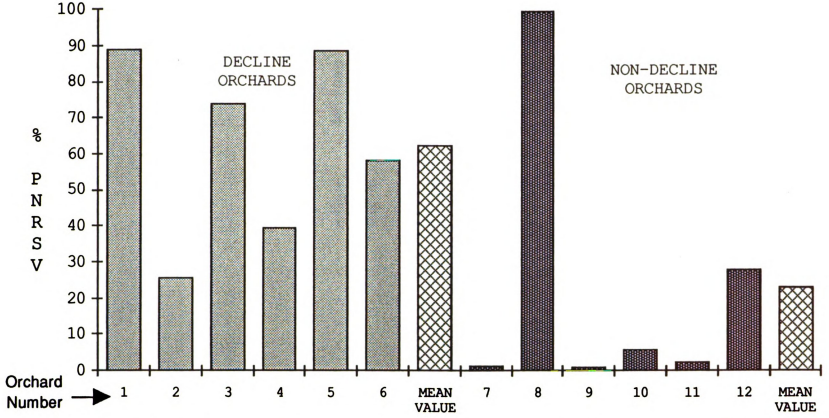

Figure 2.1 Percentage of trees ELISA-positive for PNRSV by orchard (200 trees per orchard) showing mean values of infection for decline and non-decline categories

Table 2.3 Incidence of trees ELISA-positive for prune dwarf virus in six declining and six non-declining sour cherry orchards in southwest Michigan.

Orchard Number	Orchard Status	# of Trees PDV positive / # of Trees Sampled	Incidence of Virus (%)
1	Decline	8/187	4.28
2	Decline	2/186	1.08
3	Decline	0/182	0.00
4	Decline	3/200	1.50
5	Decline	3/194	1.55
6	Decline	28/197	14.21
7	Non Decline	0/200	0.00
8	Non Decline	13/198	6.57
9	Non Decline	0/196	0.00
10	Non Decline	0/200	0.00
11	Non Decline	0/200	0.00
12	Non Decline	8/199	4.02

orchards tested. Prune dwarf virus was found at much lower levels than PNRSV, with 14.21 percent PDV infection being the highest incidence of the virus in any one orchard. Within the selected 200-tree plots, the six decline orchards had an average of 3.84 percent of the trees testing ELISA-positive for PDV while the non-decline orchards had an average of 1.76 percent of the trees testing ELISA-positive for PDV. Chi-square analysis found the incidence of trees testing ELISA-positive for PDV in the decline and non-decline orchards to differ significantly (P=.01) from the expected 1:1 ratio (Table 2.4). Figure 2.2 shows the percentage of the individual trees infected with PDV in each orchard and the overall mean percentages of the trees that tested ELISA-positive for PDV in the six decline and six non-decline orchards.

Tree Evaluation

Decline vs. Non-decline. It is useful to first compare some characteristics of the the tree evaluation data between decline orchards and the non-decline orchards without regard to virus infection. The total number of replanted trees in the decline orchards was 23.29 percent of the total number sampled, significantly higher than the non-decline figure of 2.96 percent (P =.001, chi-square procedure). Similarly, within the six 200 tree plots of the decline orchards a significantly higher percentage (4.8 percent) of the trees were dead, while in the six non-decline orchards only 0.75 percent of the trees were dead (P=.001, chi-square

Table 2.4 Chi-square analysis of PDV incidence between decline and non-decline orchards.

Treatment Comparison	Observed[a]	Expected[b]	X^2	P
	Decline: Non Decline[c]			
PNRSV positive	44:21			
PNRSV negative	1102:1172	1:1	8.85[d]	<.01

[a]Observed values are for ELISA positives (+) and ELISA negatives (-) for the compared treatments summed over six decline plots and the six non decline plots.

[b]Null hypothesis: The number of diseased and healthy trees are independent of the compared decline and non-decline status.

[c]Decline treatments consisted of 1146 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six declining orchards. Decline treatments consisted of 1193 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six non-declining orchards.

[d]A significant X^2 indicated that PDV incidence is not independent of decline and non-decline orchard status.

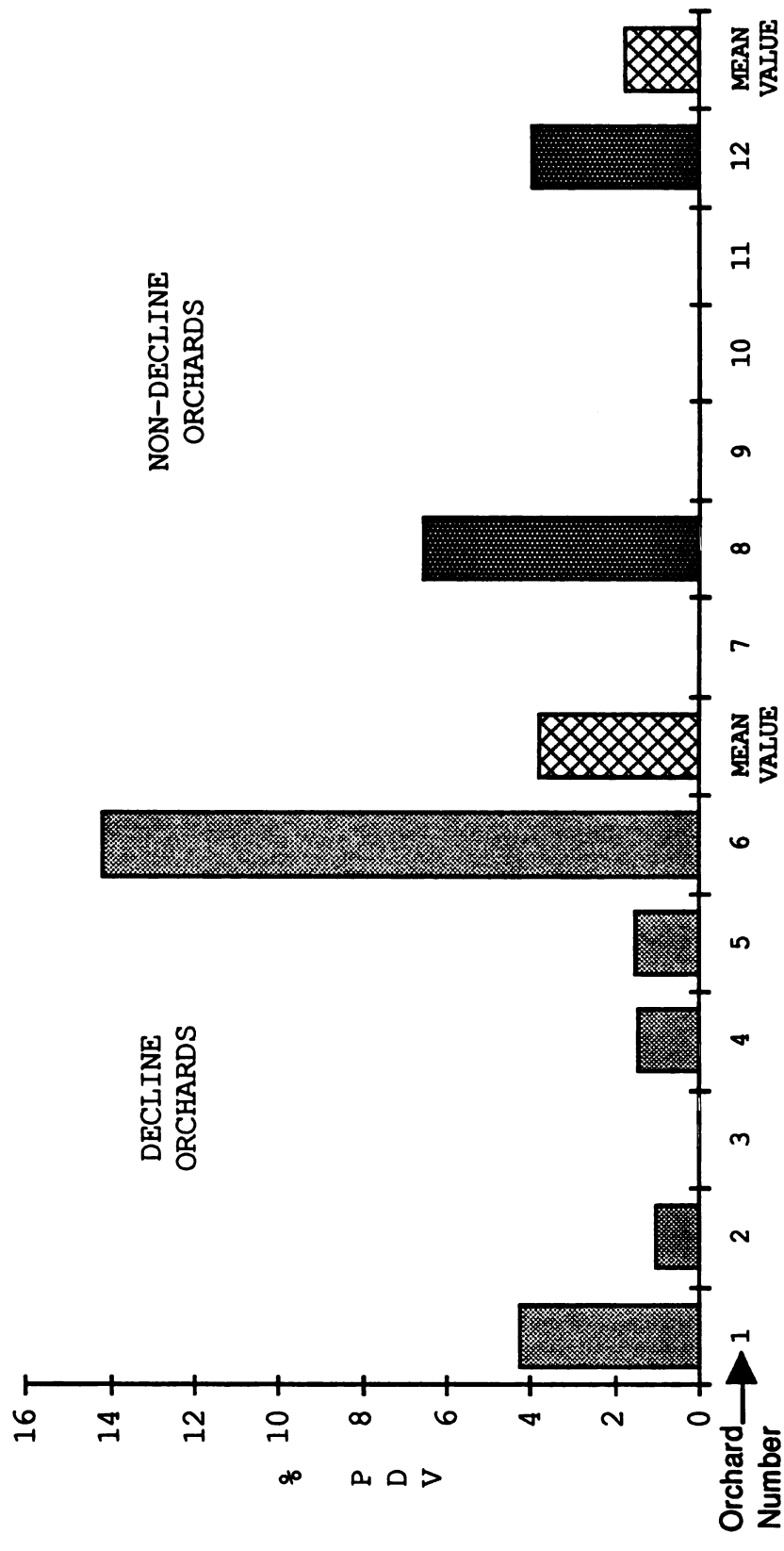

Figure 2.2 Percentage of trees ELISA-positive for PDV by orchard (200 trees per orchard) showing mean values of infection for decline and non-decline categories

procedure). Figure 2.3 compares the decline and non-decline orchards in terms of the amount of trunk damage. At all three levels - damage 1 (mild), damage 2 (severe), and total damage (mild and severe combined), the decline orchards had significantly more damage than the non-decline orchards (P=.001, chi-square procedure). In addition, the two tree evaluation characteristics of borer incidence and trunk or branch gummosis were significantly more prevalent (P =.001, chi-square procedure) in decline orchards than in the non-decline orchards.

The levels of blindwood and stunting in the decline and non-decline orchards are shown in Table 2.5. The decline orchards had significantly more blindwood than the non-decline orchards. The reverse was found to be true for stunting; the non-decline orchards were shown to have significantly more stunted trees than the decline orchards.

Comparisons of the mean vigor rating and the five individual vigor ratings, per orchard and between the decline and non-decline orchards, are shown in Table 2.6. No differences were seen between decline and non-decline in terms of the mean vigor rating. The percentage of trees given the lowest and the medium vigor rating (1 and 3) was significantly higher in the decline orchards. Significantly more trees given the high vigor ratings (4 & 5) were found in the non-decline orchards than in the non-decline orchards. When all the trees given the two low vigor ratings (1 & 2) are combined significance is shown at the P = .001 level (higher incidence for the decline orchards) and when all the

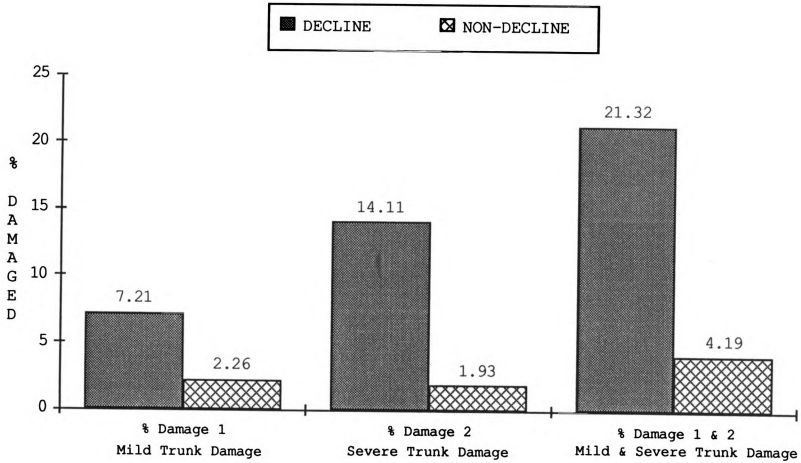

Figure 2.3 Trunk damage for 967 decline and 1193 non-decline orchard trees

Table 2.5 Percentage of stunting and blindwood for each of six decline and six non-decline orchards and mean incidence of stunting and blindwood for the decline and non-decline orchards overall.

Orchard[a] Number	% Of Trees with Stunting	% Of Trees with Blindwood
1	2.67	-
2	1.61	0.00
3	6.04	76.4
4	4.50	3.50
5	0.00	89.2
6	1.52	89.3
7	5.53	0.00
8	1.51	59.1
9	0.00	18.0
10	13.0	2.00
11	11.5	0.00
12	5.50	2.00
Orchard Status[b]	Mean incidence	Mean incidence
Decline	**3.23**	**51.62** (.001)
Non-Decline	**6.18**[c] (.01)	**13.45**

[a]Each orchard plot = 200 trees rated in 1983. Orchard 1 was not rated for blindwood.

[b]Decline orchards include orchards 1 to 6; non-decline orchards include orchards 7 to 12.

[c]Value significantly greater than the corresponding value (P=number in parenthesis) when incidence of tree evaluation characteristic is compared over orchard type by chi-square analysis.

Table 2.6 Tree vigor ratings[a] for six decline and six non-decline orchards.

Orchard Number[b]	Mean Vigor Rating	% Trees Vigor Rating "1"	% Trees Vigor Rating "2"	% Trees Vigor Rating "3"	% Trees Vigor Rating "4"	% Trees Vigor Rating "5"
1	2.88	25.65	8.38	41.88	0.00	24.08
2	3.38	0.00	0.00	81.11	0.00	18.89
3	3.40	13.92	8.76	38.14	2.06	37.11
4	3.71	6.63	3.57	44.39	2.55	42.86
5	-	-	-	-	-	-
6	2.91	14.21	0.00	75.96	0.00	9.84
7	4.02	1.00	1.00	45.50	0.00	52.50
8	2.84	18.69	1.52	68.18	0.00	11.62
9	3.93	0.00	0.51	52.53	0.00	46.97
10	4.00	5.50	6.00	24.50	11.50	52.50
11	2.94	13.64	4.04	69.19	3.03	10.10
12	3.93	3.02	6.03	45.23	0.00	50.25
Orchard status[c]						
Decline	**3.2**	**12.2**[d(.001)]	**4.2**	**55.7**[(.05)]	**0.1**	**27.0**
Non Decline	**3.6**	**7.0**	**3.2**	**50.8**	**2.4**[(.05)]	**37.4**[(.01)]

[a]Vigor rating system: 1 = tree excessively stunted; branches sparsely foliated,few fruit spurs 2 = tree slightly stunted; low fruit spur/shoot growth ratio 3 = tree growth average;slightly more shoot growth to fruit spur growth 4 = tree growth average to above average; nearly equal ratio of fruit spur/shoot growth 5 = tree growth above average; branches full; fruit spur/ shoot growth equal or better.

[b]Each field plot consisted of 200 trees; all live trees were rated for vigor in 1986. Orchard # 5 was not rated for vigor.

[c]Decline orchards (orchards 1 to 6) included 944 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchard (orchards 7 to 12) included 1193 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[d]Value is significantly greater (P= number in parenthesis) than corresponding value when the total incidence of each vigor level is compared over orchard status by chi-square analysis.

trees given the two higher vigor ratings (4 & 5) are combined, significance is also shown at the $P = .001$ level (higher incidence for non-decline orchards).

Prunus Necrotic Ringspot Virus. The same tree evaluation characteristics were considered with reference to the PNRSV survey data. The incidence of trees singly infected with PDV was too low overall to statistically associate the presence of PDV with any of the tree evaluation data. In addition, the age of the trees was considered in regard to virus infection. Figure 2.4 shows the variation of infection by both PNRSV and PDV viruses with age. These data were taken from six orchards in the study which had a variation in tree ages present.

Table 2.7 compares the stunting, blindwood,tree death and replant percentages for four different categories. One category, (A) compares the trees that tested ELISA-positive for PNRSV to the trees that tested ELISA-negative for PNRSV in the decline orchards. Another catergory, (B), compares the trees that tested ELISA- positive for PNRSV to the trees that tested ELISA-negative for PNRSV in the non-decline orchards. A third category, (C), compares all the decline orchards to all the non-decline orchards without regard to virus infection. The fourth category,(D), compares all the trees (decline or non-decline) that tested ELISA-positive for PNRSV to all the trees that tested ELISA-negative for PNRSV.

As Table 2.7 indicates, blindwood incidence was consistently and significantly higher for the decline orchards and for the PNRSV-ELISA positive trees. Similarily,

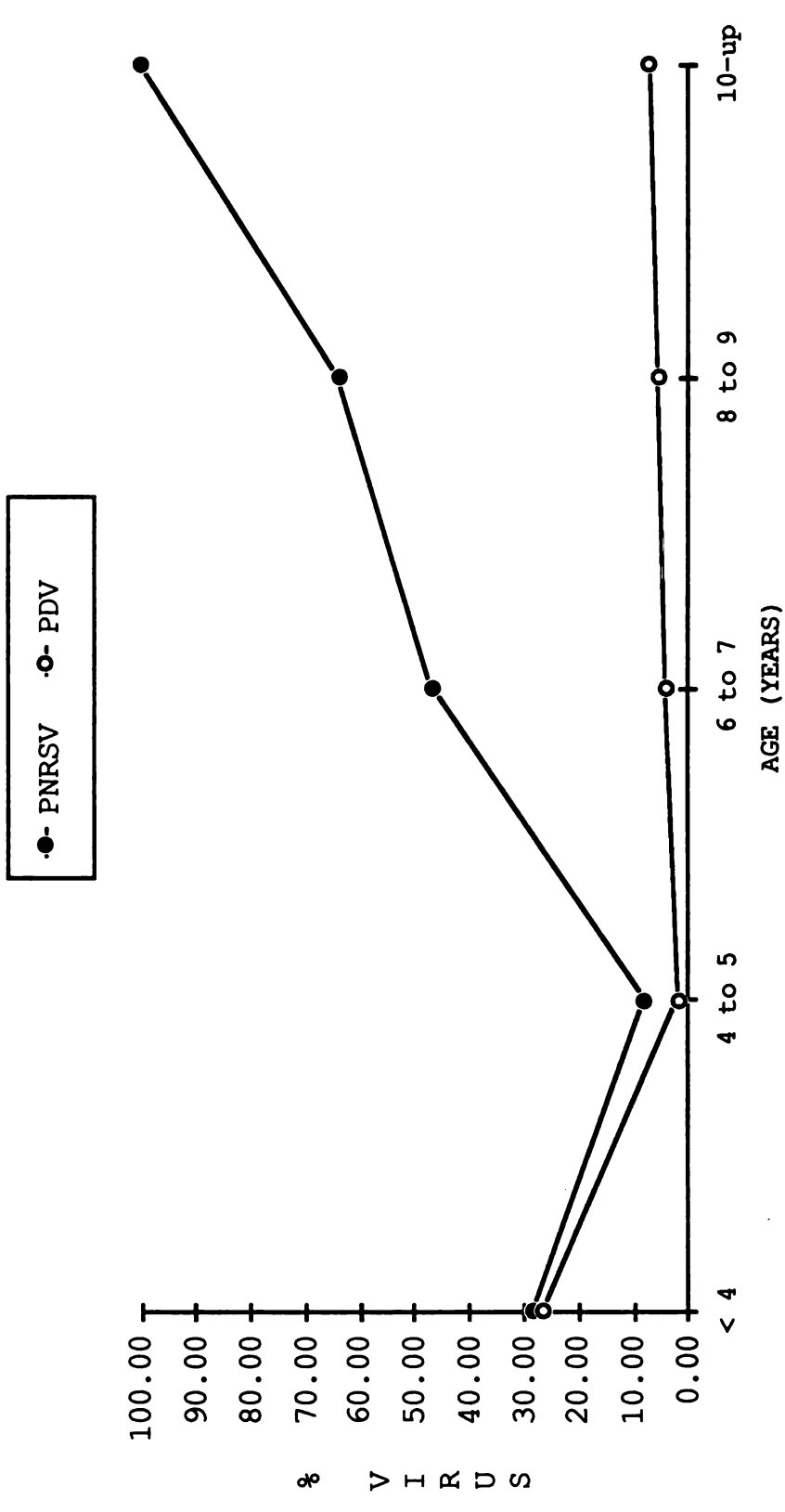

Figure 2.4 Percentage of PNRSV and PDV infection in 1200 trees by tree age

Table 2.7 Percent tree replant,stunting,blindwood,and tree death for trees ELISA-positive for PNRSV and trees ELISA-negative for PNRSV between and within decline and non-decline orchards.

	% Of Trees with:		% Of Trees:	
Category	Stunting	Blindwood	Dead	Replanted
(A)				
+[a]/decline[b]	2.65	96.17[c](.001)	2.76	18.20
-/decline	2.33	25.51	2.20	25.25(.05)
(B)				
+/non-decline	2.91	44.36(.001)	2.32(.05)	13.31
-/non-decline	7.19(.05)	4.25	0.11	2.94
(C)				
Decline PNRSV + & -	2.53	56.69(.001)	2.46(.001)	20.74(.001)
Non-Decline PNRSV + & -	6.20(.001)	13.50	0.42	3.10
(D)				
All PNRSV positives	2.73	78.83(.001)	2.32(.01)	13.31(.05)
All PNRSV negatives	5.63(.001)	10.24	0.75	9.80

[a]+ = PNRSV-ELISA positive, - = PNRSV-ELISA negative.

[b]Decline orchards included 944 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock;Non-decline orchards included 1193 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[c]Value is significantly greater than the corresponding values (P= number in parenthesis) when total incidence of a characteristic is compared within categories by chi-square analysis.

the amount of tree death per orchard was significantly higher for the decline orchards and for the trees with PNRSV when compared to the non-decline orchards and the PNRSV negative trees. Since the virus data and the tree evaluation data were taken in subsequent years, it was possible to record deaths of trees known to have tested ELISA-positive for PNRSV. The numbers of replanted trees was also found to be significant, with the numbers of replants in the declining orchards higher than in the non-declining orchards, but the relationship of replants to PNRSV ELISA-positive or ELISA-negative trees was inconsistent. Table 2.7 also indicates that incidence of stunting was significantly higher in the non-decline orchards and that the incidence of stunting was significantly higher for all the trees without PNRSV infection.

The comparison of trunk damage within the four categories is shown in Table 2.8. In terms of trunk damage, the non-decline orchards were significantly healthier (more trees rated damage = 0) than the decline orchards and significantly less damage was present in PNRSV-negative trees. The total damage incidence (mild and severe damage combined) showed damage to be positively associated with both decline orchards and with the presence of PNRSV infection.

Vigor ratings given to the orchard trees are compared within the four categories in Table 2.9. The incidence of tree vigor rating 3 was only significant in comparison (C), with the decline orchards having significantly more trees rated at this level than the non-decline orchards. The

Table 2.8 Percent trunk damage[a] on trees ELISA-positive and ELISA-negative for PNRSV between and within decline and non-decline orchards.

Category	% Trees Damage Category "0"	% Trees Damage Category "1"	% Trees Damage Category "2"	Total Damage Category "1 & 2"
(A)				
+[b]/decline[c]	70.22	8.45	18.75[d](.001)	27.20(.001)
-/decline	85.53(.001)	5.63	6.86	12.49
(B)				
+/non-decline	95.27	2.18	2.18	4.36
-/non-decline	95.32	2.29	1.85	4.14
(C)				
Decline				
PNRSV + & -	75.05	7.08(.001)	13.34(.001)	20.42(.001)
Non-Decline				
PNRSV + & -	95.31(.001)	2.26	1.93	4.19
(D)				
All PNRSV positives	78.63	6.34(.01)	13.18(.001)	19.52(.001)
All PNRSV negatives	92.31(.001)	3.32	3.39	6.71

[a] Trunk damage rating system : 0 = no visible trunk damage, 1 = visible mild trunk damage, 2 = visible severe trunk damage.
[b] + = PNRSV-ELISA positive; - = PNRSV-ELISA negative.
[c] Decline orchards included 944'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchards included 1193 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.
[d] Value is significantly greater (P = number in parenthesis) than the corresponding value when damage classes were compared within category by chi-square analysis.

Table 2.9 Tree vigor ratings[a] for trees ELISA-positive and ELISA-negative for PNRSV between and within decline and non-decline orchards.

Category	Mean Vigor Rating	% Trees Vigor Ratings 1 & 2	% Trees Vigor Rating 3	% Trees Vigor Ratings 4 & 5
(A)				
+[b]/decline[c]	2.23	22.61[d](.001)	51.84	22.06
-/decline	3.38	7.1	57.35	31.62(.01)
(B)				
+/non-decline	3.11	17.46(.001)	51.27	23.27
-/non-decline 44.55(.001)	3.73	3.15		48.04
(C)				
Decline PNRSV + & -	3.13	15.60(.001)	52.98 (.05)	25.56
Non-Decline PNRSV + & - 39.65(.001)	3.59	6.87		48.78
(D)				
All PNRSV positives	3.00	20.88(.001)	51.65	22.47
All PNRSV negatives 40.57(.001)	3.62	4.76		50.94

[a] Vigor rating system:1 = tree excessively stunted; branches sparsely foliated,few fruit spurs 2 = tree slightly stunted; low fruit spur/ shoot growth ratio 3 = tree growth average;slightly more shoot growth to fruit spur growth 4 = tree growth average to above average; nearly equal ratio of fruit spur/ shoot growth 5 = tree growth above average; branches full; fruit spur/ shoot growth equal or better.
[b] + = PNRSV-ELISA positive, - = PNRSV-ELISA negative.
[c] Decline orchards included 944'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchards included 1193 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.
[d] Value is significantly greater (P = number in parenthesis) than the corresponding value when vigor classes were compared within category by chi-square analysis.

incidence of poor tree vigor (ratings 1 and 2) was significant for the categories (C) and (D). Significantly more decline than non-decline trees and more trees ELISA-positive for PNRSV than trees ELISA-negative for PNRSV were rated at these lower levels. The incidence of the high vigor ratings (4 and 5) was shown to be significant in all four categories. Categories (A),(B) and (D) all indicated that significantly more PNRSV-free trees were given these high vigor ratings. Category (C) indicated that significantly more non-decline trees were given a 4 or 5 rating.

Nursery Survey for PNRSV and PDV

The results of the ELISA surveys for PNRSV and PDV at the four nurseries are shown in Table 2.10. Prunus necrotic ringspot virus or PDV was detected at three out of the four nurseries tested, though at generally lower levels than what was detected in the commercial orchards. Overall, 4.4 percent of the 1086 one-year old trees and rootstocks tested at the four nurseries were ELISA positive for either PNRSV or PDV. As in the commercial orchards, the majority of these (95.8 percent) were PNRSV-ELISA positive while considerably less (4.2 percent) tested PDV-ELISA positive. Figure 2.5 indicates the percentages of nursery samples ELISA-positive for either PNRSV or PDV. In addition, one out of the 460 total budwood source trees sampled at nursery B tested ELISA-positive for PDV.

Table 2.10 One-year old trees and rootstocks ELISA-positive for PNRSV or PDV at four nurseries.

Nursery	Number Sampled	Variety	Number PNRSV positive / Percent PNRSV positive	Number PDV positive / Percent PDV positive
"A"	120	Mont.[a]/Mazzard	0/0.0	0/0.0
	120	Mont./Mahaleb	0/0.0	0/0.0
	60	Mazzard	0/0.0	0/0.0
	60	Mahaleb	0/0.0	0/0.0
"B"	100	Mazzard	0/0.0	1/1.0
	100	Mahaleb	0/0.0	0/0.0
	30	Turkish Mahaleb	0/0.0	0/0.0
	259	Mont./Mahaleb	0/0.0	0/0.0
"C"	200	Mont./Mahaleb	35/17.50	0/0.0
"D"	46	Mont./Mahaleb	11/23.91	1/2.17

[a]Mont. = 'Montmorency'

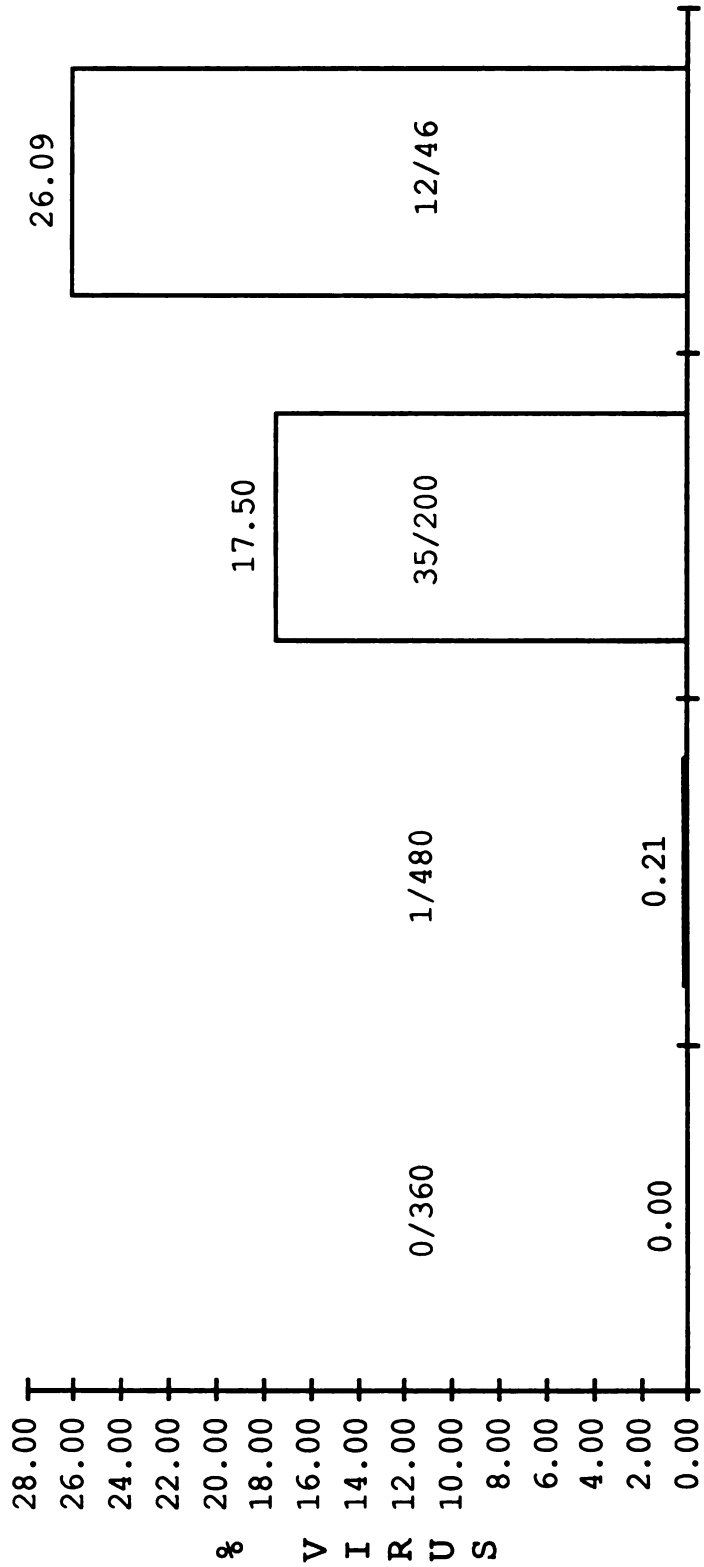

Figure 2.5 Percentage of nursery tree samples ELISA-positive for Ilar virus (either PNRSV or PDV)

DISCUSSION

Chi-square analyses indicated that there is a relationship between the decline and non-decline orchards and level of infection by two ilarviruses. The exact nature of this relationship however, was not entirely defined by the results of this study. For example, consider the level of PNRSV in field #8 in Figure 2.1. Although this was a fairly vigorous, productive non-declining orchard, the level of PNRSV infection was the highest of all twelve fields tested. While noting that this orchard could be an exception, it indicates, along with the presence of PNRSV in the other non-declining orchards, that PNRSV infection is probably not the sole factor in tree decline. Overall levels of PDV infection, while significantly higher in the decline orchards than in the non-decline orchards, were simply too low to account for the scope of the decline problem.

In addition, the age data presented in Figure 2.4 show how both the PNRSV and the PDV incidence of infection increase with tree age. This would be expected with any pollen-borne virus prevalent in the growing region. Aside from relatively high infection in the youngest tree age group (< than 5 years old and due to nursery infection) the older a tree is the more likely it is to be infected with either virus. In these six orchards at least, and for PNRSV in particular, any tree over 8 years of age was as likely to be infected as it was not.

The tree evaluation data give further insight to the possible nature of the relationship of PNRSV to decline. By comparing all decline and all non-decline orchards (Figure 2.3, Tables 2.5,2.6) it is seen that the overall health of the decline orchards is lower than that of the non-decline orchards, as would be expected. More trees in the decline orchard had died and had been replanted. Decline orchards also contained more trees of low vigor and less trees of high vigor than the non-decline orchards and more trees exhibiting blindwood. Levels of trunk damage were also significantly higher in the decline orchards than in the non-decline orchards. Incidence of borer injury and trunk gumosis, both factors which could be associated with trunk damage, were also significantly higher in the decline orchards than in the non-decline orchards. All of these findings validate the orchard selection and they characterize the overall decline and non-decline conditions.

When the tree evaluation data is considered in terms of the PNRSV survey, the relationship of virus to decline can be seen. To fully understand the results, however, some aspects of the four way comparison tables should be noted.

The comparison category (C) gives information on a given characteristic's relationship to the orchard decline or non-decline status. If, for any characteristic, the entire decline group is significantly different from the entire non-decline group, we can regard the decline status as being associated with the characteristic in some way. The comparisons of all the PNRSV positives to all the PNRSV

negatives without regard to decline or non-decline status [category (D)] gives similar information in terms of the influence of viral infection. It must be realized, however, that this last category can be biased. The group of trees ELISA-positive for PNRSV will in general contain more trees from the decline orchards since they have been shown to have a higher rate of infection with this virus. Any significance seen in the comparison of decline to non-decline [category (C)] could then be a contributing factor or 'tag along' in a comparison of the PNRSV positive group to PNRSV negative group [category (D)]. The other two comparison categories of PNRSV infection within decline [category(A)], and PNRSV infection within non-decline orchards [category(B)], can avoid this situation. In the decline comparison (A), for example, all trees are from the same orchard type; the only known difference is virus infection. A significance in this category then could indicate that PNRSV infection or lack of PNRSV infection could be associated with a particular characteristic. We can not, however, rule out other factors that may be occurring and contributing such as macro- and micro-site factors, environmental influences and various pathogenic interactions, viral or otherwise.

Tables 2.7 and 2.9 indicate that the characteristics of blindwood and tree vigor were both positively associated with PNRSV presence. Keitt and Clayton (1943) noted that PNRSV infection reduced spur formation in sour cherry. Blindwood - an excess of sparsely foliated or fruited growth - could be a similar effect of PNRSV infection. Other factors such as

pruning methods, nutrient status, and environmental conditions must be considered, however in interpreting the association of blindwood and PNRSV infection.

Overall reduction in vigor, in terms of reduction of growth and yield has been noted in sour cherry by Lewis (1951), Way and Gilmer,(1963) and Davidson and George (1965). Our results indicate that PNRSV infection was associated with those trees in the study that were of low vigor and lack of PNRSV infection was one characteristic of those trees that were very healthy and vigorous. It is likely the decline in vigor shown by the tree evaluation data for the PNRSV-positive trees and in the decline orchards is a part of the total decline syndrome. Perhaps the virus first initates lowered spur/fruit production and reduces overall vigor. The reduced vigor could then predispose the trees to invasion by other pathogens and weaken the ability of the tree to withstand unfavorable abiotic conditions.

To look directly at possible yield reduction, another characteristic often associated with PNRSV (and PDV) infection in sour cherry, was out of the scope of this project. The best indirect way to approach yield through the context of this study was through the combination of blindwood incidence and overall tree vigor. High blindwood/low vigor could indicate that the fruiting system of the tree was reduced. The incidence of high blindwood and lower vigor occurred significantly in both the decline versus the non-decline orchards overall and in the PNRSV positive trees from decline orchards versus the PNRSV negative trees

from decline orchards. The decline orchards and the trees that tested positive for PNRSV would then be expected to have lower fruit production than the non-decline orchards or the trees that tested negative for PNRSV. This lowered production would have resulted from the decreased fruit set on infected trees or the high incidence of trees of low vigor, which had a higher rate of vegetative growth than fruit spur formation.

The tree evaluation data also reveal interesting information about trunk damage. Overall, the decline orchards had significantly more trees with trunk damage than the non-decline orchards. In addition, the overall damage that occurred in the decline orchards was more severe that the overall damage to trees in the non-decline orchards. These findings indicate that trunk damage could be a part of the decline syndrome. Since within both the decline and non-decline categories there was significantly higher incidence of trunk damage for the trees with virus; trunk damage was also associated with PNRSV infection.

The trunk damage rated for this study was damage caused by mechanical harvesting machines. Brown et al. (1982) have shown that damage to sour cherry by harvesting equipment is due to improper clamp pressures, decreased bark strength and exacerbated by high water levels in the bark. Although it is not known to do so, physiologically, PNRSV may have affected the trees in such a way as to promote trunk damage. Similarly, the virus may have induced lowered vigor which then decreased the trees ability to withstand any damage.

Yet, it is also possible that the lower vigor seen for the decline orchards and for the PNRSV infected trees is partly due to the overall higher levels of trunk damage from these same groups. Without a full investigation of many interacting factors on a tree by tree basis it is not possible to separate all the implications of trunk damage and its' relationship to virus and vigor. Perhaps, other research in progress on this issue will further elucidate the relationship of these factors and decline.

Stunting was positively associated with the trees without PNRSV infection as well as with the non-decline orchards. It should be noted however, that the total number of trees affected was relatively low. The declining orchards would have been expected to have more stunting than the non-decline orchards. It is possible that more of the stunted trees in the decline orchards had been removed, since the levels of replants for the decline orchards were much higher. The association of stunting with PNRSV-negative trees is not in agreement with Milbrath (1950) who showed an association of virus free trees with taller and sturdier growth. Millikan (1955) however, noted that although a growth reduction occurred with PNRSV infection, it was due to a reduction of the number of branches and not due to stunting. Further investigation of micro-site factors - soil type and structure, soil and root levels of nematodes - as well as concise differences in branch growth and tree height would be needed to reveal other causal factors relevant to this finding.

In considering PNRSV and PDV in relation to the decline problem it is important to note that both viruses have been known to be present in Michigan cherry plantings since the trees were first planted in the state. The decline phenomenon is only recent, however, and this indicates that other factor(s) may be invloved. Although early decline symptoms and high levels of infection by PNRSV may occur at relatively the same age in cherry, currently there is no evidence to directly and singularily correlate this virus with the decline syndrome, mostly due to other possible influencing/ interfering characteristics.

It has been proven that both PDV and PNRSV have a deleterious effect on tart cherry growth and yield (Rassmussen and Cation, 1942; Parker et al.; 1959; Cochran et al., 1950). It was shown by this study that the declining orchards are more severely infected with both viruses than were the non-declining orchards, which suggests that the presence of virus and declining orchard status is related. It is likely that virus - PNRSV particularily - plays a part, perhaps as a predisposition agent, in the decline problem. Prunus necrotic ringspot virus, acting to induce overall low vigor, could also be an important contributing factor in a cumulative disease process. Perhaps what alone is a slight problem or strain to a tree becomes, in conjunction with enviromental, cultural or other pathogenic factors, a more lethal component.

There was a large variation (from zero to 26.09 percent) of virus infection levels between the nurseries

tested. At nursery A, no virus was detected in the 240 1-year-old trees tested or in 120 rootstocks, while nursery D had a relatively high incidence of virus infection of 12 out of a total of 46 1-year-old trees tested. When questioned, the nursery operators reported that a variety of virus control measures (including none) were practiced at each nursery when obtaining budwood for propagation purposes, which could account for the wide variation in virus infection. Operators at nursery C, for example, reported a complete lack of testing of budwood for either PNRSV or PDV prior to selection and the same was suspected but unconfirmed for nursery D. One of the primary sites of budwood for Nursery C was an orchard included in the field survey of this study which had a 74 percent PNRSV infection rate. The nursery operators claimed to use only healthy trees from this orchard for budwood but the variability of, or the complete lack of symptomatic tissue on PNRSV-infected trees would make it difficult to avoid infected trees. In general, all of the trees in commercial orchards that were planted from nursery stock from either nursery C or D would be expected to have higher PNRSV or PDV infection at an earlier age, since the infected stock would serve as a local inoculum source.

The nursery controls at nursery A and B, in contrast, included testing the budwood trees at regular intervals and obtaining budwood trees from virus-free certified nurseries. Equally important was that these nurseries controlled virus in their rootstocks and/or purchased similarily certified roostock materials. As shown in a study by Fleisher et al.

(1964) of 'Mazzard' and 'Mahaleb' rootstocks, certification of rootstock is important for control of these viruses. In that study, levels of infection of rootstock by PNRSV, for example, varied from 0.2 to 35 percent in unregistered seedlings while the highest level of PNRSV detected in registered seedlings was 2.7 percent.

The orchard industry's general premise that all sour cherry plantings in southwest Michigan will eventually be infected with PNRSV may be somewhat true, yet as shown in this study the amount of infection at earlier ages is markedly different for some orchards. The orchards planted with infected stock are disadvantaged since both viruses have been shown to cause reduced vigor and stunting in young trees. Both Millikan (1955) and George and Davidson (1965) have shown that the effect on growth and vigor is more severe the younger the trees are at the time of infection. Cleaning up all nurseries would improve the chances of giving these trees a better start, even though the current levels of virus in established plantings cannot ensure a virus-free life. Controls such as purchasing virus free nursery stock and treating young trees with gibberellin in the first years of flowering to prevent early infections (Hull and Klos, 1962) should be implemented. The most effective control of planting disease resistant stock is not yet available but preliminary work in Washington state by Howell and Mink (1988) may eventually lead to biological control of PNRSV. These researchers have begun studies in sweet cherry with an apparently non-pathogenic strain of PNRSV which may offer a

form of natural protection from PNRSV infection. Such a possibility increases the importance of attempts to prevent virus infection in the nursery and in the orchard.

III. TOMATO RINGSPOT VIRUS

MATERIALS AND METHODS

Field and Nursery Survey for TmRSV

Experiments were conducted to investigate the level of TmRSV in declining and non-declining cherry trees in southwest Michigan. Within each of the twelve 200 tree plots selected for PNRSV and PDV testing, fifty trees were selected in a grid pattern for TmRSV testing. Cambial samples were taken from each tree at or below the graft union. A 1.0 cm diameter boring tool was used to remove circular bark pieces from each tree. Four samples per tree were taken at equidistant locations around the tree. The borer cylinder was hammered through the bark and the cylinder of tissue was removed. The cylinder of tissue consisted of an outer bark piece which was discarded and the cambial tissues which were collected. In addition any cambial tissue remaining on the exposed wood of the tree trunk was collected. Samples were rinsed with water and the four samples per tree were combined and placed in cold PBS-Tween/PVP buffer [approximately a 1:5 dilution (v/w)] and stored on ice.

At three area nurseries, cherry trees were tested for the presence of TmRSV. At one nursery, 60 trees consisting of 'Montmorency' on 'Mazzard' rootstock and 60 trees consisting of 'Montmorency' on 'Mahaleb' rootstock were tested. At the second nursery two hundred trees consisting of 'Montmorency' on 'Mahaleb' rootstock were tested and at a third, 200 trees consisting of 'Montmorency' on 'Mazzard'

rootstock and 200 trees consisting of 'Montmorency' on 'Mahaleb' rootstock were tested. The trees were all 1 or 2 years old, and sampling was done with a 0.5 cm diameter boring tool due to the small diameter of the trees.

Tree Evaluation

As noted in Chapter 2, each tree was evaluated for various growth and health characteristics. Trees were rated for presence of blindwood and stunting; mechanical harvesting damage to the trunk portion of the tree, if present was noted as mild or severe. Trees were also rated for overall vigor, with a rating of 1 given to a non-vigorous, stunted or sparsely foliated or fruited tree, often with excessive bare spaces on the branches. A rating of 5 was given to an extremely vigorous tree with an even ratio of shoot growth to fruit spur formation. In each orchard the number of dead and replanted trees was noted. These various features of each tree were then compared in terms of orchard status (decline and non-decline) and TmRSV presence.

Distribution of TmRSV within Cherry Trees

Studies were initiated to investigate the distribution of TmRSV within individual cherry trees. Six trees that had tested ELISA-positive for TmRSV were selected for testing for TmRSV distribution. On each tree, cambial samples were removed from the north, south, east and west sides of the trunks, at or below the graft union. Samples were also taken in 5 cm increments along the trunk with five samples taken

above and five samples taken below the graft union. Each sample was assayed separately. In addition, six to eight young leaf samples and four root samples were taken from each tree. If available, sucker leaf tissue was also sampled.

In another distribution test, 150 greenhouse-grown 2-year-old trees that had been inoculated with a TmRSV infected bark chip were tested 9 months post-inoculation. The infected bark chips were obtained from mature cherry trees naturally infected with TmRSV. On each inoculated 2-year-old tree, four 0.5 cm diameter cambial tissue samples were taken near the graft location; four to six leaf and flower bud samples and one to three root samples were also taken.

In another study, five trees that had previously tested TmRSV-positive were re-tested at regular intervals over a period of 68 days. Four cambial tissue samples were combined for each tree on each test date.

TmRSV, Dandelion and Nematode Study

At each of three decline and three non-decline orchard plots, five TmRSV-positive and five TmRSV-free trees were selected to study the relationship between the infection of trees and surrounding dandelion by TmRSV and the population density of *Xiphinema* species. At each orchard, the dandelion populations around the 10 trees were sampled for ELISA testing for TmRSV. Six dandelions at the dripline of the tree canopy and four near the trunk of the tree were selected at each site. One to five leaf and flower samples and one to

three root samples were combined for each dandelion tested.

Soil and tree root samples were also taken at each tree. Young feeder roots were taken from three separate locations approximately 6 to 12 inches below ground and tested by ELISA for TmRSV. Soil samples for each tree were made up of eight subsamples equally distributed around the tree. In the laboratory, 100 cc-portions of each soil sample were processed for nematode counts by the density gradient centrifugation-flotation technique (Jenkins, 1959). Extracted nematodes of the species *Xiphinema*, *Criconemella*, and *Pratylenchus* were identified and counted with the aid of a dissecting microscope. Endoparasitic nematodes were extracted by the Shaker technique (Southey, 1970), detailed as follows: One gram of washed roots was suspended in a 50 ml mix of mecuric chloride (10ppm) and dihydrostreptomycin sulfate (50ppm) and then shaken at 100 rpm for 48 hours. The extracted *Pratylenchus* species were identified and counted.

Symptom Expression Study

Three decline fields were studied to compare symptom expression with detection of TmRSV. In each of the three fields, the fifty trees that were tested for TmRSV were examined for typical stem pitting and grooving symptoms associated with PSP in peach. An oval patch of bark approximately 3 cm by 5 cm was cut out from each tree and the bark peeled back to reveal the inner bark. Invaginated pitting and longitudinal grooves were recorded if present.

ELISA Procedure for TmRSV

In all cases the samples to be tested for TmRSV were kept cold throughout the collection, storage and virus assay procedure. All bark, root and leaf samples were assayed within 6 hours of collection. Bark and root samples were cut into small pieces, diluted 1:5 (w/v) in PBS-Tween/PVP buffer (extraction buffer), and ground up with a Tekmar Tissumizer or with a mortar and pestle, and then strained through cheesecloth. Cherry orchard dandelion leaf samples were diluted 1:10 (w/v) in extraction buffer and ground up with a Tissumizer.

Samples were assayed for TmRSV by $F(ab')_2$ ELISA (Barbara and Clark, 1982), in flat-bottomed polystyrene Microelisa® substrate plates with 96 wells per plate. Antiserum to TmRSV was prepared from virus purified from infected raspberry tissue (See page 85 for details). The $F(ab')_2$ ELISA procedure consisted of the following steps. **1)** 200 µl of 1.0 mg/ml TmRSV-IgG-$F(ab')_2$ fragments in sodium carbonate buffer, pH 9.6, were placed in all 96 wells. The plates were then incubated in plastic bags for 4 hours at 37° C. **2)** Plates were emptied and rinsed with a phosphate buffered saline solution, pH 7.4 with 0.5 % (v/v) polyoxyethylene sorbitan monolaurate (Tween 20). This procedure was repeated at least three times with at least 3 minutes between rinses. **3)** A 200 µl sample of tissue extract was placed in each plate well. Each plate included four to eight healthy control wells, two to three diseased control

wells and one to two wells containing extraction buffer only. The remaining wells were filled with test samples, which were each replicated twice on the same Microelisa® plate. Filled plates containing sample extracts were stored in plastic bags at 5° C overnight. **4**) After rinsing as per step 2, 200 µl of a solution containing 1.5 µg/ml of TmRSV IgG in extraction buffer was placed in each well. The plates were then incubated in plastic bags for 3 hours at 37° C. **5**) After rinsing as per step 2, 200 µl of horseradish peroxidase-labeled protein-A conjugate (Zymed Laboratories, So.San Francisco, CA 94080) at a concentration of 2.0 µg/ml in extraction buffer was added to each well. The plates were then incubated in plastic bags for 3 hours at 37° C. **6**) After rinsing as per step 2, 200 µl of a solution containing freshly prepared O-phenylene diamine (dihydrochloride) (Sigma P-3808 Sigma Chemical Co, St Louis, MO 63178) at a concentration of 0.7 mg/ml in substrate buffer (7.3 g Na_2HPO_4, 5.11 g citric acid, 0.4 mls H_2O_2) was added to each well. Plates were incubated at room temperature and scored visually and spectrophotometrically for an amber color reaction. Absorbance was measured at 490 nm with a Microelisa® minireader. Plates were zeroed on an empty well and readings were made of each plate at 30 minutes and 60 minutes after substrate addition. Photometrically, samples were considered ELISA-positive if the mean absorbence at 490 nm of each of the duplicate sample wells was greater than the mean A_{490} nm value of the healthy control samples plus three

standard deviations.

Assay Comparisons for TmRSV Detection

Variations of enzyme linked immunosorbent assay (ELISA) were evaluated for use in detecting TmRSV in plant tissues. In 1985, the double antibody sandwich (DAS) form of ELISA (Clark and Adams, 1977) was compared to the heterologous antiglobulin double antibody sandwich (HADAS) form of ELISA (Voller et al, 1978; Bar-Joesph and Malkinson, 1980) and to an indirect method of ELISA using $F(ab')_2$ fragments of immunoglobulin (Barbara and Clark, 1982). In 1987, the $F(ab')_2$ method was compared to the dot-ELISA (Banttari and Goodwin, 1985) method using antibody coated nitrocellulose membranes.

Virus Preparation. Tomato ringspot virus was obtained from field-infected raspberry (Rubus idaeus L.) plants. Cultures of TmRSV were maintained under greenhouse conditions in 'National Pickling' cucumber (*Cucumis sativus* L.) and *Chenopodium quinoa* Willd.

Tomato ringspot virus was purified for ELISA and serum production using 'National Pickling' cucumber as the propagation host. The purification procedure used was developed from a variation of a protocol by Dias (1975). All procedures were carried out at 4^{o} C. One hundred grams of infected tissue was homogenized in a cold waring blender in 250 ml of 0.05 M phosphate buffer, pH 8.0, containing .02 M 2-ME and .02 M ascorbic acid. The homogenate was then

squeezed through four layers of cheesecloth and the mixture was then centrifuged in an IEC no 872 rotor at 10K rpm for 20 minutes (low speed centrifugation). Ammonium sulfate (15 percent w/v) was added to the supernatant. The mixture was stirred overnight and was then given a second and third low speed centrifugation. The virus particles in the supernatant were then pelleted by ultracentrifugation in a Beckman no. 30 rotor at 28K rpm for 2.5 hours. Pellets were resuspended in 1.0 ml of 0.01 M phosphate buffer, pH 7.5, and stored at 4° C overnight. The virus was further purified by another low speed centrifugation, and clarified by a high speed centrifugation (Beckman 40 rotor ,38K rpm 90 min) of the resulting supernatant. The resulting pellets were then resuspended in 200 µl of 0.05 M phosphate buffer, pH 7.5 and stored at 4° C overnight. Linear log sucrose density gradients (0 -30 %) were prepared with buffer and left to equilbrate overnight. Sucrose gradients were loaded with 0.6 ml of virus preparation per tube and centrifuged in Beckman SW-41 rotor at 38 K rpm for 90 minutes at 4° C. Gradients were fractionated and the virus band collected using an ISCO density gradient fractionator and UV analyzer (Instrumentation Specialities Co., Lincoln NE 68504). The sucrose fractions containing virus were diluted 3:1 (v/v) with .01M sodium phosphate buffer and centrifuged in Beckman no.40 rotor at 38K rpm for 4 hours. Pellets were resuspended in 0.01 M phosphate buffer pH 7.5, using a glass rod and allowed to stand overnight. Concentration of the virus

preparation was determined at A_{260nm} using a Gilford spectrophotometer.

Root and cambial tissues of sour cherry used in these experiments were sampled as previously mentioned (pages 77 and 78). Leaf and flower tissue from *Chenopodium quinoa*, 'National pickling' cucumber and field grown dandelions and roots from dandelions were also sampled. Samples consisted of 0.5 to 1.0 gm of tissue in various dilutions of cold extraction (for ELISA) or grinding buffer (for dot-ELISA). The tissue was ground by hand with a mortar and pestle or with a Tissuemizer. After grinding, samples were strained and stored at 4° C until further use.

Antiserum Production. Tomato ringspot virus antiserum was produced in New Zealand white rabbits or in White Leghorn hybrid laying hens. Rabbits were immunized by intramuscular injections of 1.0 ml solutions (0.5 mg/ml TmRSV) emulsified (1:1 v/v) in Freund's complete adjuvant (Difco Products Co., Detroit, Mi 48232) for the first injection and Freund's incomplete for subsequent injections. A total of three injections were made on a 10-12 day schedule. Rabbits were bled from the marginal ear vein one week after the last injection and at 7 to 10 day intervals for a total of four bleedings. Rabbit serum used was from the third or fourth bleeding. The blood was collected and left for 3 hours at room temperature. The clear serum was pipetted off and stored at 4° C until further use. Serum titers were determined by gel double diffusion tests [8% agarose (Sigma

type I)] using TmRSV infected cucumber as the test antigen.

Chickens were immunized by two separate wing vein injections (70 ug/ml and 100 ug/ml TmRSV respectively) on day 1 and day 6 and one intramuscular breast injection on day 13 (700 ug/ml TmRSV, using Freunds' Incomplete adjuvant). A total of 10 eggs were collected on days 17, 20, 23, 28, 31, 32, 35, 37 and 39. Antibody used was from the yolk of eggs collected on days 32, 35 and 37 and combined.

Immunoglobulin Preparation. Anti-TmRSV-globulins (IgG) from immunized rabbits were prepared by precipitation from antisera with ammonium sulfate (Clark and Adams, 1977). Ten ml of saturated ammonium sulfate was added dropwise to 1:10 dilution (v/v) of serum in distilled water and mechanically stirred for 30 to 60 minutes. After centrifugation at 6K rpm (Beckman no.40 rotor) for 5 minutes, the pellet was collected and resuspended in 2.0 ml (v/v) half-strength PBS. This globulin fraction was dialyzed three times against 500 ml half-strength PBS and passed through a 5 cm column of DEAE-cellulose (Sigma) in a 10 ml glass pipette. Half-strength PBS was used to elute the gamma-globulin fraction from the column. Two ml protein fractions were collected by monitoring the effluent spectrophotometrically at A_{280nm}. The first fractions with a peak absorbing at A_{280nm} were collected and spectrophotometrically adjusted to a 1.0 mg/ml concentration.

Anti-TmRSV-chicken globulins (IgG) were obtained from the eggs of immunized hens as described by Bar-Joesph and

Malkinson (1980). Egg yolks were mixed with PBS, (20ml/yolk) and centrifuged (10K, 20min). The supernatant was mixed 1:1 (v/v) with glycerol for storage at -20°C.

The $F(ab')_2$ fragments of immunoglobulin were prepared by pepsin digestion. Rabbit-produced anti-TmRSV-IgG (1µg/ml in 0.07 M sodium acetate pH 4.0 containing 0.05 M NaCl) was digested by the addition of pepsin (Sigma) diluted 1:1000(v/v) in distilled water (45 mg/ml) and incubated overnight at 37° C. The $F(ab')_2$ fragments were separated from the products of digestion by dialysis against three changes of PBS.

Conjugation of Immunoglobulin. Alkaline phosphatase (AP) (Sigma Type VII-S) and hydrogen peroxidase (HRP)(Sigma) was coupled to gamma-globulin by the gluteraldehyde method of Avrameas (1969) as described by Clark and Adams, (1977). A 1.0 ml aliquot of AP or HRP was centrifuged at 6K rpm in a Beckman no. 40 rotor for 5 minutes. To the pellet, 1.0 ml of purified anti-TmRSV gamma-globulin was added followed by dialysis three times against PBS, pH 7.4. Conjugation of enzyme to the IgG-fraction was facilitated by the addition of glutaraldehyde (Sigma,electron microscope grade) to a final concentration of 0.05 % (v/v) for 4 hours at room temperature. Glutaraldehyde was removed by dialysis against PBS three times and conjugated-anti-TmRSV-IgG was stored with 1.0 % bovine serum albumin (BSA) (w/v) at 40° C.

Hydrogen peroxidase-labeled rabbit-anti-chicken-IgG was obtained from U.S. Biochemical Co. (Cleveland, OH 44122).

Protein A, (from *Staphylococcus aureus*) conjugated to HRP and AP was purchased from Cooper Biomedical (Malvern, PA 19355) and Zymed Laboratories (So.San Francisco, CA 94080), respectively.

DAS-ELISA. Assays were performed as described on page 42, however, both AP- and HRP-conjugated-IgG systems were tested. For the AP system, the coating antibody was used at 0.5 µg/ml, and for the HRP system the coating antibody was used at 1.0 µg/ml. Both conjugates were used at a dilution of 1/800 (v/v) in extraction buffer. The substrate for the HRP-labeled antibody was O-phenylene diamine in OPD substrate buffer. Plates were read after 15 and 30 minutes using a Dynatech Microelisa® minireader with a 410 nm (AP) or 490 nm (HRP) filter.

HADAS-ELISA. The assay procedure was carried out in Microelisa® polystyrene plates and consisted of the following steps: **1)** A 200 µl solution containng 1.0 µg/ml rabbit-produced anti-TmRSV-IgG in sodium carbonate buffer, pH 9.6 was placed in each well. The plate, wrapped in plastic was incubated at 37° C for 1.5 hours. **2)** Plates were emptied and rinsed with a phosphate buffered saline solution, pH 7.4 with 0.5 % (v/v) polyoxyethylene sorbitan monolaurate (Tween 20). This procedure was repeated at least three times with at least 3 minutes between rinses. **3)** A 200 µl sample of tissue extract was placed in each plate well. Each plate included four to eight healthy controls wells, two to three diseased control wells and one to two wells containing extraction buffer only. The remaining wells were filled with

test samples, which were each replicated twice on the same Microelisa ® plate. Filled plates containing sample extracts were stored in plastic bags at 5° C overnight. **4)** After rinsing as per step 2, a 200 µl sample containing a 1:500 (w/v) dilution of chicken-produced anti-TmRSV-IgG in PBS containing 2.0 % BSA was placed in each plate well. The plate, wrapped in plastic was incubated at 37° C for 1.5 hours. **5)** After rinsing as per step 2, a 200 µl sample containing HRP-conjugated rabbit anti-chicken-IgG diluted 1:1000 (v/v) in PBS was placed in each plate well. The plate, wrapped in plastic was incubated at 37° C for 1.5 hours. **6)** After rinsing as per step 2, a 200 µl sample containing a solution of O-phenylene diamine (0.7 mg/ml) in OPD substrate buffer was place in each plate well. Plates were allowed to incubate and after 15 and 30 minutes were read using a Dynatech Minireader equipped with a 490nm filter. Photometrically, samples were considered ELISA-positive if the mean absorbance at 490nm of the duplicate sample wells was greater than the mean A_{490} nm value of the healthy control samples plus three standard deviations.

F(ab')$_2$ ELISA. The protocol for the F(ab')$_2$ assay was reported on page 81. In addition, a similar system using AP-conjugated protein-A was tested. The enzyme substrate for the AP-protein-A moeity was a solution of p-nitrophenyl phosphate (1.0 mg/ml) in substrate buffer [10% diethanolamine (v/v) ,pH 9.8].

Dot-ELISA. The method of Bantarri and Goodwin (1985) was used. The assay was carried out on nitrocellulose membranes (NCM BioRad 4.5 µm, BioRad Laboratories Richmond, CA 94804). All wash steps consisted of five six-minute rinses on a gyratory shaker in a solution of Tris-buffered saline containing 0.05 % Tween 80 (TBS-T80 buffer). All incubations were in a covered pyrex dish. The NCM was handled with forceps or with gloves. Assay steps were as follows: **1)** The NCM was incubated four hours at room temperature with a solution of 60 µl anti-TmRSV-IgG in 60 ml sodium carbonate buffer, pH 9.6.(a 1:1000 v/v dilution) **2)** The NCM was washed and then transferred to 60 ml TBS-T80 containing 3.0 % BSA (w/v) (blocking step) and incubated 1.5 hours at 37° C. **3)** Sample extracts [tissue ground in TBS-T80 containing 10 % PVP (grinding buffer)] were added to the NCM which was placed in a BioRad Bio Dot® apparatus. Samples were incubated overnight at 4° C. **4)** After removal from the apparatus, the NCM was washed and then transfered to 60 ml of a 1:800 (v/v) solution of AP-conjugated anti-TmRSv-IgG in TBS-T80 and incubated 3 hours at room temperature. **5)** The NCM was again washed, then incubated for 30 minutes to 1 hour in 60 ml of a substrate solution [6.0 mg/ml fast red salt (Sigma)] in 0.2M Tris HCL pH 8.2, filtered,combined in a 1:1 ratio with 0.1 % AS-MX phosphate (Sigma) in 0.2 M Tris HCL. **6)** The NCM was then rinsed with distilled water, air dried or blotted dry and read visually for color reaction. Samples were considered positive if a red to pink dot appeared and negative if no dot appeared.

RESULTS

Field and Nursery Survey for TmRSV

Tomato ringspot virus was detected in trees from eight out of the twelve southwest Michigan orchards sampled. The highest level of infection was in orchard number three, a declining orchard, where 50 percent of the trees tested ELISA-positive for TmRSV. Incidence of TmRSV for the individual orchard blocks is shown in Table 3.1. The six decline orchards had an average of 23.67 percent of the trees that tested ELISA-positive for TmRSV while the non-decline orchards had an average of 9.67 percent of the trees that tested ELISA-positive for TmRSV. Chi-square analysis found the incidence of trees testing ELISA-positive for TmRSV in the decline and non-decline orchards to differ significantly (P=.001) from the expected 1:1 ratio (Table 3.2). Figure 3.1 shows the percentage of trees infected in individual orchards and the overall mean percentage of trees that were detected as ELISA-positive for TmRSV in the six decline and six non-decline orchards.

No TmRSV was detected in any of the 720 nursery trees tested at the three area nurseries.

Tree Evaluation

The individual tree evaluation data can be considered in relation to TmRSV infection. Table 3.3 compares the stunting, blindwood, tree death and tree replant percentages

Table 3.1 Incidence of trees ELISA-positive for tomato ringspot virus in six declining and six non-declining sour cherry orchards in southwest Michigan.

Orchard Block Number	Orchard Condition	Number of Trees TmRSV positive / Number of Trees Sampled	Incidence of Virus(%)
1	Decline	23/50	46.00
2	Decline	9/50	18.00
3	Decline	25/50	50.00
4	Decline	2/50	4.00
5	Decline	8/50	16.00
6	Decline	4/50	8.00
7	Non Decline	4/50	8.00
8	Non Decline	8/50	16.00
9	Non Decline	0/50	0.00
10	Non Decline	0/50	0.00
11	Non Decline	10/50	20.00
12	Non Decline	7/50	14.00

Table 3.2 Chi-square analysis of TmRSV incidence between decline and non-decline orchards.

Treatment Comparison	Observed[a]	Expected[b]	X^2	P
Decline/ Non-Decline[c]	+71:29 -229:271	1:1	20.17[d]	<.001

[a]Observed values are for ELISA positives (+) and ELISA negatives (-) for the compared treatments summed over the sampling dates.

[b]Null hypothesis: The number of diseased and healthy trees are equal between the compared treatments.

[c]Decline treatments consisted of 300 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six declining orchards. Decline treatments consisted of 300 individual 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock from six non-declining orchards.

[d]A significant X^2 indicated that TMRSV incidence is not independent of decline and non-decline treatments.

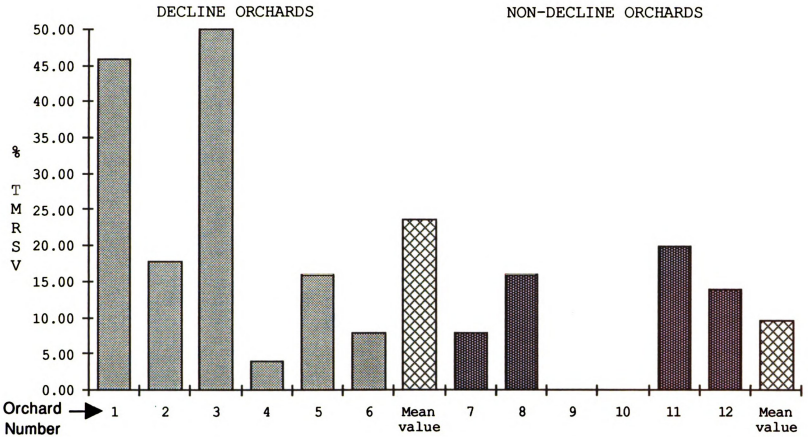

Figure 3.1 Percentage of trees ELISA-positive for TmRSV by orchard (50 trees per orchard) showing mean values of infection for decline and non-decline categories

Table 3.3 Percent tree replant, stunting, blindwood, and tree death for trees ELISA-positive for TmRSV and trees ELISA-negative for TmRSV between and within decline and non-decline orchards.

Category	% Trees with Stunting	% Trees with Blindwood	% Trees that were Dead	% Trees that were Replanted
(A)				
+[a]/decline[b]	0.00	57.14	3.17	12.69
-/decline	3.74	58.28	2.67	10.16
(B)				
+/non-decline	3.45	6.90	0.00	0.0
-/non-decline	5.54	12.92	0.37	1.85
(C)				
Decline				
TmRSV + & -	2.80	58.00[c](.001)	2.80(.05)	10.8(.001)
Non-Decline				
TmRSV + & -	2.67	6.17	0.17	0.83
(D)				
All TmRSV positives	1.08	41.30	2.17	8.69
All TmRSV negatives	4.80	31.44	1.31	5.24

[a]+ = TmRSV-ELISA positive, - = TmRSV-ELISA negative

[b]Decline orchards included 250 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchards included 300 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[c]Value is significantly greater than the corresponding values (P = number in parenthesis) when total incidence of a characteristic is compared within categories by chi-square analysis.

in four different categories. One category, (A) compares the trees that tested ELISA-positive for TmRSV to the trees that tested ELISA-negative for TmRSV in the decline orchards. Another category, (B) compares the trees that tested ELISA-positive for TmRSV to the trees that tested ELISA-negative for TmRSV in the non-decline orchards. A third category, (C) compares all the decline orchards to all the non-decline orchards without regard to TmRSV infection. The fourth category, (D), compares all the trees (decline or non-decline) that tested ELISA-positive for TmRSV to all the trees that tested ELISA-negative for TmRSV. According to chi-square analyses, the decline versus non-decline category was the only category that was shown to have significant differences in terms of the levels of these four characteristics. The levels of blindwood, tree death and replants were all significantly higher in the decline orchards than in the non-decline orchards. Levels of tree stunting between decline and non-decline orchards did not differ significantly.

Vigor ratings given to the orchard trees are compared within the four categories in Table 3.4. In comparison category (A), significantly more decline trees that tested ELISA-positive for TmRSV than decline trees that tested ELISA-negative for TmRSV were given the low vigor ratings 1 and 2. Similarly, in the overall decline versus non-decline category (C), the decline trees had significantly more trees given the low vigor ratings 1 and 2 than did the non-decline trees. Also when the trees that tested ELISA-negative for

Table 3.4 Tree vigor ratings[a] for trees ELISA-positive for TmRSV and trees ELISA-negative for TmRSV between and within decline and non-decline orchards.

Category	Mean Vigor Rating	% Trees Vigor Rating 1 & 2	% Trees Vigor Rating 3	% Trees Vigor Rating 4 & 5
(A)				
+[b]/decline[c]	2.41	38.09[d](.001)	49.20	9.5
-/decline	2.05	18.18	63.10(.05)	16.04
(B)				
+/non-decline	3.03	10.35	79.31(.05)	10.34
-/non-decline	3.55	9.6	50.92	38.70(.01)
(C)				
Decline				
TmRSV + & -	3.78	23.20(.001)	59.60	14.40
Non-Decline				
TmRSV + & -	3.51	9.67	53.67	36.00(.001)
(D)				
All TmRSV positives	2.61	29.34(.001)	58.69	9.78
All TmRSV negatives	3.29	13.10	55.89	29.47(.001)

[a]Vigor rating system: 1 = tree excessively stunted; branches sparsely foliated, few fruit spurs; 2 = tree slightly stunted; low fruit spur to shoot growth ratio; 3 = tree growth average; slightly more shoot growth to fruit spur growth; 4 = tree growth average to above average; nearly equal ratio of fruit spur to shoot growth; 5 = tree growth above average; branches full; fruit spur to shoot growth equal or better.

[b]+ = TmRSV-ELISA positive, - = TmRSV-ELISA negative.

[c]Decline orchards included 250 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchards included 300 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[d]Value is significantly greater (P = number in parenthesis) than the corresponding value when vigor classes were compared within category by chi-square analysis.

TmRSV were compared to the trees that tested ELISA-positive for TmRSV [comparison category (D)], the latter had significantly more trees rated at these two lower vigor levels. The incidence of high vigor ratings 4 and 5 was shown to be significant in three of the four categories. Categories (B) and (D) indicated that significantly more TmRSV-free trees were given these high vigor ratings. Category (C) indicated that significantly more non-decline trees were given a 4 or 5 rating.

Levels of trunk damage within the same four comparison categories are indicated in Table 3.5. As was true in previous trunk damage comparisons, the decline orchards overall had significantly more trunk damage than the non-decline orchards. Trees that tested ELISA-positive for TmRSV also had significantly more trees rated with damage than did the trees that tested ELISA-negative for TmRSV. Tomato ringspot virus was also significantly associated with total damage within the non-decline orchards.

Another comparison that can be made for the TMRSV-positive trees is that of incidence of PNRSV and PDV. These comparisons are shown in Table 3.6. Overall, PNRSV infection was significantly higher in the trees from the decline orchards than in the trees from the non-decline orchards,[category (C)] as was previously shown. In addition, the TmRSV-positive trees had significantly more trees also infected with PNRSV than did the trees without TmRSV [category(D)]. Within the decline orchards as well [category (A)], significantly more trees that were

Table 3.5 Percent trunk damage[a] on trees ELISA-positive for TmRSV and trees ELISA-negative for TmRSV between and within decline and non-decline orchards.

Category	% Trees Damage 0	% Trees Damage 1	% Trees Damage 2	Total Damage 1 & 2
(A)				
+[b]/decline[c]	69.87	6.34	23.80	30.14
-/decline	74.86	9.62	14.43	24.05
(B)				
+/non-decline	89.66	3.45	6.90	10.35[d(.05)]
-/non-decline	94.46	2.58	1.85	4.43
(C)				
Decline				
TmRSV + & -	73.60	8.80(.001)	16.80(.001)	25.6(.001)
Non-Decline				
TmRSV + & -	94.00(.001)	2.67	2.33	5.00
(D)				
All TmRSV positives	76.08	5.43	18.47(.05)	23.90(.01)
All TmRSV negatives	86.46(.05)	5.45	6.98	12.43

[a]Trunk damage rating system : 0 = no visible trunk damage; 1 = visible mild trunk damage; 2 = visible severe trunk damage.

[b]+ = TmRSV-ELISA positive; - = TmRSV-ELISA negative.

[c]Decline orchards included 250'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline torchards included 300 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[d]Value is significantly greater (P = number in parenthesis) than the corresponding value when damage classes were compared within category by chi-square analysis.

Table 3.6 Percent PNRSV and PDV ELISA-positives[a] among trees ELISA-positive for TmRSV and trees ELISA-negative for TmRSV between and within decline and non-decline orchards.

Category	% PNRSV	% PDV
(A)		
+[b]/decline[c]	80.28[d(.05)]	0.00
-/decline	63.76	3.93
(B)		
+/non-decline	37.93	0.0
-/non-decline	23.62	1.11
(C)		
Decline TmRSV + & -	67.67[(.001)]	3.00
Non-Decline TmRSV + & -	12.50	0.50
(D)		
All TmRSV positives	68.00[(.001)]	0.00
All TmRSV negatives	42.00	2.40

[a]All trees tested by DAS-ELISA in 1983, 1984.

[b]+ = TmRSV-ELISA positive; - = TmRSV-ELISA negative.

[c]Decline orchards included 250'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline orchards included 300 'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock.

[d]Value is significantly greater (P = number in parenthesis) than the corresponding value when PNRSV and PDV ELISA-positives were compared within category by chi-square analysis.

ELISA-positive for TmRSV were ELISA-positive for PNRSV than were the trees that tested ELISA-negative for TmRSV.

Distribution of TmRSV within Cherry Trees

The distribution of TmRSV in cambial tissues around the union of six naturally infected TmRSV positive trees is reported in Table 3.7. When the cambial samples from the four locations on the tree were combined, in all six cases TmRSV was detected. Individually, the four cambial samples from a given tree were not always positive. No cambial samples were positive for tree C when individually tested, although the combined sample was positive for TmRSV. Trees B,D,E had one of the four samples positive individually while trees A and F had three of the four positive individually. No virus was detected in the leaf and root or sucker samples taken from these six trees. Of the samples taken above and below the graft union only those five centimeters below the union were positive and not in all six trees. No samples taken more than five centimeters below the graft union or those taken above the graft union were positive for TmRSV.

Table 3.8 shows the results of the testing of 150 greenhouse grown TMRSV-bark chip inoculated 2-year old trees. Overall, the incidence of virus detected was relatively low, with 4.16 percent the highest level of virus in any of the tissues tested. The sucker and cambial tissues had the most TmRSV positive samples.

When five trees that had tested ELISA-positive for TmRSV were tested repeatedly over a three month period, TmRSV

Table 3.7 TmRSV distribution in cambial tissues of 11-year-old naturally infected cherry trees.

TREE	SAMPLE[a] North	South	East	West	N-S-E-W Combined	Healthy Mean
A	.15[b](+)[c]	.15(+)	.15(+)	.05(-)	.13(+)	0.04
B	.10(+)	.06(-)	.05(-)	.04(-)	.14(+)	0.04
C	.06(-)	.06(-)	.06(-)	.07(-)	.13(+)	0.04
D	.06(-)	.05(-)	.06(-)	.11(+)	.13(+)	0.04
E	.02(-)	.03(-)	.03(-)	.10(+)	.18(+)	0.02
F	.02(-)	.18(+)	.26(+)	.11(+)	.14(+)	0.02

[a]North, South, East, West samples consisted of 2 cm plug of cambial tissue from respective location on trunk.

[b]Number is A_{405nm} value of the sample.

[c](+) - positive for TmRSV, (-) - negative for TmRSV, by ELISA. Samples were considered positive if the mean A_{405nm} value of the of two replicate wells exceeded two times the mean value of five healthy sample wells.

Table 3.8 TmRSV distribution and detection in 'Montmorency' trees on 'Mahaleb' rootstock nine months post-inoculation[a] with TmRSV-positive wood chip.

	TISSUE TYPE			
	Cambial	Leaf/Flower/Bud	Root	Sucker
Number Positive [b]				
Number Sampled	3/150	1/150	0/150	3/72
Virus Incidence (%)	2.00	0.66	0.00	4.16

[a]Trees were inoculated at one year of age by inserting at the graft union two 1.5 centimeter wood chips taken from the graft union of a tree that had tested ELISA-positive for TmRSV.

[b]Samples considered positive if the mean A_{405nm} value of two replicate wells was over two times the mean of healthy controls.

detection was variable (Table 3.9). One tree (B), although positive before the study, failed to yield a TmRSV-positive response at any of the five sampling dates. The other four trees were positive on at least two but not more than four of the five sampling dates. On any given sampling date the highest rate of TmRSV detection was four out of the five trees tested. Figure 3.2 graphically illustrates the varibility of virus detection over the three month period and within the five trees.

TmRSV, Dandelion and Nematode Study

Table 3.10 compares the characteristics of this study for the TmRSV-positive and the TmRSV-negative trees within the decline and non-decline groups selected for this experiment. In addition, the overall decline and non-decline groups without regard to virus infection are compared and the TmRSV-positive group is compared to the TmRSV-negative group. The characteristics shown in this table are mean vigor, the mean percent positive dandelion for each group, and soil and root nematode levels measured. It is only in the category of overall decline versus non-decline status [category(C)], that statistical significance is seen. The trees in the decline group had significantly more of the surrounding dandelions TmRSV-positive than did the non-decline group. The non-decline group however, had significantly more *Criconemella* nematodes and significantly more *Pratylenchus* per 100cc of soil.

Table 3.9 TmRSV detection in cambial tissues from five naturally infected cherry trees at intervals over a 68-day period.

	SAMPLE[a]					
TREE	Day 1	Day 11	Day 20	Day 34	Day 68	Totals
Healthy Mean	**0.085[b]**	**0.09**	**0.06**	**0.09**	**0.11**	
A	0.56(+)[c]	0.24(+)	0.18(+)	0.30(+)	0.17(-)	4/5
B	0.10(-)	0.12(-)	0.09(-)	0.14(-)	0.15(-)	0/5
C	0.21(+)	0.68(+)	0.12(-)	0.20(+)	0.09(-)	3/5
D	0.19(+)	0.43(+)	0.16(+)	0.27(+)	0.09(-)	4/5
E	0.20(+)	0.69(+)	0.07(-)	0.11(-)	0.09(-)	2/5
Day Total	4/5	4/5	2/5	3/5	0/5	

[a]Each sample consisted four cambial samples taken at four equidistant locations at or below graft union, combined. All trees were tested positive for TmRSV at least once before day 1.

[b]Number = A_{405nm} value, each value is average of two replicate wells

[c](+) - positive for TmRSV, (-) - negative for TmRSV , by ELISA. Samples were considered positive if the mean A_{405nm} value of two replicate wells exceeded two times the mean of value of five healthy sample wells.

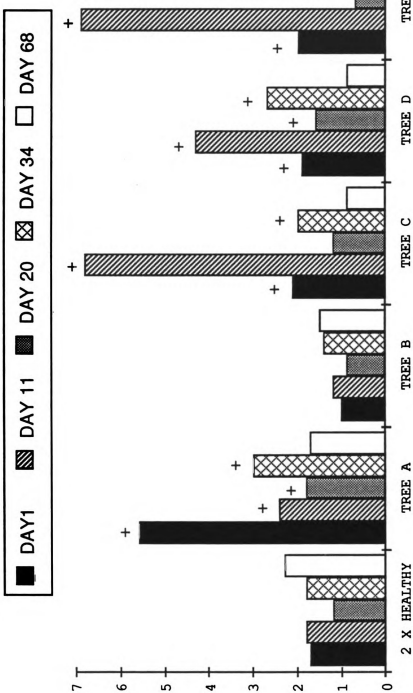

Figure 3.2 ELISA values for five 11 year-old previously ELISA-positive (+) cherry trees tested five times over a 68 day interval

Table 3.10 Mean vigor of cherry trees and mean percent TmRSV-positive dandelions and nematode density surrounding 30 cherry trees in three decline and three non-decline orchards.

			Nematode Density[c]			
	Mean	Mean % Positive	*Xiphinema*	*Criconemella*		*Pratylenchus*
Category	Vigor[a]	Dandelion[b]	soil	soil	soil	root
+[d]/decline[e]	2.87	2.00 %	20.27	0.80	0.13	18.73
-/decline	2.50	1.73 %	25.93	0.80	1.80	3.80
+/non-decline	3.20	0.33 %	51.00	10.33	2.87	2.47
-/non-decline	3.00	1.27 %	29.93	5.87	3.07	1.53
Decline TmRSV + & -	2.68	1.87 %[f(.01)]	23.10	0.80	0.97	11.27
Non-Decline TmRSV + & -	3.10	0.80 %	40.47	8.10[(.01)]	3.0[(.01)]	2.00
All TmRSV positives	3.03	1.17 %	35.63	5.57	1.50	10.60
All TmRSV negatives	2.75	1.50 %	27.93	3.33	2.43	2.67

[a] Vigor rating system:1 = tree excessively stunted; branches sparsely foliated,few fruit spurs; 2 = tree slightly stunted; low fruit spur to shoot growth ratio; 3 = tree growth average;slightly more shoot growth to fruit spur growth; 4 = tree growth average to above average; nearly equal ratio of fruit spur to shoot growth; 5= tree growth above average; branches full; fruit spur to shoot growth equal or better.
[b] 10 dandelions / per tree were tested for TmRSV by $F(ab)'_2$ ELISA.
[c] Per 100cc soil or 1 gram root.
[d] + = TMRSV-ELISA positive, - = TMRSV-ELISA negative.
[e] Decline treatment included 30 (15 TmRSV positive/15 TmRSV negative)'Montmorency' trees on 'Mazzard' or 'Mahaleb' rootstock. Non-Decline treatment included 30 'Montmorency' trees (15 TmRSV positive/15 TmRSV negative) on 'Mazzard' or 'Mahaleb' rootstock.
[f] Value is significantly greater than the corresponding values (P = number in parenthesis) when total incidence of a characteristic is compared within categories by chi-square analysis.

Symptom Expression Study

The results of the comparison of symptom expression to TmRSV detection are shown in Table 3.11. Approximately one-half of the trees that were ELISA-positive for TmRSV infection also showed cambial pitting and grooving symptoms. Approximately one-third of the trees ELISA-negative for TmRSV infection, in contrast, did have cambial pitting and grooving symptoms.

Assay Comparisons for TmRSV Detection

Tables 3.12 and 3.13 reveal the results of the two assay comparisons. Table 3.12 indicates the relative sensitivity of different immunoassay methods and conjugated enzymes in detecting TmRSV. While the DAS-ELISA alkaline phosphatase system could detect TmRSV in cucumber at the highest dilution (1:10000) it failed to detect TmRSV in infected tree cambial tissues. All three indirect ELISA systems - $F'(ab)_2$ alkaline phosphatase and horseradish peroxidase, and HADAS-ELISA, could detect TmRSV in cucumber and cambial tissues with a similar sensitivity.

Table 3.13 shows the relative sensitivities of $F'(ab)_2$-ELISA and dot-Blot immunoassay in detecting TmRSV, both purified and in various tissues. Both systems could detect 0.01 µg of TmRSV but the $F'(ab)_2$-based system was more sensitive at detecting TmRSV diluted in healthy sap. Dot-ELISA did not detect TmRSV in cherry roots and was not as

Table 3.11 Expression of cambial pitting and grooving symptoms vs. TmRSV infection in 'Montmorency' cherry.[a]

VIRUS STATUS	Total Number/ Number Sampled (percent)	SYMPTOMS: Pitting and/or Grooving	SYMPTOMS: No Pitting or Grooving
POSITIVE for TMRSV[b]	57/150 (38.0%)	23/57 (40.3%)	34/57 (59.6%)
NEGATIVE for TMRSV	93/150 (62.0%)	35/93 (37.6%)	58/93 (62.4%)

[a]150 'Montmorency' on 'Mazzard' or 'Mahaleb' rootstock sour cherry trees.
[b]Trees tested for TmRSV by F'(ab)2 ELISA in 1985 . One sample equaled four 5 cm cambial discs taken at or below the graft union. Samples were considered positive if the mean A_{405} value of two replicates exceeded the mean of four replicates of healthy controls.

Table 3.12 The relative sensitivity of different immunoassay methods and enzyme labelled gamma-globulins in detection of tomato ringspot virus in infected cherry tissue.

Immunosorbent assay method[b] and enzyme used for y-globulin conjugation	The highest sample dilution at which TmRSV was detected[a]		Inner bark and cambium	
	Cucumber leaves[c]	Inner bark and cambium[d]	A_{405nm} healthy mean	Number positive / Number sampled
DAS-ELISA, peroxidase	600	5	.125	2/11
DAS-ELISA, alkaline phosphatase	10000	-	.16	0/11
F'(ab)$_2$-ELISA, peroxidase	3000	10	.075	6/11
F'(ab)$_2$-ELISA alkaline phosphatase	3000	10	.115	2/11
HADAS-ELISA, peroxidase	4000	10	.18	2/11

[a]ELISA absorbance values at least twofold higher than the absorbance values of comparable controls (TmRSV-free tissue) on the same microtiter plates.

[b]DAS-ELISA = double antibody sandwich method of ELISA; F'(ab)$_2$ = F'(ab)$_2$-based indirect ELISA; HADAS = heterologous double antibody sandwich method of ELISA.

[c]Artificially inoculated with TmRSV.

[d]Naturally infected with TmRSV.

Table 3.13 Relative sensitivity of $F(ab')_2$ ELISA and dot-Blot immunoassay in detecting TmRSV.

	DETECTION LIMIT[a]	
VIRUS SOURCE	dot-Blot	$F'(ab)_2$
Purified TmRSV	.01 µg/ml	.01 µg/ml
Purified TmRSV in sap of healthy cherry root	100 µg/ml	.01 µg/ml
Cherry roots	no detection	1:10[b]
Dandelion roots	1:100	1:1000
Cucumber leaves	1:10,000	1:100,000

[a]Level at which the ELISA absorbence value (A_{405nm}) was at least two times the comparable healthy tissue value or where red pigment was seen on the dot-Blot nitrocellulose membrane.

[b] Ratios indicate the dilution level of sample:extracting buffer (gm:ml).

sensitive as F'(ab)$_2$-ELISA in detecting TmRSV in dandelion roots and cucumber leaves.

DISCUSSION

In the twelve orchards sampled for this study, the levels of TmRSV infection found were variable. Infection levels overall were lower than the orchard infection levels of PNRSV but higher than orchard infection levels of PDV. The orchards tested ranged in levels of TmRSV infection from zero to 50 percent of the fifty trees per orchard tested. All six of the decline orchards tested had some TmRSV infection present, and the decline orchards had over twice the TmRSV infection levels than did the non-decline orchards.

While overall the level of TmRSV found in this study was not extremely high, the levels found could be important due to the potentially serious effects of TmRSV on tree health. Even though TmRSV is somewhat localized at the base of infected fruit trees, studies in apple and peach show the virus can cause serious translocation problems as a result of tissue disorganization at the graft union (Tuttle and Gotleib, 1982). This can result in the death of the canopy, weakened root systems, and related winter injury (Lister et al., 1980; Stouffer, 1969). Associations have also been made with willowy, sparse growth habits (Stouffer et al., 1969), although other researchers have not found this to be a consistent feature of TmRSV infection. In our study there were both decline and non-decline orchard trees found to be infected with TmRSV. A preponderance of the TmRSV infected trees were of decline status, however, pointing to an

association of viral infection and poor tree health.

As noted by Cummins and Gonsalves (1982), there are many factors that can influence the transmission, maintenence, reproduction and recovery of TmRSV and some of these factors could have affected the results of the TmRSV field survey. In particular, tree to tree differences in establishment of vascular tissues after grafting, and variations in defense mechanisms could have affected the susceptibility of trees to TmRSV, as well as the recovery of TmRSV from plant tissues. Trees that were all equally exposed to the virus in the field may, due to individual variability, show differences in infection levels. Mircetich and Hoy (1981) noted that TmRSV was easily detected in the early stages of infection with Prunus brownline disease in plum and prune, and less efficiently detected as the trees became symptomatic and were dying. This phenomenon may also be true with TmRSV infection in cherry.

In addition, methods of TmRSV detection in the orchard trees may have affected the results. In this study, the fifty selected trees in each orchard were tested once. This was done to obtain a 'moment in time' test for virus in the trees, and to ascertain virus levels in a relative sense. Since TmRSV is known to be irregularly distributed, it can be speculated that repeated testing of the trees for TmRSV could have resulted in higher infection levels in the orchards sampled.

The distribution of the TmRSV-infected trees within the individual orchard blocks appears to be random in nature.

Many more samples, perhaps the entire 200 tree block instead of the chosen 50, would need to be taken in order to clearly establish any viral distribution patterns. When the pre-plant fumigation status for each orchard was noted, it was found that both of the orchards that had zero percent infection with TmRSV had been pre-plant fumigated. The orchard with 46 percent TmRSV infection, however, also had been pre-plant fumigated. Perhaps a inefficient fumigation job was done in this case. If one assumes an equal efficiency of the fumigation in all cases, this could indicate that TmRSV levels in the orchards may be associated with factors other than original *Xiphinema* levels in the soil at planting.

The lack of TmRSV infection at the three nurseries may indicate that at least some of the TmRSV infection in southwest Michigan is due to field infection and not from propagative sources. Due to the irregular distribution of TmRSV in cherry, and the primary localization of virus at or below the graft union, the spread of this virus through budding of scion wood to rootstocks is probably relatively infrequent. It should also be noted in this study that the total sample size was rather small (250 trees). The sampling method used, in effect, sacrificed the trees tested, so a larger sample size was not feasible.

In considering the individual tree evaluation data, it is important to note that this TmRSV study represents a subsample of the PNRSV/PDV test group, with fifty and not two hundred trees virus tested per orchard. The tree replant,

tree death, and blindwood data that were shown to be significant in Table 3.3 were also significant for the decline and non-decline fields overall, when two hundred trees were rated at each orchard. These factors seem to be positively associated mainly with the decline status of the orchard and not in particular with TmRSV (or PNRSV/PDV) infection of the trees. At the least, these results verify that declining orchards have more dead, replanted and poor appearing trees than non-decline orchards.

The vigor of the trees, in contrast, appears to be somewhat virus related. More of the trees rated at the lower vigor levels of 1 and 2 were decline trees than were non-decline trees. Also more TmRSV-positive trees (in decline and non-decline orchards combined) were rated at 1 and 2 than were TmRSV-free trees.

At the higher vigor levels as well, there appears to be an influence of TmRSV infection on vigor. When compared to the TmRSV-positive trees, the TmRSV-free trees show significantly more trees at the higher vigor levels 4 and 5. Tomato ringspot virus infection in other species (peach, apple, plum) is known to affect vigor by reducing terminal growth, blocking effective vascular conduction, and affecting root development (Kirkpatrick et al., 1958; Lott, 1967; Lewis et al., 1968; Barratt et al., 1968; Stouffer and Uyemoto, 1976). Our studies on cherry indicate that TmRSV infection probably does not cause noticable changes in average orchard vigor. However, TmRSV presence may be a factor in the orchard trees that are extremely unhealthy, and the lack of

TmRSV seems to be a characteristic of the orchard trees that are considered extremely healthy.

Trunk damage, similar to what was seen with the PNRSV infection data, was positively associated with TmRSV-infected trees. It is interesting to note that even though the TmRSV-negative group is over four times as large, (450 trees) the smaller TmRSV-positive group (100 trees) still has twice as much trunk damage. As with PNRSV infection/trunk damage associations, no significant conclusion can be drawn from these results without further investigation on a tree by tree basis. Speculation may be made on the influence of any compromised health status (such as virus infection or trunk damage from harvesting) and the effect of any further stress, (such as harvesting damage or viral infection, respectively) on trees. Tomato ringspot virus, which can affect the strength of the union in some fruit trees, may, through effects on the conductive tissues or on cambial/bark strength promote or predispose the the trees to trunk damage. Damaged cherry trees may be predisposed to or less likely to recover from viruses due to their weakened condition, as Rosenberger et al., (1983) found to be the case with apple. Perhaps TmRSV and harvesting machine mechanical damage combine with still other factors to create an interaction that puts the trees in a overall stressed/declining state.

Prunus necrotic ringspot virus infection was also significantly associated with TmRSV infection, and again, only speculation can be made on this association without further investigation. There is nothing in particular about

these two viruses that would predict their association in nature. Orchards with high numbers of TmRSV-susceptible dandelions located nearby older PNRSV infected fields would certainly encourage the development of trees with multiple infections. One virus may induce a weaker condition overall and then allow increased infection and disease development by the other virus. Prunus necrotic ringspot virus has been shown to significantly affect the vigor and growth of trees especially when infected while young. Tomato ringspot virus, through field infection, may possibly occur on these already weakened trees with greater frequency. Specific tests designed to deduce the true relationship would need to be done to determine whether this virus to virus relationship is random or truly significantly related.

The experiment which investigated the distribution of TmRSV at the union of TmRSV infected trees revealed what appears to be an uneven distribution pattern of the virus within the trees. In these naturally infected trees, it appears that samples a few centimeters apart can be different in virus presence. In addition, no TmRSV was found in the roots or the leaves of these same trees. Bitterlin et al.(1988), Cummins and Gonsalves (1986), and Lister et al.(1980), found similar distribution phenomena of TmRSV in other species. As Mircetich et al.(1971) noted, several plant pathogenic graft-transmissible viruses are known to have local distribution in infected fruit trees. Apple, grape, plum, as well as small fruits, e.g. blueberry and raspberry, are known to have irregular distribution of this

virus in particular. Our study agrees with Cummins and Gonsalves (1986) in that it indicates that cambial sampling for TmRSV in cherry should consist of samples from a number of locations at or below the graft union, with the actual number depending on the diameter of the tree trunk.

In the trees that were infected artificially with a wood chip from a TmRSV-positive tree, little viral transfer was found after nine months. The sucker and cambial tissues were the most reliable of the four tissues tested, although overall, little virus (4 percent of all the trees tested) was detected. One reason for the low detection could have been the length of time after inoculation. Another, more likely reason may have been that the wood chips used were subject to the same irregular distribution pattern shown in the graft union study. Further work that has been done with this virus in our laboratory used a direct bark-slash inoculation method (Bitterlin et al., 1987) with purified virus for artificial infection. It appears from both the wood chip and the purified virus studies, however, that successful rates of TmRSV inoculation of cherry may be much lower than that reported for peach, apple and plum.

The studies on detection of TmRSV over time may also have been affected by an uneven distribution of the virus in the tissues. The study itself was limited by the sampling technique, and over-sampling of a finite virus reservoir may also have affected the results. This particular study indicated that detection of TmRSV in cambial tissues is inconsistent. Other work with artifically inoculated trees

has shown that although the variablity of detection of TmRSV in the cambium is also somewhat true in regard to root tissue, the roots are the least invasive and most reliable method of repeated testing, at least in young sour cherry. Studies on virus spread or studies in which the presence of TmRSV in tissues is important would have to account for this variability in detection, possibly through repeated sampling a minimum of four times.

It is possible that TmRSV infection and distribution is unique in each individual tree. The implications of this would have to be considered in any investigations involving this virus. Only extensive testing such as that done by Bitterlin and Gonsalves (1988) in peach or Cummins and Gonsalves (1986) in plum can determine the precise locations of virus within plant tissues and the probabilities of detection in specific tissues. In comparison to our studies it appears that the distribution of TmRSV in cherry is similar to the distribution of TmRSV in plum and peach. In all cases TmRSV is best detected in the tissues below the soil line. In peach and plum, as in naturally infected cherry, the cambial tissues at or below the graft union are the most consistent source of TmRSV. Root tissues seem to be most consistent in detection of the virus in young, artificially inoculated cherry and a satisfactory source of virus in naturally and artificially inoculated peach trees. In contrast to TmRSV detection studies in apple, (Bitterlin et al., 1984) TmRSV is rarely detected in cherry leaf tissue or rootstock sprouts.

In the TmRSV infected orchard tree, dandelion and nematode study, the limiting factor seems to be the virus itself. Tree infection is, however, dependent upon actual nematode feeding. Other research would suggest, especially in the absence of known nursery infection, that TmRSV-positive trees would be associated with increased vector nematode populations and increased density of TmRSV-positive dandelions. However, in this study, population levels of nematodes were not positively correlated with the viral status of either the trees or the dandelions. Perhaps if more trees had been tested it would have elucidated a clearer relationship. In this study the number of TmRSV-positive dandelions were higher in decline orchards than in the non-decline orchards. Yet overall, the number of TmRSV-positive dandelions was lower near individual trees that were TmRSV-positive than near the trees that were TmRSV-negative. Powell (1984) had found more dandelions infected with TmRSV in orchards with trees having TmRSV-induced PSP symptoms than in orchards without PSP or in non-orchard sites. Barrat et al., (1984) suggested that a low rate of correlation between trees and adjacent weeds was likely due to a low rate of virus detection in the trees and this may have been a factor in our work as well. Overall, Barrat did find that orchard health was not correlated with infected dandelion levels. Rosenberger et al. (1983) suspected that the uneven distribution of virus in the dandelions below trees is due to variation in the weeds that survived herbicide application.

The nematode population was not significantly higher in the decline sites, or near TmRSV-positive trees. In fact the *Xiphinema* population was lower than in non-decline sites or in the TmRSV-negative trees. One possible reason that the nematodes were not present is that the declining or TmRSV-infected trees and/or roots may be less vigorous and therefore less appealing to the nematodes than are the non-declining/non-TmRSV-infected roots. Powell (1984) also found no difference in *Xiphinema* levels between the PSP infected and PSP non-infected sites. Though Braun et al., (1966) found a positive relationship between levels of *Xiphinema* and *Pratylenchus* and tree vigor, when this was investigated no such association in cherry was found. In addition, the density of *Pratylenchus* or *Criconemella* spp. alone was not associated with the health of the tree, although other studies (Parker and Mai, 1956; Okie and Reilly, 1984) have shown that *Pratylenchus* is pathogenic to cherry.

It is not clear from our study that TmRSV infection in trees is always associated with TmRSV infection in dandelion and with nematode presence. Undoubtably, the dandelions do play a role in viral spread since the nematodes do lose virus upon moulting and the dandelions are a source of re-infection. A current study (Ramsdell et al, unpublished data) shows rapid infection of healthy dandelion (through nematode feeding) when in proximity of diseased dandelion, which in an orchard setting could be a major factor in virus spread. Certainly some tree infection by TmRSV does occur

from dandelion through nematodes. Considering the combined seed and nematode transmission of TmRSV in dandelions, orchard control of this weed should be considered in controlling TmRSV, especially when other options such as nematode control are difficult and sometimes prohibitively expensive.

Rosenberger et al., (1983) and others have suggested that there does seem to be more involved in TmRSV tree infection than just virus presence. In our work as well, there were several trees surrounded by dandelions infected with TmRSV (up to 70 percent of the nearby population) and high populations of *Xiphinema,* yet the trees were free of TmRSV. Just what factors are at work in situations as these are yet to be determined. Individual genetic and physiological tree varibility, plant defense mechanisms, nematode transmission specificity, TmRSV detection limits and environmental differences may all be playing a role.

The study of symptom expression shows that cambial pitting and grooving is simply not well correlated with TmRSV infection in sour cherry. The large variation in symptom expression in cambial tisssues combined with the aformentioned virus distribution and detection problems make pitting and grooving symptoms useless as an indicator of TmRSV infection in cherry trees. Virus was either not in the tissues that had symptoms or the detection methods were inefficient. In addition, it is possible that early infections are present in some trees and yet these trees are not showing symptoms, or a particular stress is required for

symptom expression. In other cases, early symptoms could be occuring, yet there is extremely low virus titer in these trees.

Mink and Howell (1980) and AlMusa et al.(1980), also showed pitting on otherwise normal (virus free) cherry trees. Our study is also in partial agreement with Bittlerlin and Gonsalves (1988) who showed that, in peach, TmRSV was in all the symptomatic stem pitted tissues tested, but also in some non-symptomatic trees.

Lewis et al. (1968), in work with peach showed that pitting was present from the root up to 12 inches above the union. In contrast to our work, they found that ninety percent of the declining/PSP-affected trees had the pitting symptom. Rosenberger et al. (1983) noted that the best symptoms in apple were indeed pitting along with necrosis at the graft union. Although there was a failure rate of 11 percent (trees with symptoms, yet not virus) they attributed this to uneven virus distribution or low titer.

Mircetich et al.(1977) speculated that pitting was only produced by some strains of TmRSV. This is probably not a major consideration in our study, for there is no reason to believe that more than one strain of TmRSV is involved in tree infection in southwest Michigan. What may be occuring is a species difference, with cherry reacting differently, for whatever reason, to TmRSV infection than does peach, plum or apple. The latter two are found with pitting but also with a consistent brown line of necrosis that is rarely found in cherry trees. It is possible that the pitting produced in

cherry is related to TmRSV but not a consistent symptom of the virus in this species.

Assay comparisons showed that all of the systems tested were capable of detecting TmRSV in plant tissues. The $F(ab')_2$- ELISA peroxidase system, however, gave the lowest background (non-infected tissues) levels and detected TmRSV in more cases than the other systems. $F(ab')_2$- ELISA/ peroxidase was the assay of choice based on speed, ease of use for large scale testing and ease of quantification. Hoy et al.(1984) found indirect ELISA to be most reliable in the detection of TmRSV. Barbara and Clark (1982), also reported that $F(ab')_2$- ELISA improved detection of virus due to lowered background. As noted by Powell (1984), indirect ELISA is more sensitive when viral concentration is a factor - which was important in our studies due to the irregular distribution of TmRSV in cherry tissues.

Dot-ELISA is often more sensitive or as sensitive as direct or indirect ELISA for detecting plant viruses (Banttari and Smith, 1984). In this study, there seemed to be an interference by the cherry root extract and this may have affected the results. The root extract tended to stick to the NCM which could have interfered with subsequent binding steps. Intial attempts to reduce this interference by increased washings with TBS-T80 and adding Trition X to the wash solution were not successful. Further work on this problem as well as use of anti-TmRSV antibodies cross-reacted with healthy cherry antigens may increase the detection

efficiency of dot-ELISA in detecting TmRSV.

SUMMARY

The decline syndrome in sour cherry is a dynamic and complex disorder. Trees in an orchard are subject to a variety of biological, environmental and cultural stresses at all stages of growth. Young trees are susceptible to soil pathogens and affected by poor soil structure, while older, maturing trees suffer cankers, mycoplasma-caused X-disease, borer insects, cold injury and damage from mechanical harvesting machines. Any number of interactions of these and other factors may be occuring in decline, with a synergistic total effect on tree health.

Viruses can infect trees at all ages and can reduce their capacity to carry out physiological functions. While rarely the cause of outright death, viral induced reductions in tree health and vigor may lower tree survival when the trees are subjected to other biotic and abiotic stress. Prunus necrotic ringspot and PDV have been shown to reduce the growth and yield of sour cherry trees. Tomato ringspot virus has been implicated in many serious diseases of tree fruits, primarily through effects on the conductive systems of the affected trees. Our surveys have shown that the incidence of all three of these viruses is significantly higher in declining southwest Michigan orchards than in non-declining southwest Michigan orchards. Further, our studies indicate that an association may be occuring between viral infection (by PNRSV and TmRSV) and poor tree vigor, blindwood, tree

death and tree replant incidence. Interestingly, both viruses are also associated with trunk damage. While the correlative nature of the data precludes determination of the precise nature of the associations that are occuring, these results indicate some ways that these two viruses may be acting as contributors to the decline phenomenon. Significant associations of any characteristic with viral presence also indicates areas where further studies, with individual trees in controlled environmental conditions (as opposed to orchards in varying natural conditions) could be focused.

Peach tree short life (PTSL), a serious problem in peach cultivation is similar to decline in its complexity. Detailed studies have led to the conclusion that PTSL results from a complex interaction of abiotic and biotic factors. Use of susceptible cultivars, production factors e.g. pruning and soil fumigation, presence of nematodes, and individual orchard site characteristics all increase the susceptibility of peach tress to injury and pathogens. Important to the control of PTSL has been a continuing cooperative effort in the southeastern United States that has resulted in a 10-point program for controlling the problem and for increasing overall longevity of economically productive peach orchard (Ritchie and Clayton,1981; Reilly et al,1986).

A similar combined effort on the part of many researchers may eventually result in a similar plan for decline-free cherry cultivation in Michigan. Attempts are in progress to associate the known biological, environmental and cultural

contributers to stone fruit decline so that a Decision Support System may be developed for growers to use as part of their management strategy to help reduce decline incidence in orchards.

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