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Master's degree in Botany & Plant Pathology

Donald C. Ramsdell
Major professor

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AN ANATOMICAL STUDY OF PRUNE BROWN LINE DISEASE AND DETECTION OF TOMATO RINGSPOT VIRUS IN PLUM

By

Karpura V Kommineni

A THESIS

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

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1996

ABSTRACT

AN ANATOMICAL STUDY OF PRUNE BROWN LINE DISEASE AND DETECTION OF TOMATO RINGSPOT VIRUS IN PLUM

By

Karpura V Kommineni

An anatomical study of bark, at the graft union of Tomato ringspot virus (TmRSV) infected, Prune Brown line diseased plum trees revealed wound areas demarcated by necrophylactic periderm. ELISA and northern hybridization were used to detect TmRSV in roots and rootstock sucker leaves of plum trees in the field. There was no significant difference between the results obtained with the two types of assays, in case of root samples. There was a significant difference in case of sucker leaf, where northern hybridization detected a higher percent infection. Chi-square analysis of the ELISA results on root or bark samples of nematode and slash inoculated 'Stanley'/Myrobalan 29C, showed a significant difference. Silver enhanced protein-A-gold labeling of bark of the BL, scion, rootstock and sucker leaves, was distributed on cell wall and cytoplasm, with a major portion seen in the axial phloem of bark and in bundle sheath of leaf.

In dedication to

my father Sri K.V.Raja Rao

and

my aunt Smt S.Madhavi and uncle late Sri S.R.Mohana Rao

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INTRODUCTION

Tomato ringspot virus (TmRSV) causes Prune Brown line (PBL) disease in prune and plum trees. The PBL disease has been reported in California (Mircetich and Hoy, 1981) and in the North Eastern United States (Brase and Parker, 1955; Cummins and Gonsalves, 1986*a*). Prune Brown line disease is characterized by the presence of a brown line (BL) at the graft union. The BL can be seen when the bark at the graft union is cut open, to expose the inner bark tissue. The BL is reported to be a result of necrosis of the phloem and cambial tissue (Hoy and Mircetich, 1984). Other symptoms of PBL include, chlorotic flecks on the rootstock sucker leaves, an inverted shoulder on the scion and a constricted appearance of the rootstock below the graft union.

Prune Brown line has been reported to be the result of an hypersensitive reaction of a resistant scion to TmRSV infection in a susceptible rootstock (Hoy and Mircetich, 1984). Cummins and Gonsalves (1986*a*) have reported that PBL is analogous to Apple Union Necrosis and Decline disease in apple trees, that is also caused by TmRSV. They suggested that PBL is the result of a hypersensitive reaction of a resistant scion to TmRSV infection in a susceptible but tolerant rootstock. An anatomical study of the PBL disease has not been done so far. Tomato ringspot virus has not been localized in the bark tissue of PBL-affected plum trees, with immunolabeling.

Tomato ringspot virus is a member of the nepovirus group of plant viruses. The virus has a bipartite genome of single stranded positive sense RNA (Stace-Smith, 1984). *Xiphinema americanum* Cobb 1913 is the natural transmission vector of the virus in Eastern North America. Cell to cell movement of the nepoviruses in the plant host has been suggested to occur through the plasmodesmata, in tubules lined with virus particles (de Zoeten and Gaard, 1969; Lucas and Gilbertson, 1994).

The objectives of this research work were (1) to compare nematode and slash inoculation treatment effects on the onset of PBL disease in 'Stanley'/Myrobalan plum, and to determine whether natural transmission or mechanical inoculation is more effective in systemic infection and the onset of the disease. (2) To compare ELISA and northern hybridization methods of TmRSV detection to determine if one is more efficient than the other in detecting TmRSV in bark, root and rootstock sucker leaf tissue of nematode and slash inoculated trees. (3) To study the histopathological changes at the graft union area of PBL-affected trees, in an attempt to understand the anatomical changes that occur in the early stages of the BL formation. (4) To localize TmRSV in the rootstock sucker leaf samples and the bark samples from the graft union of PBL-affected trees, so as to determine the distribution of the virus within the infected tissue. (5) To determine if there is a difference in brown line formation among five scion/five rootstock combinations in a field test planting.

Chapter I

Prune Brown Line Disease

Literature Review

A. Description of the disease

Tomato Ringspot virus (TmRSV) causes Prune Brown line disease (PBL) in plum and prune trees (Cummins and Gonsalves, 1986*b*; Mircetich and Hoy, 1981). The PBL disease is diagnosed by the presence of a narrow strip of brown necrotic tissue, a brown line (BL), at the graft union of prune and plum trees. The brown line is a result of an hypersensitive reaction of the scion to TmRSV infection in the rootstock. The brown line is caused by the necrosis of the cambial and phloem tissues (Mircetich and Hoy, 1981). The brown line spreads around the union causing girdling, decline and eventual death of the tree (Hoy and Mircetich, 1984). The trees sometimes develop an inverted shoulder of the scion at the graft union and chlorotic spots on the leaves of the rootstock suckers (Brase and Parker, 1955; Mircetich and Hoy, 1981). The name 'Prune Brownline' was used in 1981 by Mircetich and Hoy. They also studied the association of TmRSV with PBL and established TmRSV as the causal agent of PBL. The disease is also referred to as 'Stanley' Constriction and Decline (SCAD) (Cummins and Gonsalves, 1986*a*).

1. Early records of decline in prune trees

One of the early records of this condition in prune trees was made in 1955 in a study of declining 'Stanley' prune trees in New York (Brase and Parker, 1955). The 'Stanley' cultivars were propagated on Myrobalan rootstock (*Prunus cerasifera* Ehrh.).

The declining trees were distinguishable from healthy trees by the presence of a prominent inverted shoulder at the graft union and a constriction in the rootstock just below the union. In severe cases the bark at the union was necrotic and disrupted. Brase and Parker also observed chlorotic flecks on the leaves of the rootstock suckers in some of the trees.

Another report of 'decline' in 'Stanley' prune trees on Myrobalan rootstock was published by Kirkpatrick et al. (1958). The trees in this case also had symptoms similar to the above report such as constriction of rootstock below union and an inverted shoulder at the union.

2. PBL can be induced by various isolates of TmRSV

Hoy and Mircetich (1984) studied the role of five isolates of TmRSV in inducing PBL. The five isolates of TmRSV associated with various diseases of *Prunus* spp. were peach yellow bud mosaic (PYB), cherry leaf mottle (CLM), California stem pitting, *Prunus* stem pitting and prune brownline isolates. French prunes (*Prunus domestica* L.) on Lovell peach, Myrobalan plum (29C) and Marianna 2624 plum rootstocks were graft inoculated at the rootstock with root chip inoculum from orchard trees that were naturally infected by the five isolates. The five isolates were readily transmitted to French prune trees on Myrobalan plum and Lovell peach rootstocks. A brown line developed at the graft union at the end of three growing seasons. Tomato ringspot virus was not transmitted to the prune trees on Marianna 2624 plum rootstock and no brownline developed in this case.

3. Sources of inoculum

a. Transmission by the natural vector

Tomato Ringspot virus is vectored by the dagger nematode *Xiphinema americanum* Cobb 1913 (Hoy and Mircetich, 1984; Teliz et al., 1966, 1967; Stace-Smith, 1984). In California, *X. californicum* Lamberti and Bleve-Zacheo., is the species most commonly associated with fruit trees (Hoy et al., 1984). Hoy and Mircetich (1984) demonstrated the successful transmission of TmRSV to peach and Myrobalan plum rootstocks by the TmRSV nematode vector, *X. californicum*. However, they did not test the ability of *X. californicum* to transmit TmRSV to Marianna 2624 clonal rootstock. In Eastern North America, *X. americanum* is the most widespread species of *Xiphinema* (Hoy and Mircetich, 1984; Rosenberger et al., 1983; Teliz et al., 1967). However, other species of *Xiphinema* may be important, e.g. *X. rivesi* Dalmasso (Forer et al., 1984).

i. Transmission of TmRSV by different life stages of X. americanum

The transmission of TmRSV by different life stages of *X. americanum* was studied by Teliz et al. (1966). The life cycle was separated into four larval stages and an adult stage. The first larval stage was the smallest in size, with the developing odontostyle in the basal portion of the functioning stylet. The second and third larval stages had the developing odontostyle enclosed in the esophageal wall posterior to the basal portion of the stylet. The fourth larval stage had the rudiments of the reproductory system defined. A mixture of larvae and adults was added at the root zone of TmRSV

inoculated cucumber seedlings (800±17/pot with one seedling per pot). The nematodes were allowed an acquisition access period (AAP) of 12 days. Then the virus infected cucumber plants were cut at the soil line and healthy cucumber test seedlings were planted in the same pot. The nematodes were allowed an inoculation access period of 15 days. At the end of the IAP, the roots of the test cucumber seedlings thus inoculated with TmRSV were washed and tested by mechanical inoculation on *Chenopodium amaranticolor* Coste and Reyn., for symptom expression and on cucumber seedlings for serological identification by immunodiffusion assays. The first three larval stages and the adult stage gave a 100% transmission of TmRSV.

ii. Specificity of transmission

Xiphinema is an ectoparasitic nematode and feeds on the roots by inserting its stylet into the roots. The apical tips of the roots are the preferred sites for feeding. The virus particles accumulate as a monolayer in the cuticle lining the lumen of the odontophore, the slender oesophagus and the oesophageal bulb (Taylor and Robertson, 1977). It has been postulated that the bound virus is released from the specific site in the nematode as a result of a pH change when the nematode salivates (McElroy, 1977; Stace-Smith and Ramsdell, 1987). An hypothesis has been proposed that nepovirus transmission is linked to the interaction between the vector and the viral protein coat. The specificity of transmission reflects the specific association between the protein surface of the virus and the cuticle at the site of retention within the nematode vector. The less serologically related the viruses, the more unlikely it is that the same nematode will vector them (McElroy, 1977).

b. Planting Material

When virus-infected rootstocks are used in propagation they can serve as a source of inoculum and cause PBL in the trees. Mircetich and Hoy (1981) studied the French cultivar 'Agen' of prune on Myrobalan plum rootstock in California. They showed graft transmissibility of TmRSV from PBL-affected peach and Myrobalan plum seedling to healthy 'Lovell' peach rootstock and Myrobalan plum seedlings, and to French prune trees on 'Lovell' peach and Myrobalan plum rootstocks. Root chips from PBL infected peach trees when grafted onto the rootstock or scion of French prune trees resulted in transmission of the virus to the rootstock and not to the scion. Grafting with buds from the scion of PBL-infected trees did not result in transmission of TmRSV to the rootstock or scion. Tomato ringspot virus was not detected by enzyme linked immunosorbent assay (ELISA) from the scion portion of either the experimental trees or the PBL-affected trees in the orchards (Mircetich and Hoy, 1981).

i. Susceptibility of various rootstocks of Prunus to PBL

Based upon field observations, Mircetich and Hoy (1981) suggested that a clonal rootstock, Marianna 2624 (*P. cerasifera x P.munsonia*[?] Wight & Hedr.), may be more resistant to PBL than peach or Myrobalan plum rootstocks. Hoy and Mircetich, (1984), in a study comparing peach (*P. persica*[L.] Batsch), Myrobalan 29C plum and Marianna 2624 plum rootstocks, found Marianna 2624 to be resistant to TmRSV infection when challenged with root chip inoculum from PBL-affected orchard trees.

Cummins and Gonsalves (1986*b*) studied a pool of PBL-affected trees propagated on the following rootstocks: *P. domestica* cvs. Brompton, Yellow Egg and St. Julian A; *P. cerasifera* Ehr. cv. Myrobalan B; and five Myrobalan seedling lines. A Chi-square analysis revealed no differences among rootstocks for incidence of infection or for lateral distribution of infection within an individual tree.

ii. Are some cultivars more susceptible?

Brase and Parker (1955), in a comparison of three prune cultivars, Fellenberg, Stanley and Abundance, propagated on Myrobalan seedling rootstock with chlorotic fleck, found that constriction of the rootstock below the union was seen on the Stanley cultivar and not on the other two cultivars. Another symptom observed was alternating long ridges and depressions on the rootstock immediately below the union resulting in an appearance of angularity in cross section. The authors suggested that there may be some factor in Stanley that causes the angularity of the stock without serious injury. They also suggested that when this factor combines with the one that causes chlorotic fleck, a constriction is formed at the bud union, roots develop poorly and some of the trees die.

c. Alternate Hosts

Common orchard weeds also serve as resorvoirs of TmRSV. In a cooperative field study involving apple and peach orchard sites in Pennsylvania, New York and Indiana, it was found that 21 weed species in 12 families were infected with TmRSV in one or more locations. Some of the common weeds that were TmRSV-positive were herbaceous annuals e.g. the common chickweed (*Stellaria media* (L.)Villars.) and lamb's quarter (*Chenopodium album* L.), herbaceous perennials such as the dandelion (*Taraxacum*

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officinale Weber), broadleaf plantain (*Plantago major* L.) and sheep sorrel (*Rumex acetosella* L.) (Powell et al., 1984; Stace-Smith and Ramsdell, 1987). The TmRSV-infected weeds serve as a source of virus inoculum for the nematodes to transmit to healthy commercially important trees in the orchards.

B. Virus Detection

1. Enzyme linked Immunosorbent Assay

The enzyme-linked-immunosorbent-antibody assay (ELISA) is by far the most widely used serological diagnostic tool in plant virology (Clark, 1981; Miller and Martin, 1988). The direct Double Antibody Sandwich (DAS)-ELISA is the commonly used method of ELISA, in which untagged antibody is bound to a solid phase, e.g. wells of a polystyrene microtiter plate. The test sample, enzyme-conjugated-antibody, and the enzyme substrate are added sequentially, while unbound material is removed after each step by washing. In a positive test, the substrate solution becomes colored, and the intensity of the color, determined spectrophotometrically, is proportional to the amount of conjugate bound and, consequently, to the amount of antigen present. In a variation of the method, indirect ELISA (I-ELISA), the specific primary antibody is used untagged and the bound antibody is detected using an enzyme-conjugated secondary antibody reactive with the immunoglobulin species of the primary antibody (Miller and Martin, 1988). Reduced specificity for detecting strains of Tobacco mosaic virus (TMV) was found using this procedure (Clark, 1981).

a. *Reliabilty of ELISA for detection of woody plant viruses*

ELISA has been used for the detection of woody plant viruses in different kinds of plant samples e.g. leaf, root and bark. The sensitive detection of the virus by ELISA is dependent on the quality of the antisera available, the plant part sampled and its condition, the titer of the virus in the sample, the time of sampling and where on the plant the sample was taken.

i. Titer of virus and uneven distribution in the tree

Even in instances where the visual symptoms of the disease have progressed to an advanced stage, it may be difficult to detect the virus in woody plants. In apple trees with Apple Union Necrosis and Decline (AUND) disease caused by TmRSV, when the inner bark of the rootstock from trees with the graft union symptoms was sampled, TmRSV was not found in 11% of the trees (Rosenberger et al., 1983). The failure to detect TmRSV was probably due to uneven distribution of virus within rootstock, due to low virus titer, or due to interfering compounds in the tissue.

ii. The season when the test is done

The time of the year when the sampling is done is also important. TmRSV was detected in the leaves of the scion of orchard peach trees in May and in the roots and bark in July (Bitterlin et al., 1984). In another study, TmRSV was more efficiently detected in the leaves of peach and apple seedlings after a period of dormancy (Forer et al., 1984). The authors suggested that the dormancy induced the movement of the virus from the roots to the leaves.

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iii. Site of sampling on the tree

Tomato ringspot virus was detected in 100% of bark samples by I-ELISA when samples were taken directly below the brownline. The BL on the affected trees was seen on just one side of the circumference of the tree, and when samples were taken up to 15cm around the circumference of the tree from the end of the brownline, TmRSV was detected in only 28% of the trees. No TmRSV was detected in 56% of the samples taken >15cm from the end of the brownline (Hoy and Mircetich, 1984).

The importance of sampling at the optimal place on the tree is also illustrated by a review of a study of *Prunus* stem pitting (PSP) disease by Bitterlin et al. (1988). Bark samples taken from the lower part of the trunk of peach trees were the most reliable tissue source for detecting the virus in infected orchard trees. The highest percentage of ELISA-positive samples were obtained from samples taken within the first 10 cm below the soil line. Sometimes ELISA-positive and ELISA-negative samples were located within 1 to 2 cm of each other. In a majority of the PSP-affected trees surveyed in this study, the graft union was not clearly discernable and coincided with the soil line in some trees. Hence, the position of the sampling is given relative to the soil line. The virus was not detected in any leaf or bark samples of the upper branches. However, TmRSV was detected in the scion bark samples taken just above the soil line. In the case of root samples, virus detection was lower in the distal portions than in the proximal portions of the tree roots.

iv. Quantity of the sample

Cummins and Gonsalves (1986*b*), in a study of PBL on 'Stanley' trees, suggested that bark samples at the graft union area be taken from at least three of the four quadrants of the trunk in order to insure detection of the virus by ELISA .

v. Type of sample assayed for the virus

Extracts from rapidly growing shoot tip leaves of healthy apple trees contain antigens that can produce an ELISA positive result (false positive) for a TmRSV assay. The unidentified antigens also formed precipitates in agar gel double diffusion tests. The authors suggested that apple shoot tips should not be tested for regulatory purposes and virus certification programs (Mink et al., 1985).

2. Northern blot Hybridization

Other techniques, e.g. nucleic acid hybridization can be used for detection of plant viruses. In nucleic acid hybridization, viral nucleic acid probes are used to hybridize with homologous viral nucleic acid sequences in a dot blot assay. When RNA probes are used in nucleic acid hybridization, the technique is called Northern hybridization. The probes for plant viruses are usually complimentary DNA (cDNA) probes, since most plant viruses have an RNA genome (Miller and Martin, 1988). Transcription vectors carrying promoters specific to either Bacteriophage SP6 or Bacteriophage T7 and T3 have

facilitated *in vitro* transcription of cloned DNA sequences when the bacteriophage-specific RNA polymerase is added to the reaction (Maniatis et al., 1982; Melton et al., 1984). Thus, ssRNA probes can be generated which are more effective in northern hybridization than nick-translated DNA probes. The RNA probes make possible RNA:DNA or RNA:RNA hybridization. The RNA:RNA duplexes are more stable than DNA:DNA or DNA:RNA duplexes. This stability allows for more stringent washing conditions of the hybrids, increasing the signal to noise ratio. There is a further advantage in that the unbound RNA and the nonspecifically bound RNA on the blot can be removed by RNase digestion. However, there is a possibility of the RNA probe binding to ribosomal RNA. Unbound or non-duplexed RNA probes also have susceptibility to RNase digestion (Melton et al., 1984).

The detection of the nucleic acid hybrid is made possible by labeling the probe with a radioactive or nonradioactive label. Some of the nonradioactive labels used are biotin-avidin conjugated to alkaline phosphatase or horse radishperoxidase (Miller and Martin, 1988), and digoxigenin that can be detected by a colorimetric or chemiluminescent method (Boehringer Manheim Corp., 1994).

a. Construction of a cDNA clone of TmRSV

A TmRSV cDNA clone comprised of regions of RNA-1 and RNA-2 of a TmRSV isolate associated with Prunus stem pitting disease and AUND in the eastern United States was constructed by Hadidi et al. (1989). This clone was inserted into the polylinker region of the SP6 transcription vector pSP64. Using the SP6 promoter flanking this region, high specific activity RNA probes can be generated by SP6 RNA polymerase.

The RNA probe can be used for detection of TmRSV in northern blot hybridization assays. As little as 1pg of TmRSV RNA was detected in a dot blot assay with a probe generated in the above manner (Hadidi and Hammond, 1989).

i. Problems in detection

Tomato ringspot virus has been detected in nectarine (*Prunus persica* [L.] Batsch) trees using transcribed RNA probes specific to RNA-2 of the virus (Powell et al., 1991). Extracts from bark removed from areas of the tree where stem pitting symptoms occurred contained detectable TmRSV RNA. Extracts from bark removed from above the stem pitted region or from the opposite side of the trunk, where symptoms were absent, did not contain any detectable RNA. The authors found hybridization to be more efficient than ELISA in detecting TmRSV from bark samples. However, ELISA was more efficient when root samples were tested. The reason for this result is not known.

Materials and Methods

Virus Purification

Tomato Ringspot virus was propagated in 'National Pickling' cucumber seedlings. The TmRSV-infected cucumber tissue was homogenized in a cold Waring blender with cold 0.5M borate buffer (0.05M borax, 0.2M boric acid), pH 7.4 (1:2 w/v). The extract was filtered through four layers of cheese cloth, frozen and slow thawed at 4°C. All futher steps were performed at 4°C. A low speed centrifugation was done at 10,000 rpm for 20 min. Ammonium sulphate at a concentration of 15g/100ml was added to the supernatant and the virus precipitated overnight. After a low (10,000 rpm for 15 min) and high speed centrifugation (28,000 rpm for 2.5 hrs), the resultant pellet was resuspended in 0.05M phosphate buffer, pH 7.5. The virus was purified through [5 to 30% (w/v)] linear-log sucrose density gradients in an SW41 rotor at 38,000rpm for 90 min. The gradients were fractionated on an ISCO Fractionater. The middle and bottom components were collected. The virus was pelleted by a high speed centrifugation at 28,000rpm for 6.5 hr and the pellet was resuspended in 0.05M phosphate buffer.

Extraction of TmRSV RNA

The purified virus particle suspension in 0.05M phosphate buffer was mixed with 10% SDS (sodium dodecyl sulphate), for a final concentration of 1% SDS, and gently vortexed for 2 min at room temperature. Phenol-chloroform extraction was done twice on this mixture, and the virus was precipitated with 3M sodium acetate (0.1 vol), pH 5.0, and chilled ethanol (3 vol). The mixture was kept at -20°C for 2 hr and the viral RNA pelleted by spinning at 14,000g for 15 min. The pellet was resuspended in TE buffer (10mM Tris

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and 1mM EDTA), pH 8.0. The concentration of the RNA was determined with an *uv* spectrophotometer by taking O.D readings at 260nm. The purity of the RNA was determined by taking readings at 260nm and 280nm.

Inoculation of Plum Trees

Nematode Inoculation for the greenhouse study

Twenty five 1-year old 'Stanley'/Myrobalan 29C plum trees were planted in five gallon plastic pots in sterilized soil, in August 1993. The nematode vector *Xiphinema americanum* was added to the soil in the root zone at the rate of 1000±15 per pot. 'National Pickling' cucumber seedlings at the rate of 5-6 per pot were planted in the same pots (Figure 1). The cucumber plants were mechanically inoculated with purified TmRSV at the cotyledon leaf stage and served as source of viral inoculum for the nematodes. As a control *Xiphinema americanum* was added to the soil of five 'Stanley' plum trees at the rate of 1000±15 per pot, and healthy cucumber seedlings served as the bait plants in this group. The plants were maintained in the greenhouse for the entire duration of the study except when they were kept in cold storage to undergo dormancy for 40 days in the winter.

Slash Inoculation for the greenhouse study

Another group of 25 'Stanley'/Myrobalan 29C plum trees were slash inoculated, in September 1993, with purified TmRSV at and a little below the graft union. Sterile razor blades were used to make cuts on the bark and simultaneously a solution of TmRSV in sucrose was dripped with a pasteur pipette on the cut areas (Figure 2). As a control, 10 plum trees were inoculated with sucrose solution in the same way as described above. This group

of plants was maintained in the greenhouse and treated in a manner similar to the nematode inoculated trees above.

Inoculation of plum trees in a field plot in Traverse City, MI.

A field plot was set up at the regional NorthWest Horticultural Research station in Traverse City, MI, in the summer of 1993. The plot was a completely randomised design of 1-year-old plum cultivars of five scions and five rootstocks (Figure 3). The plum rootstocks were Myrobalan 29C, Marianna 4001, Marianna 2624, Marianna GF8-1 and St. Julian 655-2. The scion cultivars used were Stanley, New York 58.900.12, 70031, Valor and Carolyn Harris. Another plot, identical to the above was also planted adjacent, and served as the control for the study. The plum trees in the experimental plot were nematode-inoculated with the vector, *Xiphinema americanum,* and slash inoculated with purified TmRSV in the manner detailed above.

Figure 1. Nematode inoculation of a 'Stanley'/Myrobalan 29C plum tree. Cucumber plants, 5 to 6 in number, were planted around the plum tree and inoculated with TmRSV. Nematodes added to the soil, fed on the roots of the TmRSV infected cucumber plants, acquired the virus and transmitted it to the roots of the plum tree.

Figure 2. Slash inoculation of a 'Stanley'/Myrobalan 29C tree with TmRSV. Cuts were made on the bark, at and below the graft union of the plum tree with a razor blade. Simultaneously a solution of TmSRV was dripped onto the slash injuries with a pasteur pipette, thus inoculating the tree with TmRSV.


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Figure 3. Layout of the field plot at the North West Horticultural research station in Traverse city, MI. The plot was a completely randomized design of five scions and five rootstocks. The plum rootstocks are Myrobalan 29C (Myro 29C), Marianna 4001 (Mar 4001), Marianna GF 8-1 (Mar GF8-1), Marianna 2624 (Mar 2624) and St.Julian 655-2 (St Jul 655-2). The scion cultivars are Stanley (St), New York 58.900.12. (NY), 70031, Valor (Val) and Carolyn Harris (CH). There were two plots such as the one shown here. The southernmost plot was inoculated with TmRSV and the northern plot was a non-inoculated control.

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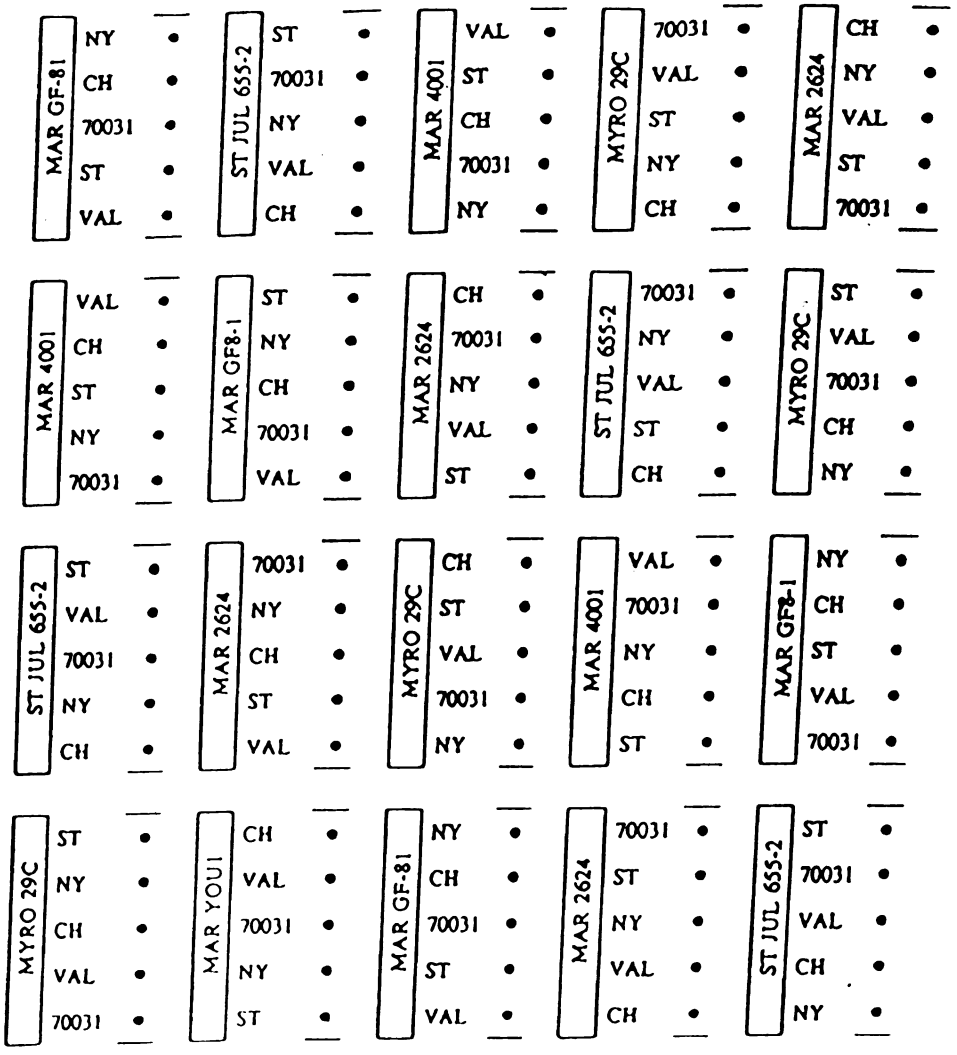
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Construction of the Riboprobe

Miniprep Plasmid DNA Isolation

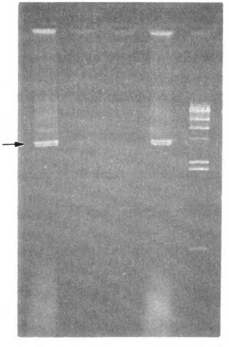
The TmRSV cDNA clone T22 with the SP6 promoter was obtained from Dr. Ahmed Hadidi (USDA, Beltsville, MD). The plasmid was amplified by culturing the host *E.coli* cells (1µl/ml medium) in 2xYT media with Ampicillin selection. The culture was grown overnight by incubating at 37°C and shaking at 400rpm. The miniprep plasmid DNA isolation was done according to the protocol obtained from Dr. Richard Allison's laboratory (Dept. Botany and Plant Pathology, Michigan State University). The culture was centrifuged at 6000 rpm for 3 min and the supernatant was aspirated. Three ml of Solution I (Maniatis, pg 1.25, vol 1) was added to the pellet containing the bacterial cells and the suspension was vortexed. The mixture was allowed to sit at room temperature for 5 min and 6ml of freshly made Solution II (Maniatis, pg 1.26) was added. The tubes were inverted to mix the contents and placed on ice. After 5 min, 4.5ml of 7.5N ammonium acetate was added and the tubes returned to ice for a further 10 min incubation. The contents were centrifuged at 14,000 rpm for 10 min. To the supernatant, 6ml of isopropanol was added and allowed to sit on ice for 1 hr to precipitate the DNA. The mixture was centrifuged at 12,000 rpm for 10 min and the pellet resuspended in 600µl of 2N ammonium acetate. The mixture was shaken for 5 min and kept on ice for 5 min. The contents were centrifuged for 5 min at 14,000 rpm. To the supernatant 600µl of isopropanol was added and incubated on ice for 1 hr to further clean the DNA. The mixture was centrifuged for 10 min at 14,000 rpm and the pellet was resuspended in 25µl of TE, pH 8.0.

Digoxigenin labeling of the riboprobe

The plasmid obtained above was linearised with Eco RI restriction enzyme (Figure 4). One μg of the template was mixed with 2μl of NTP labeling mix, 2μl of 10X transcription buffer, 2μl SP6 RNA polymerase (Genius 4 Kit, Boehringer Manheim Corporation, 9115 Hague Rd, P.O Box 50414, Indianapolis, IN 46250), and 12μl of DEPC-treated double distilled water. The transcription reaction was allowed to proceed for 2 hr at 37°C. Two μl Dnase, Rnase-free was added to the reaction and incubated for 15 min at 37°C to remove the DNA template. The trancription reaction was stopped by adding 2μl of 0.2M EDTA. The reaction was precipitated with 0.1 vol 3M sodium acetate, pH 5.0 and 3 vol chilled ethanol. The contents were mixed and kept at -20°C for 2 hours. The reaction was then centrifuged for 15 min at 13,000g. The supernatant was aspirated and the pellet washed in 70% ethanol. The centrifugation was repeated and the pellet was resuspended in 100μl double distilled water. The digoxigenin labeled probe was stored at -20°C.

Figure 4. Agarose gel electrophoresis of the plasmid T22. From the left, lanes 1 and 4: Eco R1 cut plasmid T22 (arrow), lane 5: Hind III cut Lambda DNA marker.

4



Detection of TmRSV in the Plum trees

Sampling

Bark (0.4 g) and root (0.8g) samples were obtained from each of the greenhouse plum trees for testing with ELISA and Northern hybridization. The samples were wrapped in a wet paper towel and stored on ice in the greenhouse and later stored at 4°C until further processing. The bark samples were obtained from below the graft union with a razor blade (Figure 5). Four or five cuts were made around the tree and a total of 0.4g of bark tissue was obtained. The sample was then cut into smaller pieces and half the sample processed for ELISA and the other half used for total nucleic acid extraction. Samples were also taken from the control trees and processed similarly. The root samples collected were mostly feeder roots. Whenever possible roots from around the tree were collected to get a good representative sample. In the case of the field plot trees, a 2.0g root sample per tree was obtained in August 1995. The sample was divided equally, to be tested by ELISA and Northern hybridization.

Starting with the first test in November 1993, six tests were done on the greenhouse trees for detection of the virus. The other tests were done in February, May and September 1994, and February and July, 1995.

The rootstock sucker leaves, whenever available, where tested by Northern hybridization and ELISA starting with the fifth test. The amount of sample tested per assay varied from 0.5 to 1.0g. The rootstock sucker leaves from the field plot were also tested in August 1995.

Figure 5. Collection of a bark sample from a 'Stanley'/Myrobalan 29C plum tree for testing with ELISA and Northern hybridization. Boat shaped cuts were made on the bark below the graft union of the tree and the sample collected. Four or five cuts were made around the tree to obtain a sample of 0.4g.


5

Enzyme Linked Immunosorbent Assay (ELISA)

Procedure for ELISA

Flat bottom, Immulon^R plates (Dynatech Laboratories, Alexandria, VA 22314) were coated with purified anti-TmRSV gamma globulin diluted 1:1000 (v/v) in coating buffer (0.05M sodium carbonate, sodium bicarbonate buffer, pH 9.6) at the rate of 200µl per well. The plate was placed in a plastic bag and incubated for 6 hr at 37°C. The plates were washed with PBS-tween 20 buffer (0.01M phosphate buffered saline with 0.05 % tween 20), pH 7.4, three times for 5 min each. Samples ground in extraction buffer (PBS with 0.2% chicken egg albumin, 2% polyvinylpyrrolidone and 0.05% tween 20, pH 7.4) were loaded in duplicate wells at the rate of 200µl per well and incubated overnight at 4°C. The plates were rinsed as before. The alkaline phosphatase conjugate at a dilution of 1:800 (v/v) in extraction buffer was added to the plates at the rate of 200µl per well and incubated at 37°C for 2hr. The plates were rinsed as above. The PNP (*p*-nitrophenyl phosphate) substrate (Sigma) dissolved in substrate buffer (9.7% diethanolamine, pH 9.8) at a concentration of 0.5mg/ml, was added. Two readings at absorbance 405nm were taken at 5min intervals after 10 to 15 min had elapsed following addition of the substrate. The mean and standard deviation of the A405nm readings for the healthy controls was determined. The threshold value for positive virus identification was the mean A_{405}nm value plus three standard deviations of healthy control samples. Samples greater than the threshold value were considered positive.

Quantification of the virus detectable with ELISA in leaves, roots and bark tissue

In order to determine the least concentration of TmRSV detectable with ELISA, a standard dilution series of purified virus was tested. Ten two-fold dilutions of TmRSV were made in plum root, bark or leaf extracts, starting with a concentration of 10μg of the virus in the case of leaf and bark and 50μg in the case of root extracts. The ELISA procedure as detailed above was done and the A405nm readings were recorded for all the dilutions. This allowed the estimation of the amount of virus detected in the postive samples by comparison of the readings for the positive with the standard.

Northern Hybridization

Extraction of Total Nucleic Acids

The samples were ground in liquid nitrogen and mixed with DEPC treated NaOH-glycine low salt extraction buffer(0.1M glycine-NaOH, 50mM NaCl, 1mM EDTA, 1% N-lauryl sarcosine, 2% SDS, pH 9.0), 1:2 w/v. The mixture was shaken at room temperature for 15 min and equal amounts of phenol and choloroform : isoamyl alcohol (25:1) were added. The mixture was again shaken for 5 min. A low speed centrifugation at 6,000rpm for 20 min at 4°C was done. The total nucleic acids (TNA) were precipitated in 3 vol chilled ethanol and 0.1 vol 3M sodium acetate, pH 5.0. The nucleic acids were recovered by a low speed centrifugation at 10,000 rpm for 30 min. The pellet was resuspended in 50-100μl of TE, pH 8.0.

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Dot Blotting of the TNA and Hybridization

The total nucleic acid samples were dot-blotted (5 to 10µl) onto a positively charged nylon membrane (Boehringer Mannheim Corp.) prewet with 20X SSC (3M sodium chloride, 300mM sodium citrate, pH 7.0). The nucleic acids were immobilised on the membrane by baking for 1 hr at 80°C. Prehybridization was done at 60°C for 2 hr with gentle agitation. The prehybridization solution consisted of 5X SSC, 50% formamide, 0.02% SDS, 0.1% N-lauryl sarcosine, 2% (w/v) northern blocking reagent (Boehringer Mannheim Corp.), 20mM sodium maleate, pH 7.5. The hybridization solution is the same as the above prehybridization solution with the probe added at a concentration of 20ng/10ml. The hybridization was done overnight at 60°C with gentle agitation. The hybridized membrane was washed twice with gentle agitation in 2X SSC for 15 min at 59°C, followed by a wash at 59°C with 0.1X SSC +0.1% SDS for 30 min.

Chemiluminescent detection with Lumi-Phos 530

The hybridization membrane was washed with Genius Buffer 1 (100mM Tris-HCl, 150mM NaCl, pH 7.5) at room temperature for 5 min. The membrane was blocked with the Northern blocking reagent diluted 1:5 in Genius buffer 1 for 30 min. The membrane was incubated in anti-DIG-alkaline phosphatase diluted 1:10,000 in Northern blocking reagent for 30 min. The membrane was then washed with Genius Buffer 1 for 15 min and Genius buffer 3 (110mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH9.5) for 5 min. Lumi-Phos 530 was spread evenly on the membrane placed between two sheets of acetate paper at the

rate of 0.5ml per $100cm^2$. The membrane was exposed to X-ray film after 2 hr, three times for 5, 15 and 30min.

Quantification of the viral RNA detectable with Northern hybridization

Two fold serial dilutions were made with the TmRSV RNA, with concentrations ranging between 50ng and 0.6pg. The dilution series was dot-blotted and hybridized to the TmRSV riboprobe. The intensity of the signal obtained at each concentration of the known RNA was used to compare with the signal obtained in case of the experimental sample and the amount of viral RNA in the experimental sample was thus determined.

Statistical Analysis

A Chi-square statistical analysis was done on the results obtained by testing root and bark samples by ELISA for NI and SI plum trees, to test the null hypothesis that, there is no difference between the plum trees in the two treatment groups, i.e. nematode and slash inoculation, in their response to TmRSV infection. Yates correction factor (Little and Hills, 1975) was also applied to the analysis.

The results obtained by testing the root and rootsock sucker leaf samples from the Traverse city field plot were subjected to a one way analysis of variance for a completely randomized design, to compare the variability between the results obtained with ELISA and Northern hybridization.

Results

Detection of TmRSV in the plum trees

The results obtained by testing the nematode inoculated (NI) and slash inoculated (SI) plum trees with ELISA and Northern hybridization are presented in tables 1-4 and in tables 6-9 respectively.

Detection of TmRSV with ELISA (Greenhouse Tests)

There was 32% infection of TmRSV in the roots (Table 1) and 48% infection of TmRSV in the bark (Table 2) of nematode inoculated trees at the end of six tests.

There was 84% infection of TmRSV in the roots (Table 3) and 88% infection of TmRSV in the bark (Table 4) of slash inoculated trees at the end of six tests.

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Table 1. Detection of Tomato ringspot virus in the roots of nematode inoculated plum trees with ELISA.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	+	-	-	-	-	-	+
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	+	-	-	-	-	+
5	-	-	-	-	-	-	-
6	-	-	+	-	-	-	+
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	+	-	-	-	-	-	+
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-
22	-	+	-	+	-	-	+
23	-	+	-	+	-	-	+
24	-	-	-	+	-	-	+
25	-	-	-	+	-	-	+
Total	**2**	**3**	**1**	**4**	**0**	**0**	**8**
Percent[a]	**8**	**12**	**4**	**16**	**0**	**0**	**32**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

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Table 2. Detection of Tomato ringspot virus in the bark below the graft union of nematode inoculated plum trees with ELISA.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	positive at least one time
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	+	-	-	+
5	-	-	-	+	-	-	+
6	-	-	-	+	-	-	+
7	-	-	-	+	-	-	+
8	-	-	-	+	-	-	+
9	-	-	-	+	-	-	+
10	-	-	-	+	-	-	+
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	+	+	-	+
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
17	-	-	-	+	-	-	+
18	-	-	-	-	-	+	+
19	-	-	-	+	-	-	+
20	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-
22	-	-	-	-	-	+	+
23	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-
Total	**0**	**0**	**0**	**10**	**1**	**2**	**12**
Percent[a]	**0**	**0**	**0**	**40**	**4**	**8**	**48**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

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Table 3. Detection of Tomato ringspot virus in the roots of slash inoculated plum trees with ELISA.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	-	-	-	+	-	-	+
2	-	-	-	+	-	-	+
3	-	-	-	+	-	-	+
4	-	-	-	+	-	-	+
5	-	-	-	+	-	-	+
6	-	-	+	+	-	-	+
7	-	-	-	+	-	-	+
8	-	-	-	+	-	-	+
9	-	-	-	+	+	-	+
10	-	-	-	+	-	-	+
11	-	-	-	-	-	-	-
12	-	-	-	+	-	-	+
13	-	-	-	+	-	-	+
14	-	-	-	+	-	-	+
15	-	-	-	+	-	+	+
16	-	-	-	+	-	-	+
17	-	-	-	-	-	-	-
18	-	-	+	-	-	-	+
19	-	-	-	-	-	-	-
20	-	-	+	-	-	-	+
21	-	-	-	-	-	-	-
22	-	-	-	-	-	+	+
23	-	-	-	-	-	+	+
24	-	-	-	-	-	+	+
25	-	-	-	+	-	-	+
Total	**0**	**0**	**3**	**16**	**1**	**4**	**21**
Percent[a]	**0**	**0**	**12**	**64**	**4**	**16**	**84**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

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Table 4. Detection of Tomato ringspot virus in the bark below the graft union of slash inoculated plum trees with ELISA.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	-	-	+	-	+	+	+
2	-	-	-	-	-	+	+
3	-	-	-	-	-	+	+
4	-	-	-	-	-	+	+
5	-	-	-	-	-	+	+
6	-	-	-	-	-	+	+
7	-	-	-	-	-	+	+
8	-	-	-	-	-	+	+
9	-	-	-	-	-	+	+
10	-	-	-	-	-	-	-
11	-	-	-	-	-	+	+
12	-	-	-	-	-	+	+
13	-	-	-	-	-	+	+
14	-	-	-	-	-	+	+
15	-	-	-	-	-	+	+
16	-	-	-	-	-	+	+
17	-	-	-	-	-	+	+
18	-	-	-	-	-	+	+
19	-	-	-	-	-	+	+
20	-	-	-	-	-	+	+
21	-	-	-	-	-	+	+
22	-	-	-	-	-	+	+
23	-	-	-	-	-	-	-
24	-	-	-	-	-	+	+
25	-	-	-	-	-	-	-
Total	**0**	**0**	**1**	**0**	**0**	**22**	**22**
Percent[a]	**0**	**0**	**4**	**0**	**0**	**88**	**88**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

Detection of TmRSV in roots and bark of plum trees with ELISA: Percent infection

A high level of TmRSV infection was detected in the plum trees in the tests conducted in September 1994 and July 1995 (Table 5, Figure 6). In the test conducted in September 1994, TmRSV was detected in the bark of 40% of the nematode-inoculated plum trees and in the roots of only 16% of the same group of plum trees. In the same test TmRSV was detected in the roots of 64% of the slash-inoculated plum trees. None of the bark samples from the same group of slash-inoculated trees tested TmRSV-positive. Note that a higher number of trees tested positive for TmRSV in the sample sites that are away from the site of inoculation, i.e. in the bark in case of nematode-inoculated trees and in the roots in case of slash-inoculated trees.

In July 1995 TmRSV was detected in the bark of 88% of the slash-inoculated trees and in the roots of only 16% of the slash-inoculated trees. This result contrasts with the result obtained in September 1994 for slash-inoculated trees.

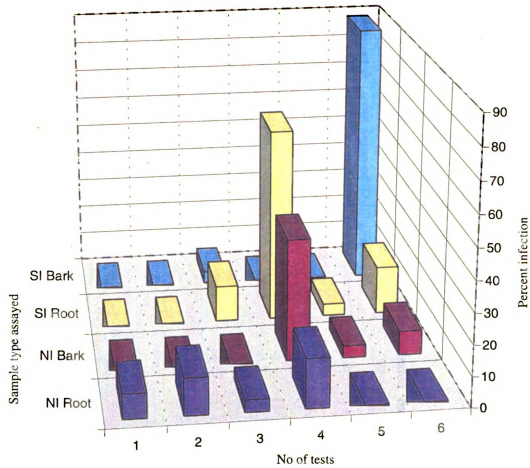
Table 5. Detection of Tomato ringspot virus in roots and bark of plum trees with ELISA : Percent infection.

Test #	NI Root	NI Bark	SI Root	SI Bark
1	8	0	0	0
2	12	0	0	0
3	4	0	12	4
4	16	40	64	0
5	0	4	4	0
6	0	8	16	88

Data in table is in percentage, as a percent of 25 plum trees tested in each test.
Test 1 : Test conducted in November 1993
Test 2 : Test conducted in February 1994
Test 3 : Test conducted in May 1994
Test 4 : Test conducted in September 1994
Test 5 : Test conducted in February 1995
Test 6 : Test conducted in July 1995

Figure 6. Detection of TmRSV in roots and bark of plum trees with ELISA: Percent infection.
NI = Nematode inoculated plum trees. SI = slash inoculated plum trees.
Test 1: Test conducted in November 1993
Test 2: Test conducted in February 1994
Test 3: Test conducted in May 1994
Test 4: Test conducted in September 1994
Test 5: Test conducted in February 1995
Test 6: Test conducted in July 1995
The data for the figure can be obtained from Table 5.

Figure 6. Detection of TmRSV in roots and bark of plum trees with ELISA: Percent infection.



Quantification of the virus detectable with ELISA

The result for the quantification of TmRSV detectable with ELISA in virus-amended plum root tissue is shown in Figure 7. The least detectable quantity of the virus was 24ng/well with an A_{405} nm value of 0.243.

The least detectable amount of the virus detectable in virus-amended plum bark tissue was 20ng/well with an A_{405} nm value of 0.363 (Figure 8).

The least detectable quantity of TmRSV in the virus-amended rootstock leaf was 39ng/well with an A_{405} nm value of 0.602.(Figure 9).

Figure 7. Quantification of TmRSV detectable with ELISA in virus-amended Plum Root tissue

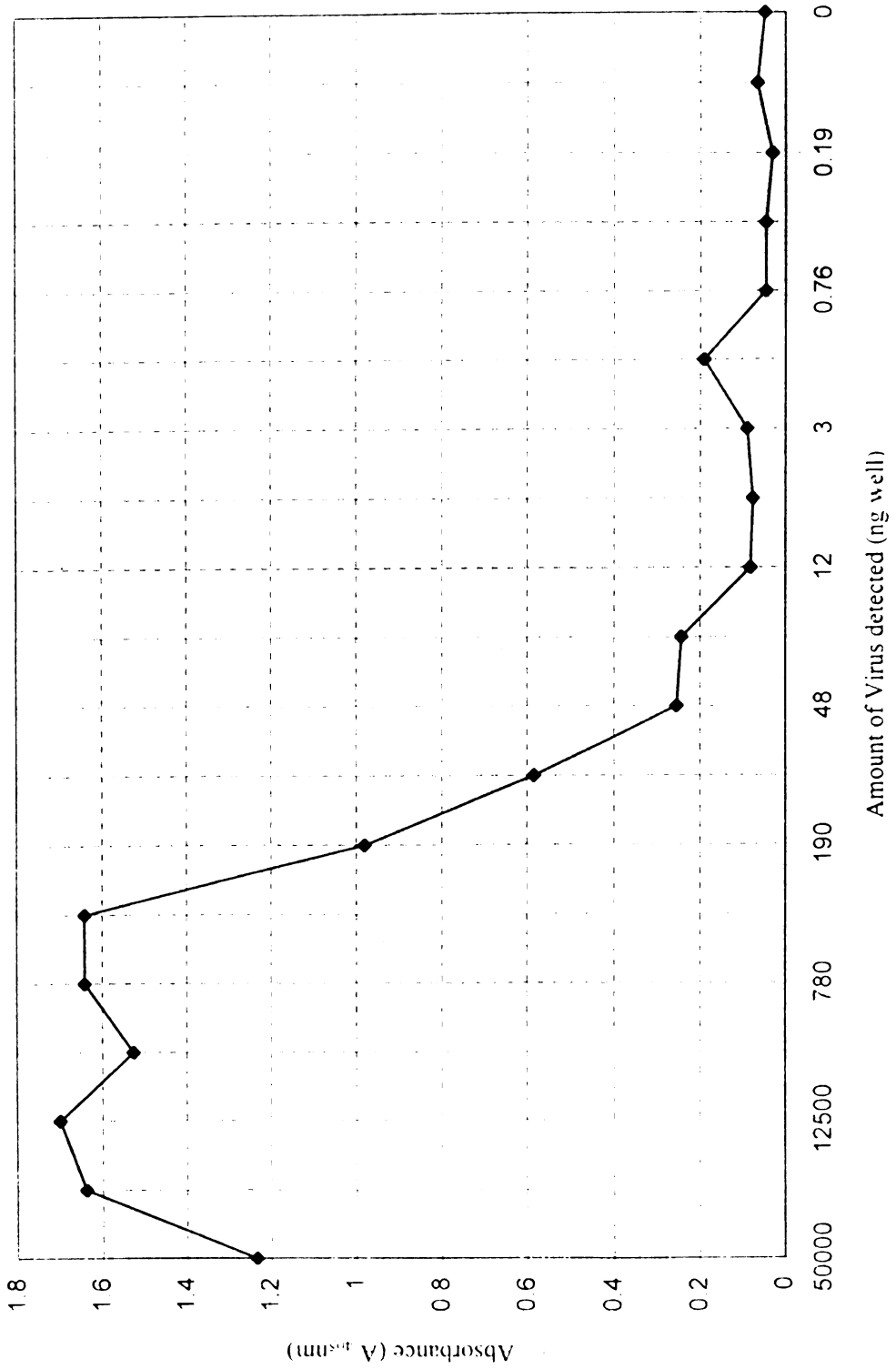


Figure 8. Quantification of TmRSV detectable with ELISA in virus-amended Plum Bark tissue

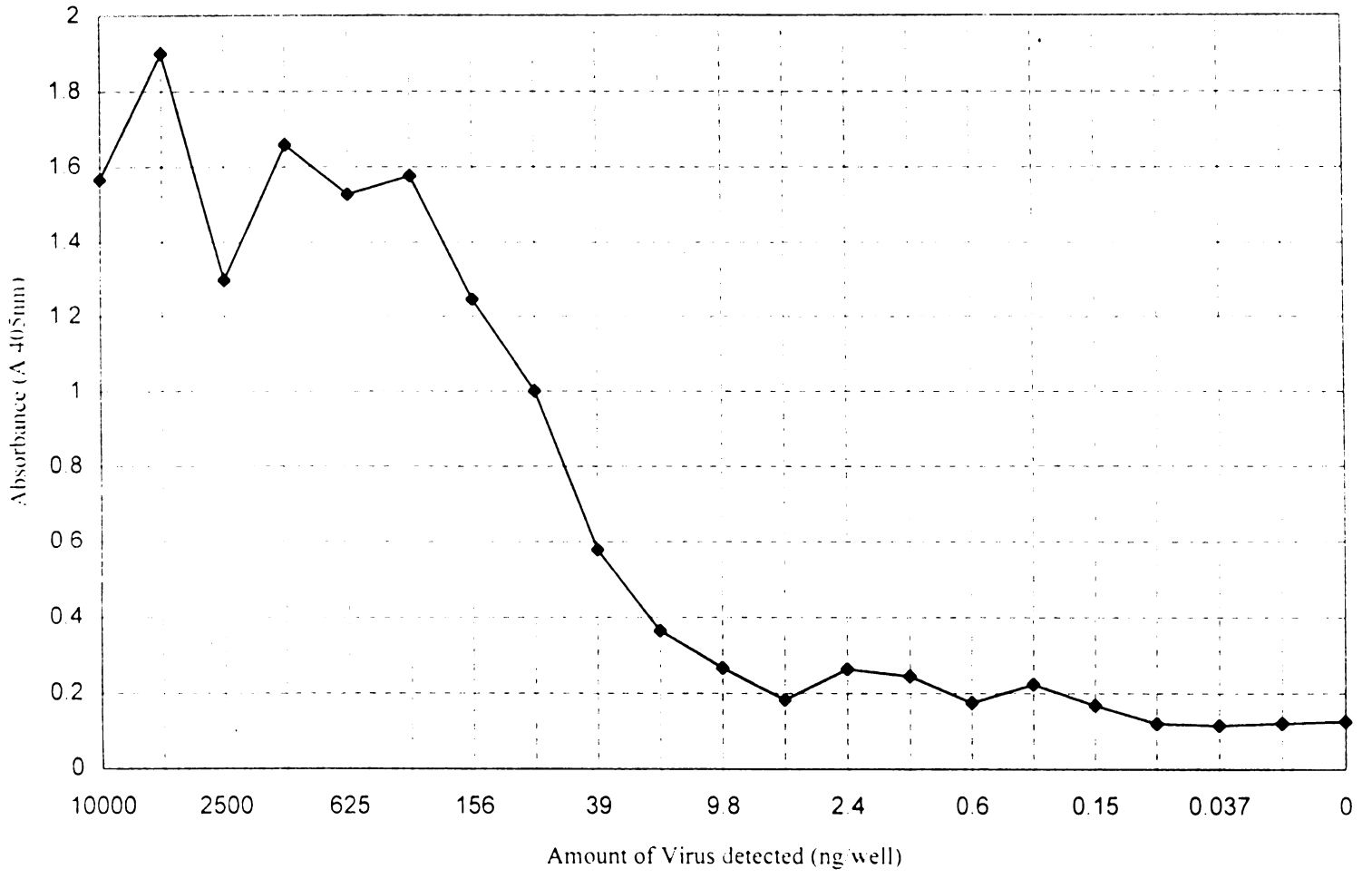
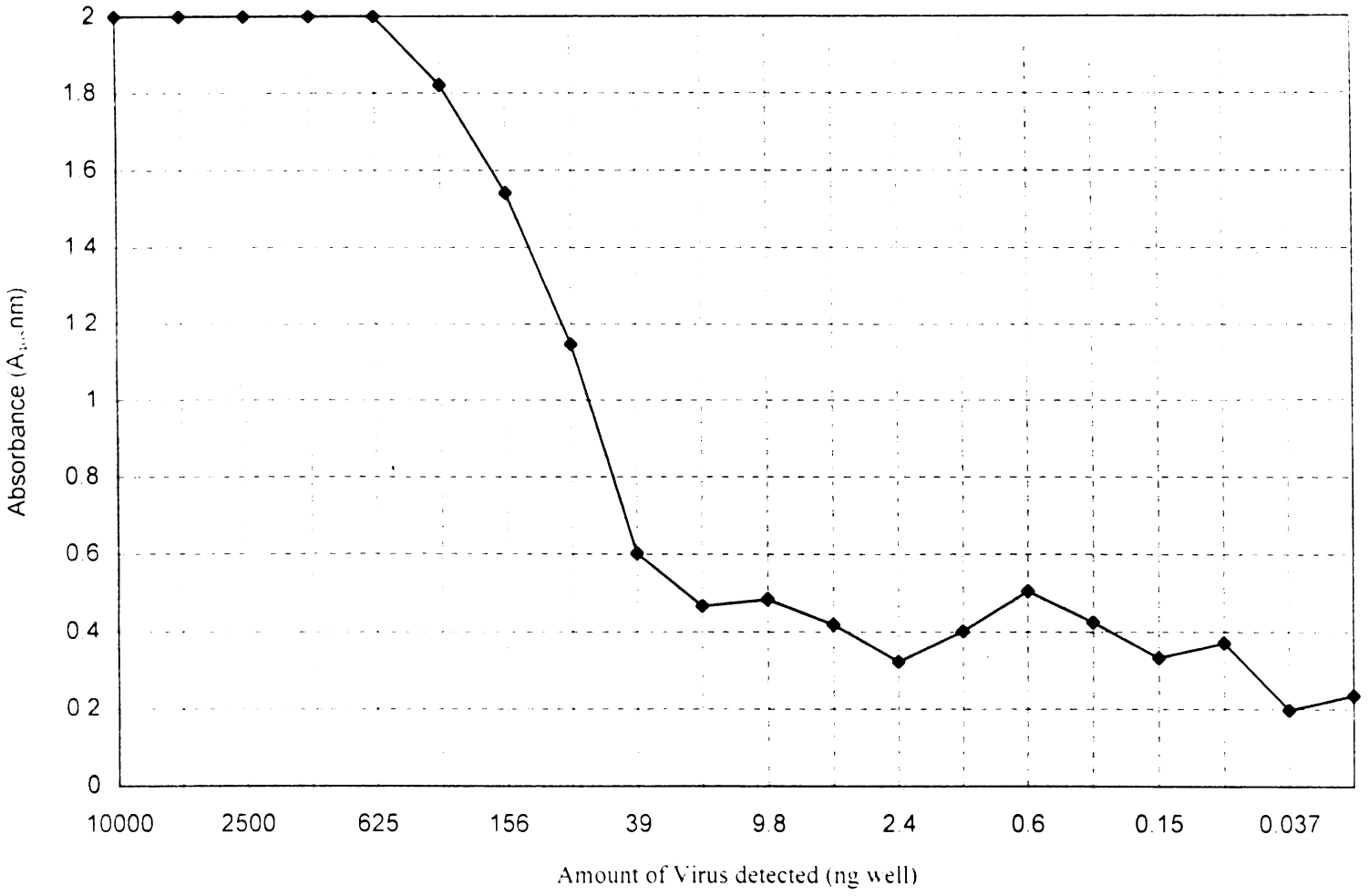


Figure 9. Quantification of TmRSV detectable with ELISA in virus-amended Plum Rootstock sucker leaf tissue



Statistical Analysis of the ELISA results

Chi-square analysis for differences between treatments

A chi-square analysis (Table 6) of the ELISA results for NI root and SI root was done. A chi-square analysis (Table 7) of the ELISA results obtained in case of NI bark and SI bark was also done. The chi-square analysis in both cases revealed a significant difference between the two treatment effects on plum trees in their response to TmRSV infection.

Table 6. A chi-square analysis of the ELISA results obtained for NI root and SI root samples.

Null Hypothesis: There is no difference between nematode inoculation and slash inoculation treatment effects on plum tree roots in their response to TmRSV infection, and any differences in results are due to chance alone.

Treatment type	TmRSV Infected	Uninfected	Total
NI Root[a]			
Observed (O)	8	17	25
Expected (E)	(14.5)	(10.5)	(25)
SI Root[b]			
Observed (O)	21	4	25
Expected (E)	(14.5)	(10.5)	(25)
Total	29	21	50

[a]: Data from Table 1
[b]: Data from Table 3

Expected infected = 29 / 50 x 25
=14.5
Expected uninfected = 21 / 50 x 25
= 10.5
Chi-square value with Yates correction = $[(O-E)-0.5]^2 / E$

= 11.83

The chi-square value is comparable to the chi-square table value with a probability of 0.001 of obtaining a value as large or larger than 10.827. Therefore the chi-square value is significant and the null hypothesis is rejected.

Conclusion: There is a significant difference between nematode and slash inoculation treatment effects on plum tree roots in their response to TmRSV infection.

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Table 7. A chi-square analysis of the ELISA results obtained for NI bark and SI bark samples.

Null Hypothesis: There is no difference between nematode inoculation and slash inoculation treatments effects on plum tree bark in their response to TmRSV infection, and any differences in results are due to chance alone.

Treatment type	TmRSV Infected	Uninfected	Total
NI Bark[a]			
Observed (O)	12	13	25
Expected (E)	(17)	(8)	(25)
SI Bark[b]			
Observed (O)	22	3	25
Expected (E)	(17)	(8)	(25)
Total	34	16	50

[a]: Data from Table 2
[b]: Data from Table 4

Expected infected = 34 /50 x 25
=17

Expected uninfected = 16 / 50 x 25
=8

Chi-square value with Yates correction = $[(O-E)-0.5]^2 / E$

= 7.4448

The chi-square value falls between the chi-square table values of 6.635 and 10.827 with probabilities of 0.01 and 0.001, that the results are due to chance alone. Therefore the chi-square value is significant and the null hypothesis is rejected.

Conclusion: There is significant difference between nematode and slash inoculation treatment effects on plum tree bark in their response to TmRSV infection.

Detection of TmRSV with Northern Hybridization

There was 52% infection by TmRSV in the roots (Table 8) and 24% infection by TmRSV in the bark (Table 9) of nematode inoculated trees at the end of six tests, as detected with northern hybridization. There was 48% infection by TmRSV in the roots (Table 10) and 12% infection by TmRSV in the bark (Table 11) of slash inoculated trees.

A result of the northern dot blot hybridization of total nucleic acids extracted from the roots of plum trees sampled from a field plot in Traverse City , MI., is shown in Figure 10. Note that the positive signal for the total nucleic acids blotted on the membrane, is in the form of an open circle (arrow).

A result for the northern dot blot assay of total nucleic acids, extracted from the bark of slash inoculated 'Stanley'/Myrobalan 29C trees during the test conducted in February 1994, is shown in Figure 11.

Quantification of TmRSV RNA detectable with Northern Hybridization

All amounts of the viral RNA dot blotted, i.e. 50ng - 0.6pg/spot, were detectable with northern hybridization (Figure 12). Note that the spots of the viral RNA are a full circle in contrast to the pattern obtained when total nucleic acids from the roots were dot blotted.

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Table 8. Detection of Tomato ringspot virus in the roots of nematode inoculated plum trees with northern dot blot hybridisation

Tree No	Test 1 09- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	+	-	-	-	-	-	+
2	-	-	-	-	-	-	-
3	-	-	-	-	-	+	+
4	-	+	-	-	-	-	+
5	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	+	-	-	-	-	-	+
16	-	-	+	-	-	+	+
17	-	-	+	-	-	+	+
18	+	-	-	-	-	-	+
19	-	-	+	-	-	+	+
20	-	-	+	-	-	-	+
21	-	+	-	-	-	-	+
22	-	-	+	-	-	-	+
23	-	-	-	-	-	-	-
24	-	-	-	-	-	+	+
25	-	-	+	-	-	-	+
Total	**3**	**2**	**6**	**0**	**0**	**5**	**13**
Percent[a]	**12**	**8**	**24**	**0**	**0**	**20**	**52**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 trees tested.

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Table 9. Detection of Tomato ringspot virus in the bark below the graft union of nematode inoculated plum trees with northern dot blot hybridisation.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-
3	-	-	-	-	-	+	+
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	-	-	-	-	-	+	+
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	+	+
10	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-
12	-	-	-	-	-	+	+
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-
20	-	-	-	-	-	+	+
21	-	-	-	-	-	-	-
22	-	-	-	-	-	+	+
23	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-
Total	**0**	**0**	**0**	**0**	**0**	**6**	**6**
Percent[a]	**0**	**0**	**0**	**0**	**0**	**24**	**24**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

Table 10. Detection of Tomato ringspot virus in the roots of slash inoculated plum trees with northern dot blot hybridization.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 07- '95	Positive at least one time
1	-	-	-	-	-	+	+
2	-	-	-	-	-	-	-
3	-	-	-	-	-	+	+
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	-	-	-	-	-	+	+
7	-	-	-	-	-	+	+
8	-	-	-	-	-	-	-
9	-	-	-	-	-	+	+
10	-	-	-	-	-	+	+
11	-	-	-	-	-	-	-
12	-	-	-	-	-	+	+
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	+	+
16	-	-	-	-	-	+	+
17	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-
22	-	-	-	-	-	+	+
23	-	-	-	-	-	+	+
24	-	-	-	-	-	+	+
25	-	-	-	-	-	-	-
Total	**0**	**0**	**0**	**0**	**0**	**12**	**12**
Percent[a]	**0**	**0**	**0**	**0**	**0**	**48**	**48**

Test 1 = Test conducted in November 1993
Test 2 =Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

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Table 11. Detection of Tomato ringspot virus in the bark below the graft union of slash inoculated plum trees with northern dot blot hybridization.

Tree No	Test 1 11- '93	Test 2 02- '94	Test 3 05- '94	Test 4 09- '94	Test 5 02- '95	Test 6 02- '95	Positive at least one time
1	-	+	-	-	-	+	+
2	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-
6	+	-	-	-	-	-	+
7	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-
11	-	+	-	-	-	-	+
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-
Total	**1**	**2**	**0**	**0**	**0**	**1**	**3**
Percent[a]	**4**	**8**	**0**	**0**	**0**	**4**	**12**

Test 1 = Test conducted in November 1993
Test 2 = Test conducted in February 1994
Test 3 = Test conducted in May 1994
Test 4 = Test conducted in September 1994
Test 5 = Test conducted in February 1995
Test 6 = Test conducted in July 1995
[a] Percent infection, as a percentage of 25 plum trees tested.

Figure 10. Northern dot blot hybridization of total nucleic acids extracted from the roots of plum trees, sampled from a Traverse City, MI., field plot in August 1995. The top blot, rows 1-4 of columns 1-4 contain total nucleic acids (TNA) extracted from the roots of TmRSV inoculated plum trees. In the bottom dot blot, from the left, column 1 is TNAs from the roots of TmRSV inoculated plum trees. Column 2: In row 1 and 2 are TNAs from the leaf of a healthy and TmRSV infected cucumber, respectively. Row 4 in column 2 and all rows in columns 3 and 4 of the bottom dot blot, contain TNAs from the roots of uninoculated plum trees.
Note the open circle pattern obtained with a TmRSV positive root sample (arrow).

10

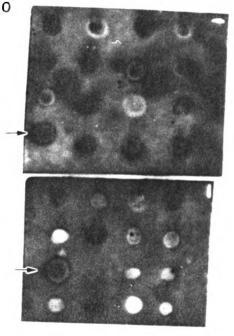


Figure 11. Northern dot blot hybridization of total nucleic acids (TNA) extracted from the bark of slash inoculated 'Stanley'/Myrobalan 29C plum trees, during the test conducted in February 1994. From left, columns 1-3 contain TNAs extracted from the bark of SI trees. Column 4: In rows 1-3 are TNAs extracted from the bark of uninoculated plum trees. In row 4 is DNA of plasmid T22, which was used as a positive control.

11

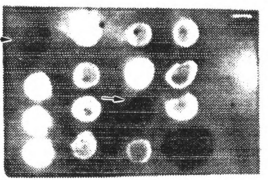
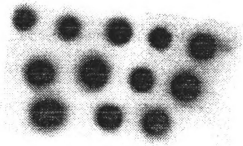


Figure 12. Quantification of TmRSV RNA detectable with Northern hybridization. Two-fold serial dilutions of purified TmRSV RNA were dot blotted. The RNA concentrations were (from the left, top row) 0.6pg, 1.25pg, 2.5pg, 5.0pg, 0.01ng, 0.03ng, 0.06ng, 0.13ng, 0.27ng, 0.55ng, 1.1ng, 2.2ng.

12



Comparison of ELISA and Northern Hybridization

A comparison of ELISA and northern hybridization in terms of detected percent infection of TmRSV in plum trees is given in Table 12. At the end of six tests ELISA was more effective than northern hybridization in detecting TmRSV in the case of NI bark, SI root and SI bark samples. Northern hybridization was more effective than ELISA in the case of NI root samples, detecting TmRSV in 52% of the trees.

A comparison of ELISA and northern hybridisation in terms of detected percent infection of TmRSV in rootstock sucker leaf samples is shown in Table 13. Northern hybridization was more efficient than ELISA in this case. In both the tests conducted in February and July, 1995, a higher percent of TmRSV-positive trees were detected with northern hybridization.

Table 12. Comparison of ELISA and northern hybridization relative to detected percent infection of TmRSV in plum trees.

Test No	NI Root		NI Bark		SI Root		SI Bark	
	ELISA	N.Hyb	ELISA	N.Hyb	ELISA	N.Hyb	ELISA	N.Hyb
1	8	12	0	0	0	0	0	4
2	12	8	0	0	0	0	0	8
3	4	24	0	0	12	0	4	0
4	16	0	40	0	64	0	0	0
5	0	0	4	0	4	0	0	0
6	0	20	8	24	16	48	88	4
Total Percent infection[a]	32	52	48	24	84	48	88	12

N.Hyb = northern hybridization

[a]: Total percent infection at the end of six tests, as a percentage of 25 plum trees tested. Data in the table are expressed in percentage and is the result obtained in the individual tests.

Test 1: Test conducted in November 1993
Test 2: Test conducted in February 1994
Test 3: Test conducted in May 1994
Test 4: Test conducted in September 1994
Test 5: Test conducted in February 1995
Test 6: Test conducted in July 1995

Table 13. Detection of TmRSV in rootstock sucker leaf samples with ELISA and northern hybridization: Percent Infection.

Sample type	Test 5		Test 6	
	ELISA	N.Hyb	ELISA	N.Hyb
NI Leaf	50	100	80	100
SI Leaf	0	20	83	100

NI Leaf : Sample from Nematode inoculated plum trees
SI Leaf : Sample from Slash inoculated plum trees
Test 5 : Test conducted in February 1995
Test 6 : Test conducted in July 1995

Testing of plum trees from field plot in Traverse city, MI.

The results obtained by testing the plum trees from the field plot in Traverse City, MI., in August 1995 are presented in Table 14. A comparison of ELISA and northern hybridization is also done in this table. ELISA appeared to be more efficient than northern hybridization in detecting TmRSV in root samples. However, a statistical analysis of the ELISA and northern hybridization results showed no significant difference between the results obtained with the two types of tests (Table 15).

Northern hybridization was more effective than ELISA in detecting TmRSV in a higher percent of the rootstock sucker leaf samples. A statistical analysis of the results obtained with ELISA and northern hybridization showed a significant difference between the results obtained with the two tests (Table 16).

A comparison of the five rootstocks planted in the Traverse city field plot in terms of percent trees detected by ELISA and northern hybridization is shown in Table 17. In all cases ELISA detected a higher percent of TmRSV infection in the roots than northern hybridization. Rootstock GF8-1 had the highest percent infection of TmRSV in the roots and rootstock Marianna 4001 had the lowest percent infection of TmRSV in the roots.

Northern hybridization detected TmRSV in a higher percentage of trees than ELISA when rootstock sucker leaf samples from Marianna 2624, Marianna GF8-1 and Marianna 4001 were tested. Eighty three percent of the rootstock sucker leaf samples from GF8-1 were TmRSV-positive with northern hybridization whereas, none were TmRSV-positive with ELISA. Similarly, 57% of Marianna 2624 and 50% of Marianna

4001 rootstock sucker leaf samples were TmRSV-positive with northern hybridization while none were TmRSV-positive with ELISA. An equal percentage of rootstock sucker leaf samples were TmRSV-positive with ELISA and northern hybridization in the case of Myrobalan 29C and St. Julian 655-2 rootstocks.

Table 14. Testing of plum trees from a field plot in Traverse City, MI. A comparison of ELISA and northern Hybridization.

Sample	ELISA (E)	Hybridization (H)	E + H[a]	E and/or H[b]
Root	31/88 (35%)	21/88 (24%)	13/88 (15%)	37/88 (42%)
Leaf	5/26 (19%)	17/26 (65%)	2/26 (8%)	20/26 (77%)
Total	36/88 (41%)	35/88 (40%)	14/88 (16%)	

Test conducted in August 1995

[a] Percentage of trees TmRSV-positive with both ELISA and northern hybridization.

[b] Percentage of trees TmRSV-positive, combining the data obtained with ELISA and/or northern hybridization.

Table 15. Statistical analysis of the results obtained by testing root samples from Traverse City, MI., for TmRSV with ELISA and Northern hybridization. Each row in the plot was considered a replication and the number of TmRSV-positive samples per row were treated as data for that row.

Treatment	Replications					Total
	1	2	3	4	5	
ELISA	7	9	7	4	4	31
N. Hyb	3	4	4	7	3	21

ANOVA

	df	SS	MS	F	Required F	
					5 %	1 %
Total	9	39.6				
Treat	1	10	10	2.70	5.32	11.26
Error	8	29.6	3.7			

Conclusion: F is not significant. There is no significant difference between the results obtained by testing root samples from Traverse City, MI., for TmRSV with ELISA and Northern hybridization.

Table 16. Statistical analysis of the results obtained by testing the rootstock sucker leaf samples from Traverse City, MI., for TmRSV with ELISA and Northern hybridization. Each row in the plot was considered a replication and the number of TmRSV-positive samples per row were treated as data for that row.

Treatment	Replications					Total
	1	2	3	4	5	
ELISA	3	0	1	1	0	5
N.Hyb	5	6	1	2	3	17

ANOVA

	df	SS	MS	F	Required F	
					10 %	5 %
Total	9	37.6				
Treatment	1	14.4	14.4	4.9	3.42	5.32
Error	8	23.2	2.9			

Conclusion: F is significant at $P = 0.10$
There is a statistically significant difference between the results obtained with ELISA and Northern hybridization when rootstock sucker leaf samples were tested for TmRSV infection.

Table 17. Testing of plum trees from a field plot in Traverse City, MI. A comparison of five rootstocks relative to the percentage of trees detected by ELISA and N. Hybridization.

Rootstock type	Root			Rootstock Leaf		
	ELISA	N.Hyb	E + H[a]	ELISA	N.Hyb	E + H[a]
M2624	38	22	17	0	57	0
M29C	35	24	12	80	80	40
GF8-1	40	15	10	0	83	0
M4001	27	22	11	0	50	0
St.Julian 655-2	33	40	27	50	50	0

Test conducted in August 1995
Data in the table are in percentage.
N.Hyb = northern Hybridization
[a] Percentage of trees that were TmRSV-positive with ELISA and northern hybridization.

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Discussion

Comparison of ELISA and Northern Hybridization

Comparing the results obtained with ELISA and northern hybridization, in the six tests conducted on the 'Stanley'/Myrobalan 29C trees, one can see that ELISA detected a higher percentage of infection of TmRSV in the NI bark, SI bark and SI root samples. Northern hybridization detected a higher percentage of TmRSV infection in case of NI root samples only. The test conducted on the roots of the plum trees from the Traverse City, MI., field plot, also show that ELISA detected a higher percentage of TmRSV infection than Northern hybridization. However, a statistical analysis of the results showed no significant differences between the results obtained with the two assays. In comparing ELISA and Northern hybridization, one must keep in mind that the two tests detect two different components of the virus. The TmRSV antibodies used in ELISA detect the coat protein, whereas, the riboprobe employed in Northern hybridization detects the RNA of the virus. Sucrose density gradients of purified TmRSV, when fractionated, show three peaks (Stace-Smith and Ramsdell, 1987). The top peak consists of the empty capsids of the virus, the second peak the capsids containing RNA-2, and the third peak corresponds to the capsids containing RNA-1. The sucrose density gradient pattern shows that overall there is an excess of viral coat protein. Therefore, one might reasonably expect ELISA to be more efficient than Northern hybridization in detecting TmRSV in virus infected plant tissue.

Northern hybridization on the other hand, is a more sensitive technique than ELISA. Hadidi and Hammond (1988), detected 1.0 pg/spot of TmRSV RNA using a riboprobe that was transcribed from a cDNA clone, similar to the clone T22 used in this study. In the present study, 0.6 pg/spot of purified TmRSV RNA was detectable with Northern hybridization, whereas, the ELISA detectable range of TmRSV in virus amended tissue was 20-39 ng/well, depending on whether it was a bark, root or leaf sample. Therefore, considering that Northern hybridization is a more sensitive technique than ELISA, it may not be out of place to compare the relative efficiency of the two techniques, in spite of the fact that there is an excess of coat protein in virus infected tissue.

One of the reasons for the poor performance of Northern hybridization may follow directly from a result stated above, i.e. Northern hybridization detected 0.6 pg/spot of purified viral RNA. The low detection of TmRSV may be due to a deficiency in the total nucleic acid (TNA) extraction procedure. It may be necessary to further purify the viral RNA from the TNAs extracted. Using an extraction procedure different from the one used in this study, Powell et al. (1991) found that hybridization was more efficient than ELISA in detecting TmRSV infection of nectarine trees when bark extracts were analyzed, but ELISA was more efficient when root extracts were analyzed. The authors did not state a reason for these differences. The buffer employed by Powell et al., 1991, was also tried in the total nucleic acid extraction from plum root and bark tissue. This buffer did not result in any improved detection of TmRSV RNA. Another probable cause,

for the low levels of detection of TmRSV in roots and bark by Northern hybridization, may be that some interfering plant compounds were precipitated with the total nucleic acids. The presence of interfering compounds, e.g. phenols and quinones, in woody plant tissue, present a problem in the extraction of nucleic acids. These compounds oxidize rapidly, causing darkening of tissue homogenates, and form complexes with plant proteins and organelles (Rezaian et al. 1987). The total nucleic acids extracted from the plum root and bark tissue may have had protein complexes with interfering compounds, precipitated along with the nucleic acids. This may help explain the diffused ring pattern obtained on the autoradiographic film when the total nucleic acids from root and bark samples were dot-blotted. Such ring patterns were absent when the total nucleic acids extracted from the rootstock sucker leaf, were dot-blotted. Faced with the possibility of unknown interfering compounds that form complexes with plant proteins in the woody plant tissue, a remedy to this problem may be to develop a total nucleic acid extraction procedure, using a variety of protein denaturing compounds in the extraction buffer. A denatured protein in the plant cell may be less likely to form complexes with the oxidized phenols, quinones or other interfering compounds. Sodium perchlorate, a dissociating and protein denaturing chaotropic salt, may be the next compound in the list of possible protein denaturing agents that needs to be tried for the successful extraction of total nucleic acids from plum bark and root tissue. Rezaian et al. (1991) have reported successful extraction of nucleic acids from grapevine leaf tissue using the above mentioned compound in the extraction buffer. Other factors that may have contributed to the low detection of the virus, are the uneven distribution of the virus in the plant, the low

titres of the virus in woody plants, and the small sample size used in this study (0.2g and 0.4g in case of bark and root, respectively).

Northern hybridization detected a higher percentage of TmRSV infection than ELISA in the rootstock sucker leaf samples from the greenhouse study and the Traverse City, MI., field plot. Statistical analysis of the results from the Traverse City, MI., field plot, showed a significant difference between the results obtained with the two assays. Extraction of total nucleic acids from the plum sucker leaf apparently is not a problem of interfering compounds. The dot-blot of the nucleic acids from the leaf samples developed a full circle pattern, as opposed to the ring pattern or empty circle, that characterizes the dot blot of the nucleic acids from the bark and root. The low detection of TmRSV by ELISA in rootstock sucker leaf samples however, is surprising. The least detectable quantity of TmRSV in virus- amended rootstock leaf was 39 ng/well with an A_{405}nm value of 0.602. This is higher than the least detectable quantity of TmRSV in virus-amended root and bark samples. Lister et al. (1980), suggested that extracts of bark may be more reliable than extracts of rootstock sprout leaves in apple trees, for a uniform and efficient detection of TmRSV by ELISA. They suggested erratic distribution of the virus in the rootstock sprouts as the probable cause. Bitterlin et al. (1984), suggested that the time of the year is also an important factor for virus detection in different parts of woody plants. Tomato ringspot virus was consistently detected in the leaves of orchard peach trees in May and in the roots and bark in July (Bitterlin et al., 1984). Plum leaf tissue may have compounds that interfere with the sensitive detection of the virus by ELISA. These

compounds may interfere with the antibody binding and thus result in decreased detection levels of the virus.

Since the results obtained with Northern hybridization do not seem to be a reliable estimate of TmRSV infection in the root and bark of 'Stanley'/Myrobalan 29C plum trees, further analysis in this study was made with the ELISA results only.

Comparison of the effects of two inoculation methods

The aim of the study, in which two groups of 'Stanley'/Myrobalan 29C plum trees were inoculated with two different methods of inoculation, was to compare the inoculation treatment effects on the onset of the PBL disease. Tomato ringspot virus is vectored by the nematode vector *Xiphinema americanum* Cobb, 1913. Nematode inoculation with *X. americanum*, using TmRSV infected cucumber as a source of inoculum, is comparable to the natural transmission mode of the virus. Slash inoculation, a mechanical transmission method, is comparable to the situation where the virus is introduced into the plant through injuries or through infected rootstock planting material. None of the trees in either group developed a BL at the end of the study. A higher percent of TmRSV infection was detected in the root (84%) and bark (88%) samples of slash inoculated plum than in the root (32%) and bark (48%) samples of nematode inoculated plum trees with ELISA. A chi-square analysis of the ELISA results obtained at the end of six tests, indicates a significant difference between the two treatment effects on 'Stanley'/Myrobalan 29C plum. In a study comparing different methods of mechanical transmission of TmRSV to peach, Bitterlin et al. (1987), found the "knife slash"

technique to give the highest and most consistent transmission rate. In the present study slash inoculation gave a higher infection rate than nematode inoculation.

High rates of TmRSV infection were detected in the fourth and sixth tests, conducted in September 1994 and July 1995, respectively. The plum trees were brought out of induced dormancy towards the end of April each year, and the season of growth for the plants was from this time onwards until the onset of the Fall season. It is notable that the high rates of TmRSV infection were detectable during this period of growth. The new Spring growth and the associated increased levels of transportation of food and water in the plant, likely enabled the virus to multiply and spread systemically. The ELISA results for the slash inoculated group of trees obtained in the fourth and sixth tests (Figure 6, Table 5), indicate that the virus is systemic. However, it is not known from this study if slash inoculation is more effective than nematode inoculation in initiating a BL at the graft union of a plum tree. Since the BL is a result of the pathogenesis of TmRSV in the plum tree, it is expected that slash inoculation will be more effective than nematode inoculation in initiating a BL.

Chapter I - Summary

Two groups of 'Stanley'/Myrobalan 29C trees each were nematode and slash inoculated to compare the inoculation treatment effects on the onset of the PBL disease. Over a 2 yr period of study neither of the two groups of plants developed a BL. A higher percentage of TmRSV infection was detected in slash inoculated trees than in nematode inoculated trees when root and bark samples were tested with ELISA. The results also indicate that the slash inoculated group of trees are systemically infected. A high percentage of TmRSV infection was detected in the period between May and September. This period corresponded with the growth period of the plants following a period of artificially induced dormancy. A chi-square analysis of the ELISA results showed significant difference between the two treatments. The results obtained when root and bark samples were tested with northern hybridization suggest a deficiency in the nucleic acid extraction procedure, probably due to interfering compounds in the root and bark, thus leading to a low recovery of the viral RNA from the test samples. In case of rootstock sucker leaf samples, the viral nucleic acid was detected by northern hybridization in a significantly higher number of samples than by ELISA, suggesting, hybridization should be used to detect the virus from rootstock sucker leaf samples.

Results of ELISA and northern hybridization on root and rootstock sucker leaf samples from a TmRSV inoculated field plot at Traverse City, MI., showed a well distributed pattern of infection. A total of 14 PBL-affected trees were noted in the field plot, when surveyed 2.5 yr and 3yr after inoculation. An anatomical study of bark sampled from the BL region is presented in chapter II.

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Chapter II

An Anatomical study of the BL and Immunolocalization of TmRSV

Literature Review

I A. Hypersensitive response

Prune Brown line disease is the result of an hypersensitive response (HR) of the resistant scion to TmRSV infection in a susceptible rootstock (Hoy and Mircetich, 1984).

According to R.K.S.Wood, an hypersensitive response is a general phenomenon that develops in plants infected by obligate, "intermediate" types of fungi, such as *Phytophthora infestans* and the facultative fungi (Goodman and Novacky, 1994). The infection of the host is followed by the rapid death of both the invaded and some neighboring cells. Goodman and Novacky (1994) said that HR involves the extremely rapid death of only a few host cells which limits the progression of the infection.

a. Virus-induced hypersensitive response

In virus-induced HR the rate at which cells die as a consequence of exposure to a virus is crucial. If relatively few host cells are inoculated and die before extensive virus replication and transcellular migration occurs, both necrosis and localization develop rapidly. However, if cell death is slow, permitting both replication and systemic movement, it is apparent that although necrosis develops, it may be at a rate too slow to prevent local lesion size increase and systemic spread of the virus. Virus-induced HR is cytologically disruptive. An hypersensitive reaction is a cytotoxic response that develops

quickly in host cells sensitive to avirulent gene products of viral plant pathogens. As a consequence of virus infection, the hypersensitive host mounts a series of diverse and complex resistance reactions. The deposition of callose, lignin and glycoproteins may occur and these are the detectable features of resistance. The synthesis of specific antiviral factors and localising envelopments may contribute to the suppression of the viral pathogen (Goodman and Novacky, 1994).

b. Hypersensitive Reaction and Graft Incompatibility

Cummins and Gonsalves (1986) suggested that PBL is analogous to Apple union necrosis and decline (AUND) disease in apple, and that PBL is an hypersensitive reaction of a resistant scion to TmRSV infection in a susceptible but tolerant rootstock. However Tuttle and Gotleib (1985) suggested that AUND in Delicious/MM106 trees is the result of a delayed graft incompatibility between the scion and stock.

Symptoms induced by virus infections such as stem pitting in peach, brown line at the graft union in trees such as walnut, plum and peach, are similar to those seen in graft incompatibility associated with virus infection (Mosse, 1962). The symptoms are considered the result of an hypersensitive reaction of the cultivar to the virus. An hypersensitive reaction is characterized by the rapid death of recently infected cells or cells surrounding an infection site preventing spread of a pathogen. Such a varietal sensitivity to a virus can be distinguished from a graft incompatibility associated with virus infection keeping in mind that the interaction between a particular stock and scion characterizes graft incompatibility (Mosse, 1962). For example, in tristeza virus infection of sweet on sour orange trees, if the scion is removed, the rootstock will recover and it

can be subsequently grafted with other scions, although it remains infected (Mosse, 1962).

Another example of graft incompatibility due to virus infection is seen in AUND affected Delicious/MM 106 trees infected with TmRSV, where histopathological changes began after at least 8 yr of compatible growth. The relative number of ray and axial parenchyma cells increased greatly at the union and above it. Abnormal tissue interfered with mobilization or translocation of carbohydrates. Pegs of periderm-like tissue developed in the xylem at the union and less frequently in the phloem and cambium. These pegs did not resemble necrotic local lesions associated with hypersensitive reactions (Tuttle and Gotleib, 1985).

B. Wound

A wound is any external or internal injury that breaches the outer protective layers of the plant and leads to the destruction of cells in a specific area of tissue (Bostock and Stermer, 1989). Precise information about wound responses in plants is essential for understanding the processes that facilitate or interfere with the development of many diseases. Plant responses to wounding and to infection by pathogens are often similar in many ways. The corky lesions and barriers observed in diseases such as fruit russeting and the shot hole diseases affecting *Prunus* spp. share the anatomical features of wound periderms, and many of the chemical and structural barriers associated with expression of

resistance to pathogens are also induced by mechanical injury (Bostock and Stermer, 1989).

a. Wound Response

In plants, the first anatomical reaction to a wound is usually the formation of a closing, or separating layer. Its function may be to provide protection against microbial invasion and desiccation during the formation of the second and more permanent barrier, the wound periderm (Wier et al., 1996). The formation of impervious tissue is the most common feature observed in the wound responses of herbaceous and woody plants (Bostock and Stermer, 1989). Many of the responses associated with hypersensitive and non-host resistance also occur during wound healing.

i. Types of wound response

Bostock and Stermer (1989), have distinguished three types of wound response in plants:

1. Autolysis and death of cells occur at the wound surface, and cells immediately adjacent to the surface become infused with an extensive layer of lignin or other phenols.
2. In the second type of wound response cell division and proliferation take place at the wound surface in addition to the changes mentioned above.
3. The third type of wound response is the most complex and entails autolysis and death of cells adjacent to the wound surface. A redifferentiation of parenchyma cells present at the time of wounding occurs to form an impervious, lignosuberised boundary zone. Meristematic activity of cells internal to the boundary zone leads to the formation of a suberized wound periderm.

b. Wound Healing

The majority of mature, vacuolate plant cells, without being separated from the mother organism, are capable of rejuvenation, i.e., they can be induced to divide and to resume growth, though to different degrees in various cells types, and also depending on the location of the plant. Under comparable conditions of growth, one and the same plant type usually exhibits some uniformity of reaction, but considerable differences occur between various plant types (Bloch, 1941).

Wound healing is of the following types (Bloch, 1941):

(1) In the terminal growing regions of the shoot and the root, the outermost, completely undifferentiated parts of the indeterminate meristem may reproduce parts lost by injury completely and more or less directly from cells abutting on the cut surface.

(2) Further distant from the apex the responses become more complicated. Wound repair here is often affected by secondary meristems such as phellogen, cambium and sometimes calluses from primary or secondary tissue. Original differentiation is influenced in various ways by both inner and outer conditions, and may become inhibited as well as accelerated.

(3) In plants or organs of low reactivity or in matured zones, cells of the ground tissue may not divide at all. It has been found, however, that in many cases redifferentiation, such as specialised thickening and chemical changes in the wall, may still be induced, and the tissue pattern near the injury may thus become partly restored.

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C. Types of periderm

The development of natural and wound periderms was considered to be the same except for stimulus and sites of occurrence (Esau, 1965; Srivastava, 1964). However Mullick proposed a classification of periderms in woody plants to distinguish two types of periderms (Mullick, 1971; 1976, and Mullick and Jensen, 1973):

a. Necrophylactic periderm: Such a periderm includes wound periderms, certain sequent periderms, and impervious layers formed beneath resin blisters and around abscission scars (Mullick, 1971). This type of periderm is distinguished from a normal periderm by its pigmentation and site of occurrence (Mullick and Jensen, 1973). The necrophylactic periderm has reddish purple pigments in most woody plants and is distinct from the brown colored natural periderms. In instances where there is no reddish purple pigmentation, the necrophylactic periderm has pigments that distinguish it from the natural periderm. Chromatographic analysis of the reddish purple pigments revealed several classes of pigments which have not been identified. However the majority of the pigments are non-anthocyanic and anthocyanic pigments constitute only a minor component of this class of pigments (Mullick, 1969). A necrophylactic periderm always forms adjacent to dead tissue, whenever necrotic tissues are present, irrespective of the causal agent of injury, providing some protection to living tissue from the necrotic tissue. Necrophylactic periderm formation is preceded by the formation of a non-suberized impervious tissue (Mullick,1975; and Mullick and Jensen, 1976).

b. Exophylactic periderm: This class of periderms include the first periderm and sequent periderms that contain similar pigments. The exophylactic periderms provide protection to the living tissues from the environment. Exophylactic periderm formation at the site of a wound is always preceded by the formation of a necrophylactic periderm. Sometimes more than one necrophylactic periderm is formed before an exophylactic periderm forms. Once an exophylactic periderm forms, a break may occur in the reddish purple necrophylactic periderm along with the associated phellem cells, causing en masse sloughing or scaling. The reddish purple periderm cells adhering to the exophylactic periderm weather away leaving the exophylactic periderm at the surface. After this loss, the area is returned to a condition similar to its original state, covered by a periderm which is chemically and histologically indistinguishable from the original periderm (Mullick and Jensen, 1973).

D. Immunogold labeling

The principle of immunogold labeling is simple: specific primary antibodies are used as affinity probes for antigens exposed at the cut surface of the section. Colloidal gold particles conjugated to a secondary antibody or to protein-A attach to the bound primary antibody and thus serve as electron opaque markers of the antigen when examined in the electron microscope. This technique, however, is not limited to the electron microscope only. Silver enhancement of the colloidal gold by deposition of metallic silver on the gold is adopted for light microscopic visualization of the gold label (Vandenbosch, 1991).

Colloidal gold particles must be stabilized with macromolecules to avoid flocculation when placed in an electrolyte solution. A wide variety of proteins, glycoproteins and carbohydrates may be used as stabilizing macromolecules. The most common for immunocytochemistry are protein-A and secondary antibodies (Bendayan, 1984).

Protein-A-gold is widely used for immunolabeling, especially for the detection of rabbit antibodies. Protein-A derived from *Staphylococcus aureus* binds to the Fc portion of immunoglobulinG (IgG), and does not interfere with the antibody's ability to bind to an antigen. Protein-A binds with low affinity to IgGs from some species, notably goats, rats and mice (Bendayan ,1984).

a. Post-embedding labeling: Specimen preparation

In the past decade, post-embedding immunogold labeling of sectioned, resin-embedded tissues has become the most popular immunocytochemical technique .

i. Fixation

The aim of chemical fixation is to have the sample preserved in the state most near to the original life-like state of the sample. Double fixation with aldehydes and osmium tetroxide is the method commonly used in electron microscopy of plant cells. The other fixatives that have been used in the past are potassium permanganate and permanganates of sodium and lithium. The fixation with such oxidative fixatives eliminates the requirement to stain the sections. However the fixation with aldehydes is the most widely preferred method because of the superiority in the preservation of cellular structure (Roland and Vian, 1991; Flegler et al., 1993).

The chemical fixatives used should be able to create stable and numerous crosslinks in the specimen. They should rapidly infiltrate and permeate the mass of the specimen. The primary fixation step forms a crosslink with the polypeptide chains of the cell. Monoaldehydes such as paraformaldehyde (1-10%) and dialdehydes such as glutaraldehyde (1-10%) have been recognized as excellent primary fixatives for plant electron microscopy studies. Although acrolein penetrates faster than glutaraldehyde, it is avoided whenever possible because of its lacriminatory effect on the user and it's unpleasant odor (Roland and Vian, 1991). The fixation of cytoplasm in acrolein can lead to a grainy and coarse appearance.

Fixation times can vary from several hours to days either at room temperature or at 4°C, depending on the kind of sample being fixed. Difficult to infiltrate materials such as pollen, spores, wood and other plant parts that have a secondary tissue require a longer period as compared to meristematic tissue that require only a couple of hours.

The specimens once sampled should be kept in the fixative immediately and if possible, cut into smaller pieces in the fixative. Plant tissues have a lot of intercellular spaces and the gases in these spaces need to be removed during fixation. It is preferable to do the initial stages of the primary fixation under very light vacuum for this purpose. At the end of the treatment the samples should sink to the bottom of the vial. The necessity of rapid fixation is important because there may be a fast traumatic reaction induced in the tissue by handling. Cell changes may occur due to excision of tissue. Osmolarity and pH of the buffer is an important consideration. The plant cells have more than 80% of their volume as a vacuolar apparatus. The contents of the vacuole are generally acidic and

sometimes secondary products may produce precipitates due to leakage. The vacuole also introduces a heterogeneity during the sectioning: a soft component opposed to the hard cell wall. Poor fixation also leads to plasmolysis (Roland and Vian, 1991).

Secondary fixation with osmium tetroxide fixes the lipids and also improves the stabilization of the proteins started in the primary fixation step. Osmium tetroxide penetrates slowly and creates a fixation gradient from the surface to the center of the sample. Secondary fixation is sometimes omitted in immunosorbent electron microscopy as protein antigenicity is retained to different degrees according to the protein studied (Bendayan, 1984).

ii. Embedding resins

Epoxy resins, such as Spurr's or Epon/Araldite are unsurpassed for structural preservation and stability in the electron beam, compared to methacrylate resins. However, the hydrophobic nature of these resins affects antigenic preservation adversely often causing a dramatic reduction of labeling. For immunocytochemical purposes, epoxy resins have been replaced by a group of polar acrylic resins, which are hydrophilic in nature.(Bendayan, 1984; and Newman, 1989)) The Lowicryl resins were designed specifically for immunocytochemical applications. However, in practice, Lowicryl has several limitations for electron microscopy of plant tissue. In published work, ultrastructural preservation is frequently inadequate. The resin is reportedly difficult to infiltrate into some plant tissue, and it can be difficult to section.

The London Resin (LR) White has become a popular alternative to Lowicryl for immunocytochemical studies of plant tissues. An aromatic acrylic resin, it shares

Lowicryl's polarity, high antigen retention and low amount of background labeling. The LR White resin is useful for light and electron microscopy, is easy to section, and generally infiltrates well into plant tissue. Adsorption of protein-A-gold to acrylic resins is minimal. Thus, embedding in acrylic resins such as LR White makes other subjective measures, such as etching and jet washing unnecessary. In aqueous environments, sections of LR White swell enormously, although the extent to which they swell depends on the degree to which they are cross-linked. This swelling might be a contributing factor in explaining their high immunosensitivity (Newman, 1989).

b. Immunogold labeling protocol

A typical labeling protocol involves the following steps. 1) Blocking of the non-specific antibody bonding sites on the sections with blocking agents, e.g. bovine serum albumen (BSA) (1-3%), gelatin (1-5%), normal goat serum (1-5%), Tween 20 (polyoxyethylene sorbitan monolaurate) (0.05-1%) in buffered saline. 2) Incubation with the primary antibody diluted in buffered saline containing one or more of the blocking agents. The proper dilution of the antibody will help to maximize the signal and minimize the background labeling. 3) Washing with buffered saline. 4) Incubation with the protein-A-colloidal gold conjugate diluted at the appropriate dilution in buffered saline, containing a blocking agent. 5) Rinsing with buffered saline followed by a rinse with distilled water. 6) Silver enhancement for the desired time. 7) Rinsing and counterstaining (Vandenbosch, 1991).

Suitable controls should be used in the immunolabeling reaction. One of the controls that can be used is the labeling with pre-immune serum. A pre-immune serum

contains the antibodies to all the immunogens that the rabbit is immunized against prior to injections with the test immunogen. Hence use of the pre-immune serum is a good control. In the absence of pre-immune serum, samples from a healthy and uninfected comparable plant sample should be included in the immunolabeling as a control. The controls should be included in every labeling reaction.

i. Viewing of the silver enhanced colloidal gold label

The silver enhanced colloidal gold can be viewed with bright field, dark field and epipolarization microscopy. In bright field the colloidal gold is seen as black granules on the surface of the tissue. In dark field and epipolarization microscopy, the gold-silver staining is seen as bright white and silver gray spots respectively, on a dark background. However, cell identification is difficult with dark field or epipolarization microscopy. It can be facilitated by bright field examination of the same field of view (de Waele, 1989).

Materials and Methods

Anatomical Study

Collection Of Brownline Samples from field plot in Traverse City, MI.

The trees symptomatic with the BL and the trees that had tested positive for TmRSV in the roots with ELISA or Northern hybridization in August 1995, were sampled. Samples representing the graft union region were taken. The location of the graft union on the sample was measured and noted. A slight swelling in the scion at the union region was used as a marker to determine the graft union in case of asymptomatic trees. Fourteen PBL-affected trees with the BL were noted in surveys conducted in November 1995 and April 1996. (Tables 18 and 19).

Processing of the samples for anatomical study of the brownline

The bark samples were fixed in 3% paraformaldehyde in 0.5M phosphate buffer, pH 7.0. The samples were stored at 4°C until further processing. A sliding microtome was used to cut 20 to 30μm sections of the bark samples. Since the bark pieces were thin and long they were supported with pieces of cork on either side while sectioning. The sections were immediately mounted in 50% (v/v) glycerol in water, to prevent desiccation.

Table 18. Collection of brown line (BL) samples from field plot in Traverse City, MI. The field plot was surveyed for the presence of a BL in Tomato ringspot virus infected trees in November 1995 and April 1996.

	Rootstocks				
Cultivars	M29C	M2624	GF 8-1	St.Jul 655-2	M 4001
Stanley		-	BL (1)	BL (1)	-
70031	BL (3)[a]	-	BL (1)	-	BL (4)
Valor	-	-	BL (1)	BL (1)	-
Carolyn Harris	BL (1)	-	-	-	-
New York 58.900.12	BL (1)	-	-	-	-

[a] Numbers in parenthesis indicate the number of trees that had a visible brown line (BL).

Table 19. Location of the BL samples collected in the field plot in Traverse City, MI.

Scion/Rootstock	Row 1	Row 2	Row 3	Row 4	Row 5
70031/M29C	1[a]		7		13
70031/GF 8-1		12			
70031/M4001	11	3	17	9	
Valor/St.Jul 655-2	9				
Valor/GF 8-1			1		
Stanley/St.Jul 655-2				12	
Stanley/GF 8-1					8
NY58.900.12/M29C				17	
Carolyn Harris / M29C	3				

[a] Numbers in the table indicate the position of the tree, in that row, from which the sample was collected. (Note: Lowest numbers are from the Southern end of the plot and highest numbers are from the Northern end of the inoculated block).

Immunogold Labeling for Light Microscopy

Production of TmRSV Antiserum

Purified TmRSV (0.3mg) in 1.0 ml of 0.05M phosphate buffer was mixed with 1.0 ml of Freund's incomplete adjuvant and an emulsion was prepared. The emulsion was injected subcutaneously into a New Zealand White female rabbit. A second injection with 0.13mg TmRSV followed 1wk later. A third injection was done 2 wk after the second injection with 0.5mg of TmRSV. All injections were done with emulsions prepared in Freund's incomplete adjuvant. A test bleed was done 4 wk after the first injection. A second and third bleed was done at 5 and 7 wk respectively, after the first injection.

Determining the titre of the Antiserum

The titer of the antiserum was estimated with agarose gel double diffusion tests. The medium was made of 0.8% agarose, 0.85% sodium chloride and 0.1% sodium azide. Holes were punched in the medium using a Grafar gel punch (Grafar Corp., Detroit, MI). The agar in the wells was aspirated using a pasteur pipette connected by tubing to a vacuum line. Samples from TmRSV-infected cucumber and healthy cucumber tissue were ground in 0.01M phosphate buffer, pH 7.4. Two-fold dilutions of the extract were made in a 0.85% (w/v) sodium chloride solution in water. The samples were loaded into the wells and the plates were stored overnight in a plastic bag, to allow the formation of immunoprecipitin lines. The healthy leaf extract served to identify the antibodies to the plant components in the antiserum.

Fixation and Embedding in LR White

TmRSV-infected cucumber leaf tissue was fixed in varying concentrations of paraformaldehyde and processed for silver enhanced protein-A-gold labeling. A fixative of 3.5% (w/v) paraformaldehyde in 0.05M phosphate buffer, pH7.0, was determined to be suitable for preserving the antigenicity in cucumber. This result was applied in the fixation of the bark tissue of the 'Stanley' plum trees that tested positive by ELISA. The samples were taken below the graft union. Two samples were taken per tree. The petiole and leaf samples of one ELISA positive rootstock sucker were also fixed as above. All the steps in the following procedure were done at room temperature with gentle agitation. The samples were dehydrated in a graded series of ethanol in distilled water at 15, 30, 45, 60, 75, 90, 100% (v/v) concentrations for 1 to 2 hr each. Two changes were given at the 100% concentration, with the second change allowed to sit overnight. The tissue was then transferred to a graded series of LR White resin (Ted Pella Inc., P.O.Box 492477, Redding, CA) diluted in ethanol at 25, 50, 75, 100% (v/v) for 5 to 6 hr. There were again two changes in 100% LR White, with the second change lasting for 14 to 16 hr. The samples were polymerized overnight at 60°C either in Beam capsules or gelatin capsules (Ted Pella Inc.) in the dark. Semi-thin sections, 1 to 1.5μm, were cut with a glass knife on an ultramicrotome (Reichart Ultracut).

Procedure for protein A-gold labeling and silver enhancement

Semi-thin sections were mounted on glass slides cleaned with Liquinox and distilled water. Polyvinyl alcohol/vinyl trimethoxy silane at a dilution of 1:50 (v/v) was used as an

adhesive. The sections were collected with a trimmed orange stick soaked in water and floated on drops of the adhesive and flattened by placing on a warming tray at 50°C. The sections were then cured overnight at 37°C.

The sections on the glass slide were encircled with a China marker to ensure that the buffers stayed on the sections and the sections did not dry out. Prior to the labeling procedure the slides were thoroughly rinsed with tris-buffered saline (TBS, 10mM Tris-HCl, 150mM NaCl pH 7.5). The non-specific antigenic sites on the sections were blocked for 1 hr at room temperature by incubating with TBS-10% BSA (w/v) (Sigma, Catalog # 7903). The sections were then incubated with TmRSV antiserum diluted 1:250 (v/v) in TBS-10% BSA at room temperature for 1.5 hr. The incubation with the primary and secondary antibodies was done by floating the sections on the buffer. For this, the buffer was placed on a strip of parafilm and two glass capillary tubes were placed on either end of the parafilm to provide support for the slide. The glass slide was placed on top of the capillary tubes in an inverted position. The sections were floated on the buffer for the entire period of the incubation. After the primary antibody incubation, the sections were thoroughly rinsed with TBS-T [TBS-triton X-100 (0.5%) (v/v)] using a wash bottle and, directing the spray at the sections with the slide held in an inverted position. The wash was then continued by incubating the sections on an upright slide with TBS-T-BSA (10%) (v/v) for 10 min. Two such washes were followed by three 10 min washes with TBS-T-BSA (10%) only.

Protein-A-gold (10nm) (Amersham Life Sciences Incorporated, 2636 S. Clearbrook Drive, Arlington Heights, IL 60005) diluted 1:50 (v/v) in TBS-10% BSA was the secondary

antibody used. The slides were again incubated in an inverted position for 1 hr at room temperature. This was followed by four 10 min washes with TBS-T and four 10 min washes with double distilled water. The sections were covered with double distilled water until silver enhancement. The silver enhancement was done with the Intense-M kit (Amersham Life Sciences Inc.) for threex5 min, with washes in double distilled water between the silver enhancement reactions. The sections were then counterstained with 0.1% (w/v) aqueous Safranin-0. The slides were dried on a warming tray. The sections were mounted in 50% (v/v) glycerol in water, covered with a cover glass and sealed with nail polish.

Control sections were always included on each slide along with the sections from the test blocks. Every labeling experiment was repeated at least twice.

Analysis of the immunolabel with the Laser Scanning Confocal Microscope

Observations were made on a Zeiss 210 Laser Scanning Confocal microscope with a 514nm laser. Since plant cell components are highly reflective with the 488nm wavelength of light, the 514nm green laser was used in the reflection studies to minimize the intensity of reflection of the plant cell components. Transmitted images in the phase contrast mode and reflected images were collected. The images were line averaged and stored. The two stored images were colored with two different colors, the transmitted with green and the reflected with red. The colored images were then overlaid, with the reflected image on top of the transmitted image. Photographs of the overlaid images were recorded on color photographic film.

Embedding in Paraffin

Bark samples for paraffin embedding were cut into small pieces, dehydrated in a tertiary butyl alcohol (TBA) series (Berlyn and Miksche, 1976), and embedded with paraffin at 50°C by adding a button of paraffin at a time. The samples were cast in fresh paraffin in disposable plastic molds (Peel-A-Way Scientific, 1800 Floridale Ave, S. El Monte, CA).

Staining with Sudan IV

Freehand sections of bark samples from PBL-affected trees, were stained with 1% (w/v) Sudan IV in 70% ethanol in water. Destaining was done with 50% ethanol in water. The Sudan IV staining was done to confirm the presence of the periderm and to look for suberised cells in the brown line region.

Statistical Analysis for differences between the scions

A statistical analysis of the results obtained in the survey of the field plot for PBL-affected trees was done, in order to determine if there were any significant differences between the scions, whether one scion was more efficient than the other in predisposing the plum trees, that it was grafted onto, to PBL. A one way analysis of variance (ANOVA) for a completely randomized design was done comparing the scion '70031' to the scions 'Valor' and 'Stanley'.

Statistical Analysis of the immunolabelling data

The silver enhanced gold particles on the cell wall and in the cytoplasm were counted on both the experimental and control sections. The data so obtained were subjected to ANOVA for a completely randomized design.

Results

Anatomical Study

Description of a healthy graft union

Graft union samples were obtained from healthy 3-yr-old 'Stanley'/Myrobalan 29C plum trees. The plum trees were the same age as the trees that had developed the BL in the field plot in Traverse City, MI. The graft union region was identified outwardly by a slight swelling of the scion. Observations on 20 to 30μm sections of this region revealed a smooth graft union (Figure 13). The graft union was identified by the presence of a callus of cells in between the rootstock and scion tissue. Fibers and sclereids were distributed uniformly throughout the graft union, rootstock and the scion (Figure 14). Observations with plane polarized light revealed druse crystals in the inner bark of the graft union but not in the periderm or the secondary phloem layers close to the periderm. There was no unusual accumulation of starch in any tissue or regions of the graft union.

Anatomical Study of the Brown line

Bark samples with the brown line, for the anatomical study, were obtained from '70031'/Marianna 4001, '70031'/Myrobalan 29C, 'Valor'/St. Julian 655-2 and 'Stanley'/ Myrobalan 29C plum cultivars, from a field plot in Traverse City, MI. A tree with the BL at the graft union is shown in Figure 15. The bark at the graft union had been peeled off to reveal the BL. Observations were also made on bark samples obtained from a PBL-affected orchard in Paw Paw, MI. The presence of necrophylactic periderms in the BL area, demarcating zones in the bark, has prompted the use of the term 'wound response

process' in the description of the anatomical changes observed in the BL area, where the wound in this case refers to the systemic infection by TmRSV in the plum tree. The area within the demarcated zones of the BL region will be referred to as wound tissue. Periderms in varying shades of brown, purple, pinkish purple and gray were seen in the BL samples. The suberized nature of the periderm cells was confirmed by Sudan IV staining. The wound periderm surrounding the wound response tissue, will be referred to as necrophylactic periderm. Sometimes pinkish purple pigments were clearly seen outlining the wound tissue. However, this was not the case at all times. Based on the available literature (Mullick and Jensen, 1973; Mullick 1975) that (1) an exophylactic periderm formation is preceded by the necrophylactic periderm formation; (2) a necrophylactic periderm always forms abutting dead cells, the term necrophylactic periderm has been used here, to refer to the periderms of the wound tissue in the early stage of the wound response process. The term exophylactic periderm has been used in the descriptions of the advanced stages of the wound response processes. The gray colored periderm layers surrounding the wound tissue are referred to as exophylactic periderms. The normal periderm in the healthy bark sample from a 'Stanley'/70031 plum tree is gray in color (Figure 16). These criteria of similar pigmentation i.e. in the normal periderm of a healthy plum tree and some of the periderms in the BL region, and, based on the literature available, that an exophylactic periderm formed at the site of a wound is similar to the periderms that are normally formed in the plant (Mullick, 1971; Mullick and Jensen, 1973), qualified the gray colored periderms to be termed exophylactic

periderms. All the periderms of shades other than gray, i.e. brown, purple and pinkish purple, are referred as necrophylactic periderms in the description that follows.

The type of anatomical changes in the BL area of the samples observed may be grouped into two categories - an early stage and an advanced stage in the wound response process. In both the stages, the BL region was highly pigmented with black, gray, dark brown, purple and pinkish purple pigments. The pinkish purple pigments appear reddish purple in the photographs due to the quality of the photographic prints. The BL region is characterized by the presence of areas of wound tissue. The wound areas were elliptical in shape in the early stages of the wound response process. Necrophylactic periderm layers surrounded these areas. The cells in the wound tissue were mostly thin walled and rectangular in radial sections and resembled cork cells. However, these cells did not stain with Sudan IV. There were compartments within an area of wound tissue and it was not uncommon to find an elliptical area within a larger elliptical area. The walls of the above mentioned compartments were lined with necrophylactic periderms. Sometimes two elliptical regions spaced at a distance were connected by pink and dark brown layers of necrophylactic periderms. Druse crystals, arranged in rows, were observed in the BL regions of '70031'/Marianna 4001 and '70031'/Myrobalan 29C plum trees.

i. Observations on the Brown line area in 70031/Marianna 4001 plum

Bark samples with symptoms of the early and advanced stages of the wound response process were studied. In the early stages of the wound response process, elliptical areas of wound tissue were seen (Figures 17, 20). There were usually two or three such areas in the bark sample. The ellipse in the radial longitudinal section, in

Figure 17, was 3mm long and 1mm thick. These areas were surrounded by necrophylactic periderm layers. The outermost periderm layers were brown in color. The next few necrophylactic periderm layers were purple and pinkish purple in color. The cells comprising the volume of this area resembled cork cells. However, the cells did not stain with Sudan IV stain, indicating they were non-suberized. Druse crystals were seen in the elliptical BL region with plane polarized light (Figure 19). The crystals were lined in rows. For the most part the wound response tissue that made up the BL area seemed to be in the rootstock region of the graft union. However, a few layers of purple and pinkish purple tissue extending from the wound tissue and into the scion, at the graft union, were seen.

An advanced stage in the wound response process shows the widespread nature of the wound and the wound regions in such cases were larger than the elliptical areas mentioned above. The wound response region of '70031'/Marianna 4001 (Figure 18) was 3.5mm long and 1.5mm thick in radial longitudinal sections. There was disruption of tissue internal to the wound periderm. Traces of purple and pinkish purple pigments could be observed. Usually the wall of the ellipse towards the outside of the tree had broken open and there was collapse of cells within, resulting in patches of cells and open spaces. Compartments lined by necrophylactic periderm were seen in the wound tissue (Figure 18). The gray colored exophylactic periderm layers of the ellipse, that were towards the inside of the tree, were several layers thicker, than was the case when the ellipse was intact, with relatively fewer layers of necrophylactic periderm. This suggests that an exophylactic periderm had formed adjacent to the necrophylactic periderm. The

outline of the wound response area was now wavy and irregular rather than as a curve of the ellipse (Figures 18 and 21). The periderm layers were pocketing inwards and it appears as if the BL had been spreading inwards and towards the central axis of the plum tree, at the time of the sampling. The cells beneath the pocketing periderm towards the inside of the tree were discolored (Figure 21). It was not possible to distinguish the graft union due to the disruption in the tissue of the graft union region.

ii. Observations on the Brown line area in '70031'/Myrobalan 29C plum

The bark samples in this study were all at an advanced stage of the wound response process (Figures 22-28). The wound response tissue in Figure 24 was 12mm long and 1 to 3mm wide. The wound response region in Figure 26 was 5mm long and 2mm wide. The necrotic cells in the BL region were black to dark brown in color and were in a state of collapse (Figure 27). Necrosis was visible in the entire thickness of the longitudinal radial section. This suggests that the BL was spreading towards the center of the trunk. In transverse sections, the necrophylactic and wavy exophylactic periderms, and the disintegration of cells in the wound tissue could be seen (Figures 22 and 23). Layers of pinkish purple tissue were seen alternating with greenish brown tissue layers, suggesting the presence or formation of necrophylactic periderm layers that would compartmentalize the wound tissue (Figure 22). Such a compartment, lined by purple necrophylactic periderm, could be seen in the radial longitudinal sections in Figure 25. A compartment lined with necrophylactic periderms and a break in the tissue within was seen, suggesting the disintegration of cells within (Figure 23). Druse crystals were observed in the purple pigmented tissue of the BL in transverse sections also.

Figure 13. A radial longitudinal section through the bark at the graft union of a healthy 'Stanley'/Myobalan 29C plum tree.The graft union is indicated by arrows. p = periderm, Sc = scion, Rs = rootstock. 67X.

Figure 14. A plane polarised image of the healthy graft union shown in Figure 14. f = sclerenchyma fibers. 67X.

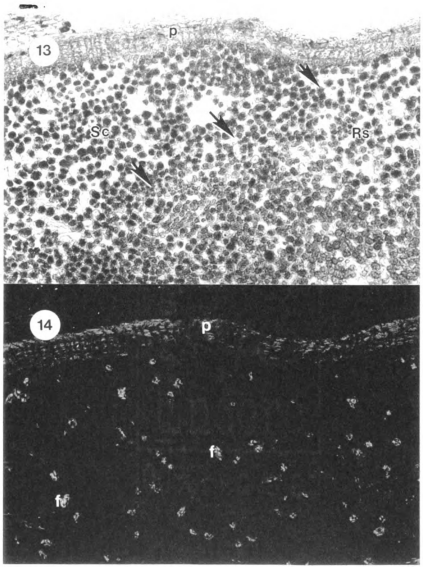

13
p
Sc
Rs
14
p
f
fd

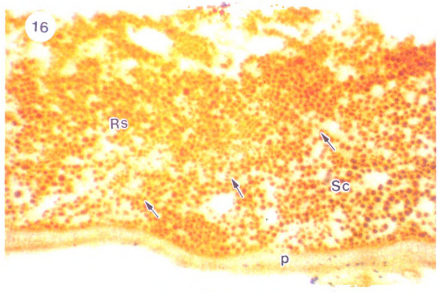
Figure 15. Brown line (arrow) at the graft union of a PBL-affected tree in the Traverse City, MI., field plot. Bark at the graft union has been peeled to show the brown line inside.

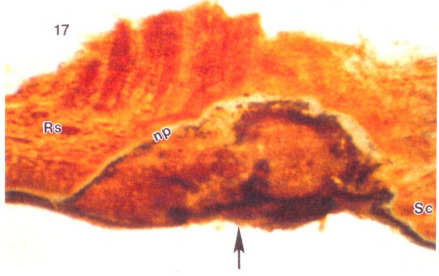

15

Figure 16. A radial longitudinal section through the graft union of a healthy 'Stanley'/Myrobalan 29C plum tree. The graft union is shown by arrows. Note the grey color of the periderm (p). Rs = rootstock, Sc = scion.

Figure 17. A radial longitudinal section through the bark at the Brown line (BL) region of '70031'/Marianna 4001 plum tree. Note the elliptical area of wound tissue that indicates the early stage of the wound response process (arrow). Sc = scion, Rs = rootstock, np = necrophylactic periderm. 30X.

Figure 18. A radial longitudinal section through the bark at the BL region of '70031'/Marianna 4001 plum tree, in an advanced stage of the wound response process. Note the infolding of the associated wound periderm layers (arrows). Also note the compartmentalisation (arrow heads) inside the wound tissue. np = necrophylactic periderm, ep = exophylactic periderm. 40X.


16
Rs
Sc
p


17
Rs
np
Sc

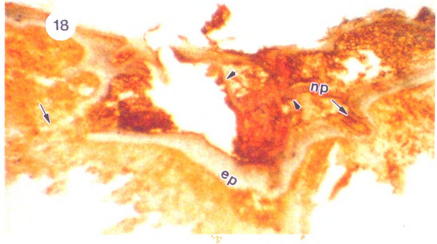

18
np
ep

Figure 19. A plane polarised image of the radial longitudinal section through the BL region of '70031'/Marianna 4001 plum tree. Fully crossed polars reveal the presence of druse crystals (arrow heads) in the wound response tissue of the BL region. 70X.

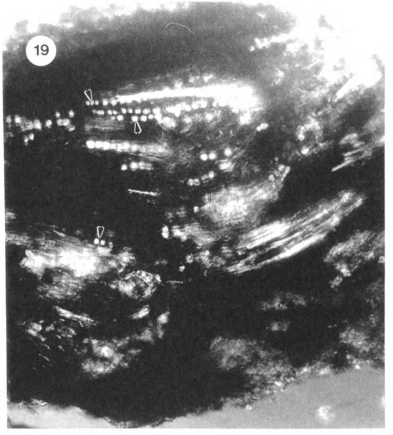

19

Figure 20. A transverse section through the BL region of a '70031'/Marianna 4001 plum tree, in an early stage of wound response. Note that the wound tissue is compact and there is no disintegration of the cell. np = necrophylactic periderm. This figure can be compared with Figure 21. 55X.

Figure 21. A transverse section through the BL region of a '70031'/Marianna 4001 plum tree, in an advanced stage of wound response. Compare with Figure 20, and note the wavy infolds (arrows) of the associated wound periderm and the disruption of the tissue within (arrow heads). 55X.

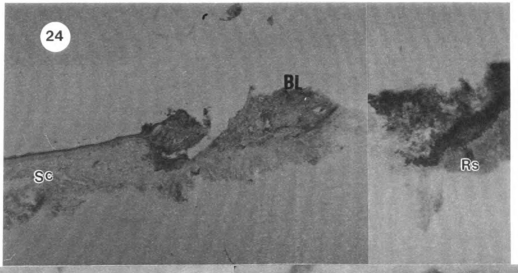
Figure 22. A transverse section through the BL region of a '70031'/Myrobalan 29C plum tree, in an advanced stage of wound response. Note the reddish purple pigmentation (arrow head), suggesting the presence of necrophylactic periderm (np). The presence of the pigments also suggests that the periderm layers that result in the compartmentalization are going to form adjacent to it. The position of the sequent exophylactic periderm (ep) suggests that it's formation resulted after several layers of necrophylactic periderm activity. 55X.

Figure 23. A transverse section through the BL region of a '70031'/Myrobalan 29C plum tree, in an advanced stage of wound response. A compartment of wound tissue is shown (arrows). Also note the collapse of the cells within. 55X.

20
np
21
22
du
ep
23

Figure 24. A radial longitudinal section through the BL region of a '70031'/Myrobalan 29C plum tree, in an advanced stage of wound response. Rs = rootsock, Sc = scion, BL = brown line. Note the extent of the BL zone. 12X.

Figure 25. A radial longitudinal view of a part of the BL region shown in Figure 24. Note the compartments (arrows) within the wound response tissue. 70X.


24
BL
Sc
Rs

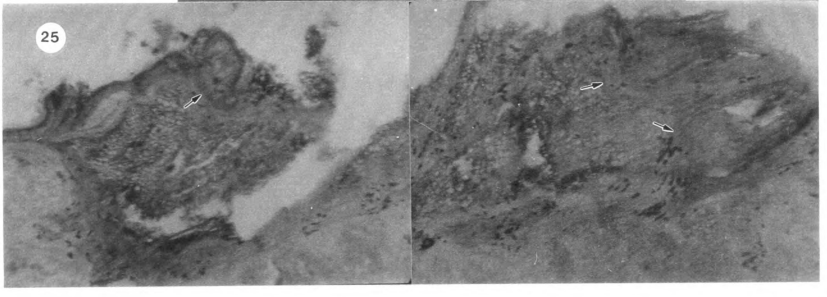
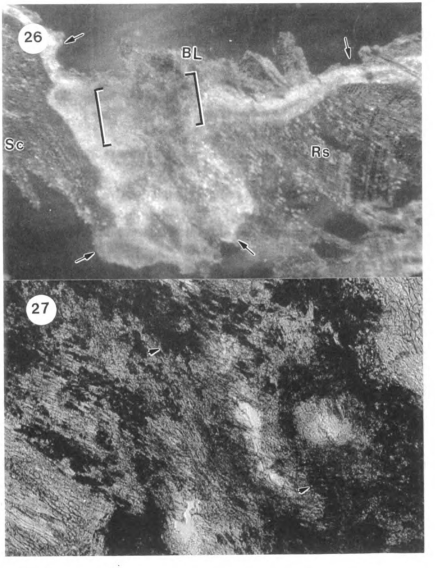

25

Figure 26. A radial longitudinal section through the BL region of a '70031'/Myrobalan 29C plum tree, in an advanced stage of BL formation. The necrotic cells of the BL region are outlined (arrows) in this dark field view of the BL region. Note that the BL is spread in the entire width of the sample. Rs = rootstock, Sc = scion. A high magnification view of the boxed area is shown in Figure 27. 27X.

Figure 27. A view of the boxed area in Figure 26. The cells in this wound response tissue have collapsed and the black cell debris (arrow heads) denoting the weathering of the dead and collapsed cells can be seen. 88X.


26
BL
Sc
Rs
27

Observations on the Brown line samples from an orchard in Paw-Paw, MI

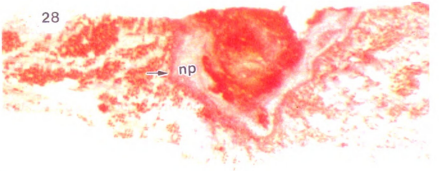
The BL samples obtained from the orchard in Paw-Paw, MI, also had areas of wound tissue similar to the elliptical areas seen in the samples from Traverse City, MI. They had a wavy outline and layers of periderm surrounding them on the outside (Figure 29, compare with Figure 28). However, it was not possible to see the color of the periderm since the samples were processed for paraffin sectioning and lost their pigmentation during the process.

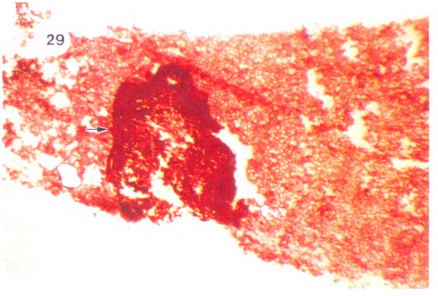
Observations on the bark samples from the greenhouse plum trees

None of the 'Stanley'/Myrobalan 29C trees in the greenhouse study had developed a brown line at the graft union to date. Semi-thin sections of the rootstock bark samples from the 'Stanley'/Myrobalan 29C trees used in the greenhouse study, revealed circular pockets of sclereids surrounded by periderm layers. These regions were well inside the natural periderm layers of the bark. However, the outer periderm layers of the circular regions were seen growing out and joining the natural periderm layers, thus suggesting that these areas constituted wound tissue. Since these samples were processed for LR White embedding it was not possible to observe the pigmentation of the periderm layers or the cells within.

Figure 28. A radial longitudinal view through the BL region of a '70031'/Myrobalan 29C plum tree. Bark samples from the BL region were processed for paraffin embedding, and 10-15μm sections were obtained and stained with 0.1% Safranin-O. The safranin stained tissue (arrow) around the wound response tissue is the necrophylactic periderm. 33X.

Figure 29. A view of the BL region of a 12-15 year-old plum tree from a PBL-affected orchard in Paw-Paw, MI. Bark samples were collected from the BL region of a PBL-affected tree, processed for paraffin embedding and the sections were stained with 0.1% Safranin-O. The elliptical wound response tissue in the BL region (arrow) is similar to the wound response tissue in Figure 17. 22X.


28
np


29

Initial stages in the Brown line formation.

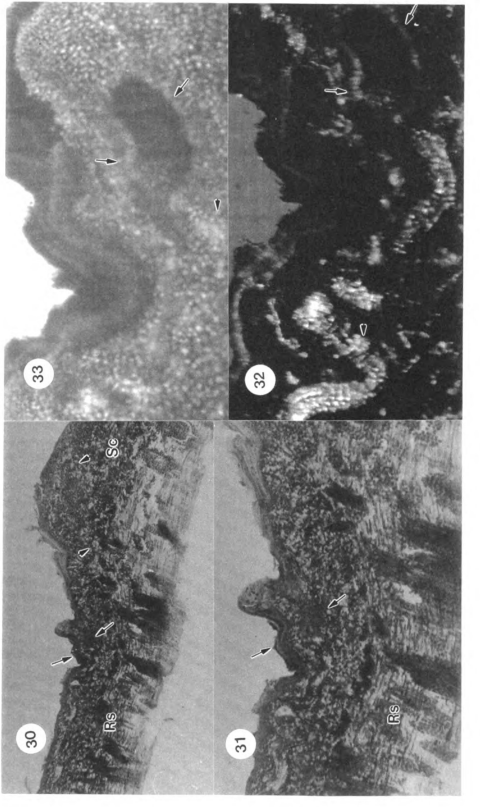
Initial stages in process of BL formation in bark can be studied by observing bark samples from the graft union of a 'Stanley'/Myrobalan 29C plum tree (Figures 30-33). The tree from which the sample was acquired did not have a visible BL; however, it was sampled, as the roots of this tree had tested TmRSV-positive in an earlier test. Crescent shaped, localized patches, 1mm in length, were seen in the bark, below the periderm (Figures 30, 31 and 33). The cells in this area were pigmented with purple and pinkish purple pigments. A little further down this area and into the rootstock region there were crescent shaped regions that were discolored. In plane polarised light, cells in some of the layers of the purple pigmented tissue could be seen (Figure 32). The crescent shaped, non-purple pigmented tissue, in the two regions further down in the rootstock, also polarised. The polarizing cells in these two non-purple pigmented regions suggest phellogen marker cell activity. It is likely that these two regions are in a process of wound response, and will form the purple necrophylactic periderms. It is likely that these regions may later on join together and form the ellipse that is characteristic of the early wound response process in the BL region. Thus it may be said that in the process of BL formation, wound response processes in the plum tree generate areas of wound tissue demarcated by wound periderms. These areas start at a point in the rootstock at the graft union and may coalesce and spread further into the rootstock.

Figure 30. A radial longitudinal section through the graft union of a 'Stanley'/Myrobalan 29C plum tree. Note the presence of two wound response regions (arrows). Sc = scion, Rs = rootstock. The graft union is denoted by arrowheads. 7X.

Figure 31. A higher magnification view of the graft union shown in Figure 30. The regions denoted by arrows were pigmented by pinkish purple pigments, denoting wound response activity. Note that these two wound tissue regions are in the rootstock of the graft. Rs = rootstock. 14X.

Figure 32. A plane polarised view of the graft union shown in Figure 31. Fully crossed polars show the birefringence of the cell layer on the outside of the wound response tissue (arrows). Note also, that additionally, two crescent areas of cells are polarising (arrow heads). The birefringent cells in the crescent areas are probably phellogen activity marker cells. This suggests that it is the site of the wound response activity next in sequence. Also note that the two probable wound response areas are in the rootstock of the graft. This figure may be compared with Figure 33. 56X.

Figure 33. A bright field transmitted view of the wound response tissue in the rootstock at the graft union of a 'Stanley'/Myrobalan 29C plum tree. Note that the the cell layers denoted by arrows are birefringent with polarised light in Figure 32. The birefringent cells in this case denote the suberised periderm layers. 66X.


30
RS
S6
31
RS
33
32

Statistical analysis

A statistical comparison of the scions '70031', 'Valor' and 'Stanley' was done, in order to see if one is significantly different than the other in predisposing the plum trees to PBL, that they are grafted on. ANOVA of the results obtained in the surveys of the field plot conducted in November 1995 and April 1996, shows that there are significant differences between '70031' *vs* 'Valor' (Table 20) and 'Stanley' (Table 21) scions.

Table 20 Comparison of the differences between the scions '70031' and 'Valor', by statiscal analysis. The results obtained by surveying a field plot at Traverse City, MI., for the presence of a BL were subjected to a one way analysis of variance, in order to see if one scion is more predisposed than the other to the formation of the BL.

Data from each of the five rows in the field plot were considered a replication. The number of PBL-affected trees with either '70031' or 'Valor' as the scion were considered as data for that row. There were three rows without any PBL-affected trees with the scion 'Valor'. The data for these rows were considered as 1 and the data in the same rows for the scion '70031' is = observed number of PBL-affected trees + 1. The corrected data are presented below.

Scion	Replications					Total
70031	3	3	2	2	2	12
Valor	2	1	2	1	1	7

ANOVA

	df	SS	MS	F	Required F 5 %	Required F 1 %
Total	9	4.9				
Treatment	1	2.5	2.5	8.33	5.32	11.26
Error	8	2.4	0.3			

Conclusion: F is significant at $P = 0.05$

There is a significant differences between the ability of the two scions in predisposing the trees, that they are grafted onto, to PBL.

Table 21 Comparison of the differences between the scions '70031' and 'Stanley' by statistical analysis. The results obtained by surveying a field plot at Traverse City, MI., for the presence of a BL were subjected to a one way analysis of variance, in order to see if one scion is more predisposed than the other to the formation of the BL.

Data from each of the five rows in the field plot were considered a replication. The number of PBL-affected trees with either '70031' or 'Stanley' as the scion were considered as data for that row. There were three rows without any PBL-affected trees with the scion 'Stanley'. The data for these rows were considered as 1 and the data in the same rows for the scion '70031' is = observed number of PBL-affected trees + 1. The corrected data are presented below.

Scion	Replications					Total
70031	3	3	2	2	2	12
Stanley	1	1	1	2	2	7

ANOVA

	df	SS	MS	F	Required F	
					5 %	1 %
Total	9	4.9				
Treatment	1	2.5	2.5	8.33	5.32	11.26
Error	8	2.4	0.3			

Conclusion: F is significant at $P = 0.05$

There is significant difference between the ability of the two scions in predisposing the trees, that they are grafted onto, to PBL.

Immunolabeling

The silver enhanced protein-A-gold labeling was seen on the BL, rootstock and scion tissue of '70031'/Marianna 4001 bark samples (Figures 36, 37, 38 and 42). Labeling was also seen on the bark samples of the BL (Figures 34, 35) and the rootstock (Figure 40) of 'Valor'/St. Julian 655-2 plum tree. Rootstock bark (Figure 39) samples and rootstock sucker leaf (Figure 45) samples from 'Stanley'/Myrobalan 29C trees, which were a part of the group of plum trees used in the greenhouse study, were also immunolabeled with the TmRSV antiserum. A major portion of the labeling was seen on the cell wall. There was sparse labeling in the periderm layers. Statistically non-significant labeling was seen on the healthy rootstock bark (Figure 41) and healthy rootstock sucker leaf (Figure 46) samples. Labeling was not seen on the BL (Figure 43) and the healthy rootstock (Figure 44) bark samples when pre-immune serum was substituted for the TmRSV serum in the labeling reaction.

Statistical analysis was done on the combined counts of the label on the cell wall and in the cytoplasm. The controls used in this case were healthy rootstock sucker leaf and healthy bark samples obtained from below the graft union of 'Stanley'/Myrobalan 29C plum trees. Two blocks of the healthy rootstock sucker leaf sample and four blocks of the healthy bark sample were sectioned. Control sections were always included in every labeling run and treated in a manner similar to the diseased sample.

Labeling of Brown line samples

Labeling was seen on the cell wall and in cytoplasm of the cells (Figures 34, 35, 36 and 37). However, a major portion of the label was seen on the cell wall. Labeling was seen in the elliptical BL areas and in the tissue surrounding it. There was little non-specific labeling on the resin. The cells with dark colored cytoplasmic contents appeared to have an affinity for the immunolabel seen in the cytoplasm. There was a small amount of labeling on the periderm layers of the BL. The statistical analysis of the labeling on the BL bark samples and on the healthy bark samples showed significant differences between the two. The F value was significant at $P = 0.01$ (Table 22).

Figure 34. Light microscopy immunolabeling of a brown line (BL) bark sample of a 'Valor'/St.Julian 655-2 plum tree. Note that there is sparse labeling (arrow) on the necrophylactic periderm (np) and the fibers (f) in the BL area. Bar = 50μm.

Figure 35. Light microscopy immunolabeling of a BL bark sample of a 'Valor'/St. Julian 655-2 plum tree. The tree sampled had a visible BL. Although the roots had tested TmRSV-negative in an earlier test, the presence of TmRSV in the BL area is shown by the presence of the gold label, shown here as red and yellow dots (arrow). Majority of the label is on the cell wall of the secondary phloem cells.

Figure 36. Light microscopy immunolabeling of the BL region of a '70031'/Marianna 4001 plum tree. The silver enhanced gold labeling (arrow) is seen as a red and yellow reflected image on the green transmitted image of the BL area. Note that there is sparse labeling of the necrophylactic periderm (np) layers in the BL region. Here again the label is on the cell wall of most of the secondary phloem cells and in the cytoplasm of some of the secondary phloem cells. The sectioning was done through the elliptical region shown in Figure 17.

Figure 37. Light microscopy immunolabeling of the BL region from a '70031'/Marianna 4001 plum tree. The silver enhanced gold label (arrow) is seen on the cell wall of the secondary phloem cells.

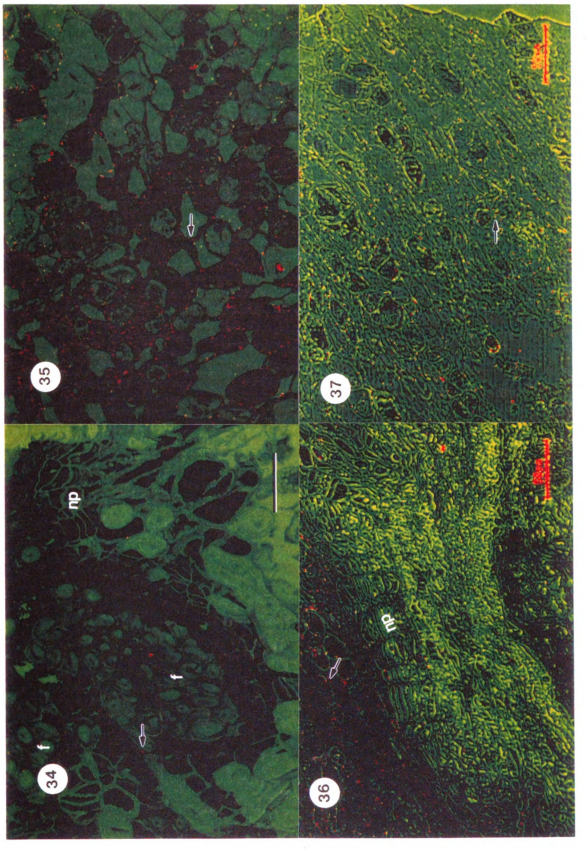

34
f
np
f
f
35
36
np
37

Table 22 Analysis of the immunolabeling on BL sections of a '70031' / Marianna 4001 tree. Brown line sections were treated with TmRSV antiserum (1:250), protein-A-gold (1:50) and silver enhanced. Four blocks were sectioned and counts were obtained from at least one section per block. The sum total of the counts on the cell wall and in the cytoplasm was statistically analyzed.

Treatments	Replications							Total
	1	2	3	4	5	6	7	
Healthy	4	35	17	7	51	49	96	259
Brown line	228	356	337	360	256	346	418	2301

ANOVA

	df	SS	MS	F	Required F 5 %	1%
Total	13	329,547.71				
Treatments	1	297,840.28	297,840.28	112.72	4.75	9.33
Error	12	31,707.43	2,642.29			

Conclusion : F is significant at $P = 0.01$

Labeling of the scion and rootstock tissue of BL infected trees.

The scion (Figure 42) and rootstock tissues (Figures 38 and 40) immediately adjacent to the BL area were also positively immunolabeled for TmRSV. The analysis of variance for the combined counts on the cell wall and in the cytoplasm was done. The results are presented in Tables 23 and 24, respectively. The F values obtained in case of the scion and the rootstock were both statistically significant at $P = 0.01$.

Figure 38. Light microscopy immunolabeling of the rootstock bark sample from a '70031'/Marianna 4001 plum tree. The portion of the bark sample adjacent to and below the BL was processed for LR White embedding and immunolabeled. Note the silver enhanced gold label (arrow) on the cell wall and the cytoplasm of the secondary phloem cells. f = fiber. Bar = 50μm.

Figure 39. Light microscopy immunolabeling of the rootstock bark sample from a slash inoculated 'Stanley'/Myrobalan 29C plum tree. Note the heavy labeling (arrow) on the axial (ax) component of the secondary phloem compared to the labeling on the radial (ra) component. Bar = 50μm

Figure 40. Light microscopy immunolabeling of the rootstock bark sample from a 'Valor'/St. Julian 655-2 plum tree. The portion of the bark sample adjacent to and below the BL was processed for LR White embedding and immunolabeling. A major portion of the silver enhanced gold label is seen on the cell wall of the secondary phloem cells (arrow). Bar = 50μm.

Figure 41. Light microscopy immunolabeling of the rootstock bark sample from a healthy 'Stanley'/Myrobalan 29C plum tree. Note the statistically insignificant labeling on the bark sample (arrows). f = fiber. Bar = 50μm.

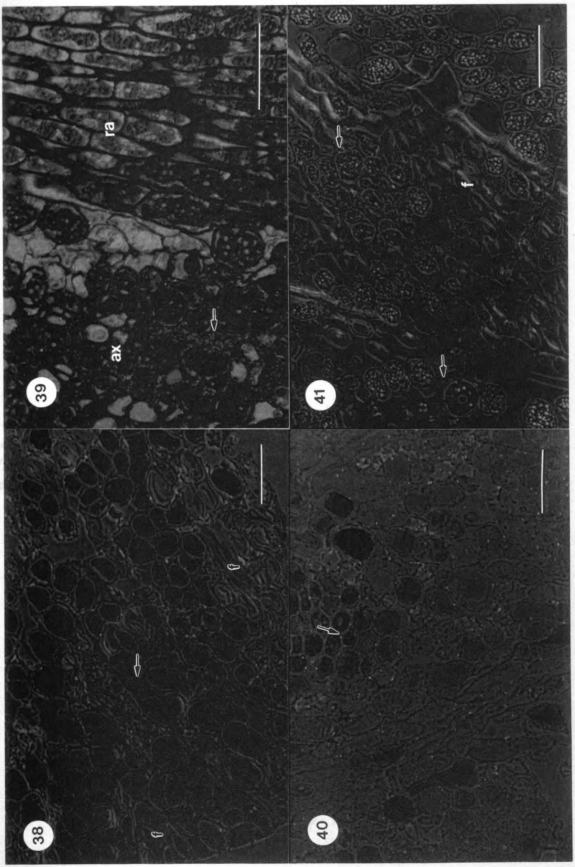

38
39
ax
ra
40
41
f

Figure 42. I
'70031'/Mari
on the cell wa

Figure 42. Light microscopy immunolabeling of the scion bark sample from a '70031'/Marianna 4001 plum tree. Note the silver enhanced protein-A-gold label (arrow) on the cell wall and cytopalsm of the bark tissue. Bar = 50μm.

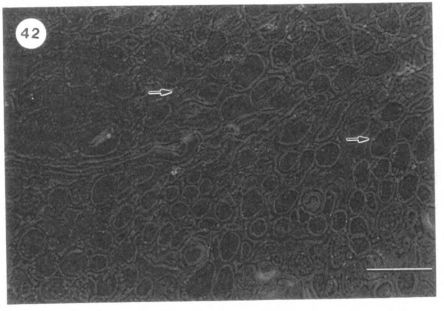

42

Table 23 Analysis of the immunolabeling on the scion tissue of a '70031' / Marianna 4001 tree. Scion tissue immediately adjacent to the BL was labeled with TmRSV serum (1:250), protein-A-gold (1:50) and silver enhanced. Three blocks were sectioned and counts were obtained from at least one section per block. The sum total of the counts on the cell wall and in the cytoplasm was statistically analyzed.

Treatments	Replications							Total
	1	2	3	4	5	6	7	
Scion	217	412	263	195	218	294	232	1831
Healthy	4	35	17	7	51	49	96	259

ANOVA

	df	SS	MS	F	Required F	
					5 %	1%
Total	13	215,600.86				
Treatments	1	176,513.15	176,513.15	54.19	4.75	9.33
Error	12	39,087.71	3,257.31			

Conclusion : F is significant at $P = 0.01$

Table 24 Analysis of the immunolabeling on the rootstock tissue of a '70031' / Marianna 4001 tree.Rootstock tissue immediately adjacent to the BL of a '70031' / Marianna 4001 tree was labeled with TmRSV (1:250), protein-A-gold (1:50) and silver enhanced. Two blocks were sectioned and counts were obtained from at least two sections per block. The sum total of the counts on the cell wall and in the cytoplasm were statistically analyzed.

Treatments	Replications						Total
	1	2	3	4	5	6	
Rootstock	340	242	725	363	272	233	2175
Healthy	4	35	17	7	51	49	163

ANOVA

	df	SS	MS	F	Required F	
					5 %	1%
Total	11	510,891.67				
Treatments	1	337,345.34	337,345.34	19.44	4.96	10.04
Error	10	173,546.33	17,354.63			

Conclusion : F is significant at $P = 0.01$

Statistical analysis of the labeling with the Pre-immune serum

Tissue from the the BL samples (Figure 43) and healthy bark (Figure 44) were treated with pre-immune serum (1:250), protein-A-gold (1:50) and silver enhanced. The sum total of the counts on the cell wall and in the cytoplasm were statistically analyzed. The F value was not significant (Table 25). Therefore, the null hypothesis that no differences exist between the labeling on the BL and healthy bark samples holds.

Figures 43 and 44. Light microscopy immunolabeling of a BL bark sample of a '70031'/Marianna 4001 plum tree (Figure 42) and a rootstock bark sample of a healthy 'Stanley'/Myrobalan 29C plum tree (Figure 43) labeled with pre-immune serum. Note the statistically insignificant labeling on both the bark samples (arrows). f= fiber, BL = Brown line area. Bar = 50μm.

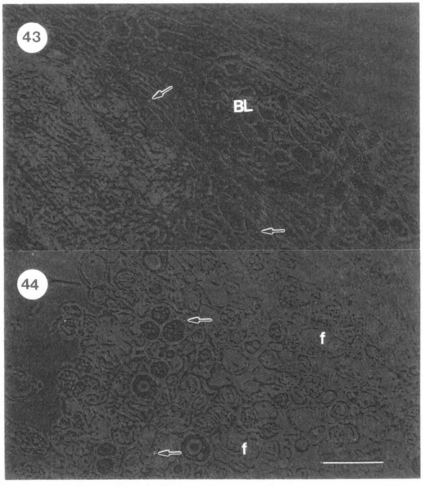

43
BL
44
f
f

Table 25 Analysis of the immunolabeling on the BL tissue treated with pre-immune serum. Brown line tissue from a '70031' / Marianna 4001 tree was treated with pre-immune serum (1:250), protein-A-gold (1:250) and silver enhanced. The sum total of the counts on the cell wall and in the cytoplasm was statistically analyzed.

Treatments	Replications			Total
	1	2	3	
Brown line	11	17	19	47
Healthy	1	5	10	16

ANOVA

	df	SS	MS	F	Required F	
					5 %	1%
Total	5	235.5				
Treatments	1	160.17	160.17	0.09	7.71	21.20
Error	4	75.33	18.83			

Conclusion : F is not significant.

Labeling of the rootstock bark sample obtained from the greenhouse study

Labeling was seen in the cytoplasm and on the cell wall (Figure 39). There was little labeling in the periderm layers. Both the axial and radial components of the secondary phloem were labeled. The statistical analysis of the sum total of the counts on the cell wall and in the cytoplasm (shown in Table 26). was significant at $P = 0.05$.

Table 26 Analysis of the immunolabeling on the rootstock tissue of a 'Stanley'/ Myrobalan 29C tree.Rootstock tissue below the graft union of a slash inoculated 'Stanley' / Myrobalan 29C tree was labeled with TmRSV antiserum (1: 250), protein-A-gold (1:50) and silver enhanced. One block was sectioned and counts were obtained from four sections. The sum total of the counts of the label on the cell wall and in the cytoplasm was statistically analyzed.

Treatments	Replications						Total
	1	2	3	4	5	6	
Rootstock	425	224	49	513	480	116	1807
Healthy	4	35	17	7	51	49	163

ANOVA

	df	SS	MS	F	Required F	
					5 %	1 %
Total	11	389,733				
Treatments	1	191,561.33	191,561.33	9.67	4.96	10.04
Error	10	198,171.67	19,817.17			

Conclusion : F is significant at $P = 0.05$

Labeling of the plum rootstock sucker leaf sample

Labeling was present in the cytoplasm and on the cell wall (Figure 45). The labeling was present in all tissue types. The bundle sheath cells were heavily labeled. There was sparse labeling in the xylem tissue. The statistical analysis is shown in Table 27. The F value was statistically significant at $P = 0.01$.

Figure 45. Light microscopy immunolabeling of a rootstock sucker leaf sample of a nematode inoculated 'Stanley'/Myrobalan 29C plum tree. Note that a significant amount of the silver enhanced gold label (arrows) is seen in the bundle sheath (bs) parenchyma cells. e = epidermis, m = mesophyll, x = xylem. Bar = 50μm.

Figure 46. Light microscopy immunolabeling of the rootstock sucker leaf of a healthy 'Stanley'/Myrobalan 29C plum tree. Note the statistically non-significant labeling on the leaf sample (arrows). bs = bundle sheath, x = xylem, e = epidermis, m = mesophyll.

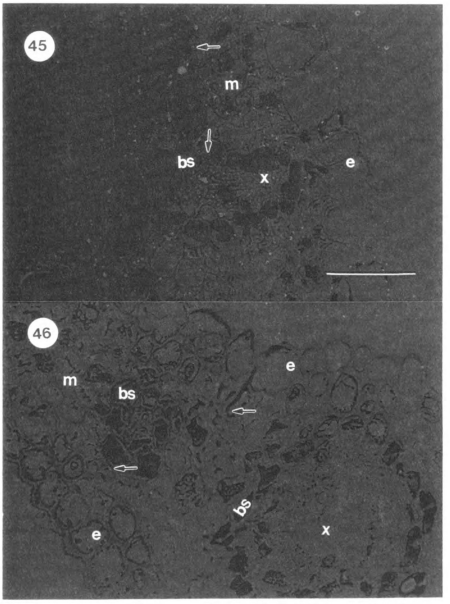

45
m
bs
x
e
46
m
bs
e
bs
e
x

Table 27 Analysis of the immunolabeling on the rootstock sucker leaf tissue .
Rootstock sucker leaf tissue from a nematode inoculated 'Stanley'/Myrobalan 29C tree was treated with TmRSV antiserum (1:250), protein-A-gold (1:250) and silver enhanced. Three blocks were sectioned and counts were obtained from at least one section per block. The sum total of the counts on the cell wall and in the cytoplasm was statistically analyzed.

Treatments	Replications							Total
	1	2	3	4	5	6	7	
TmRSV-infected	278	327	281	242	263	190	250	1831
Healthy	9	10	17	12	15	24	65	152

ANOVA

	df	SS	MS	F	Required F	
					5 %	1 %
Total	13	214,269.21				
Treatments	1	201,360.07	201,360.07	187.18	4.75	9.33
Error	12	12,909.14	1,075.76			

Conclusion : F is significant at $P = 0.01$

Discussion

The defense response of the plant: Formation of the Brown line

Plants respond in a nonspecific manner to a variety of factors that disrupt the integrity of the system. The factors that elicit the defense response may be injuries due to insects, birds, wounds, or infection by pathogens such as nematodes, virus, fungi and bacteria. Work by Mullick (1975) has shown that non-suberized impervious tissue (NIT) and necrophylactic periderm formation occurs in the bark of woody conifers such as *Abies grandis* (Dougl.) Lindl., *A. amabilis* (Dougl.) Forbes., *Tsuga heterophylla* (Raf.) Sarg., *Thuja plicata* Donn, as a nonspecific host response. The author also suggested that NIT formation may be the physiological basis of host response to diseases in the bark of conifers. Since TmRSV was immuno-localised in the bark tissue at the BL area, and also in the scion and rootstock tissue immediately above and below the BL respectively, it may be assumed that the histopathological changes seen, were due to the presence and activity of TmRSV, in other words due to infection by TmRSV. Based on symptoms seen in the plum tree at the BL area, one can distinguish stages in the defense reaction of the plant to TmRSV. In the formation of the necrophylactic periderm and demarcating the cells within, the plum tree has responded in a nonspecific manner. The elliptical areas of wound tissue seen in the BL region (Figure 17) are an illustration of this defense response of the plant. After such a demarcation has occurred, it is to be expected that a normal exophylactic periderm would form, separating the wound tissue from the inside of the plant and later on, causing a sloughing off, of all the layers that it separates from the intact plant tissue. This stage is illustrated in Figure18, where the tissues formerly

enclosed by an intact periderm are now exposed and are in a state of collapse. Necrotic cell debris can also be seen in this stage, denoting the decay in that tissue, presumably due to exposure to the environment. This would normally be expected to be the end of all apparent signs of infection having ever taken place in the plant. However, even as the wound tissue is being sloughed off, some unknown changes in the plant, presumably caused by viral activity, result in spread of the periderm inward (Figure 18). The periderm became wavy with the trough of the wave spreading inward. There are discontinuities in the periderm, thus creating channels between the cells inside and the cells formerly enclosed by the periderm. As the periderm spreads inward at several points, it creates more areas that elicit the defense reaction from the plant by stimulating the formation of a necrophylactic periderm. At this stage the BL symptoms are not only spreading inward, they are also spreading circumferentially. Eventually such discrete points of necrosis join together to result in a complete brown line, that extends the entire circumference and also weakens the graft union. Thus, the plum tree is unable to effectively defend itself, albeit passively, against the virus. In such a weakened state, the tree would be susceptible to winter injury. Winter injury in PBL-affected plum and prune trees has been reported by Brase and Parker (1955), Kirkpatrick et al. (1958), and Mircetich and Hoy (1981). The inwardly spreading BL also creates portals of entry for secondary pathogens such as fungi and bacteria, that may further debilitate the tree.

Process of BL formation

Studies by Mullick (1975), and Mullick and Jensen (1976) have shown that necrophylactic periderm develops internal to a non-suberized impervious tissue (NIT). Necrophylactic periderm can be distinguished from the sequent periderms by its pigmentation (Mullick, 1971), (Mullick and Jensen, 1973). The histopathological changes seen in the bark of 'Stanley'/Myrobalan 29C show a polarizing layer of tissue, internal to which purple necrophylactic periderm layers were seen (Figures 32 and 33). The polarizing nature of the purple necrophylactic periderm cells and the exophylactic periderm cells was shown by Mullick and Jensen (1973). The cells in the necrophylactic periderm are filled with a luminous fluid that makes it difficult to detect polarization. When the contents of the cells have flowed out, then one can distinguish the cell's outline more clearly. This may be why polarized cells were observed as a outline of the wound area. Polarized light also revealed two crescent areas in the rootstock, further down from these wound areas. These crescent areas had not developed a necrophylactic periderm yet. The polarizing cells probably denote phellogen activity marker cells. This indicates that the crescent areas are an impervious tissue zone and they will most likely develop the purple necrophylactic periderm internally. When a number of such areas with wound response symptoms are seen along the circumference of the bark, they may join together, either by merging or, through strips of necrophylactic periderm connections, and result in a continuous zone of necrotic tissue, the brown line. In a study of AUND disease (Rosenberger et al., 1983), the authors suggested that the symptoms progress from an

intermittent pitting to a continuous row of pits to a necrotic layer of tissue. The formation of the BL in the PBL disease may occur in a similar manner, with areas of wound tissue joining to form a circle of necrotic tissue around the circumference of the bark resulting in a brown line.

Druse crystals

One of the most commonly encountered calcium oxalate crystals in plants is the druse, a spherical aggregate of individual crystals (Francheschi and Horner, 1980). Presence of druse crystals has been reported in the bark and wound callus of apple (*Pyrus malus* L.), which is classified in the Rosacae family that *Prunus* also belongs to. In the present study, druse crystals were observed in the BL region. The presence of the druse crystals suggests two possibilities, (1) the crystals are a byproduct of fungal infection of the BL area and/or, (2) the crystals are performing a storage function.

Calcium oxalate crystals have been reported in wounded peach bark tissues inoculated with *Leucostoma cincta* (Pers.:Fr.) Hohn. and *L. persoonii* (Nits.) Hohn. (Traquair, 1987). These fungi are facultative parasites that invade the wounded tissues of peach trees, causing serious dieback and canker problems. Calcium oxalate crystals in close proximity to fungal hyphae have been reported in sugar beet and carrot leaf tissue infected with *Sclerotium rolfsii* Sacc. (Punja and Jenkins, 1984). Various cell wall degrading enzymes are produced during pathogenesis by soft rot fungi such as *S. rolfsii* Sacc. and canker pathogens such as *Endothia parasitica* (Murr.)And. (Troquair, 1987). An hypothesis has been proposed that oxalic acid produced by *S. rolfsii* during

pathogenesis sequesters calcium resulting in the formation of calcium oxalate crystals (Punja and Jenkins, 1984). Calcium pectate, a constituent of the middle lamella of the cell wall, is a rich source of calcium. The calcium for crystal formation in diseased tissue was presumed to be derived from the pectates of the cell wall, during cell wall degradation (Punja et al., 1984). The above hypothesis is further supported by a study showing the production of calcium oxalate crystals on potato dextrose agar medium inoculated with *L. cincta* and *L. persoonii* fungi (Troquair, 1987). The source of calcium for the production of calcium oxalate crystals was the agar. Similar crystals were produced on the agar in the absence of fungi, when oxalic acid alone was added to the agar. The druse crystals observed in the BL region may imply a fungal infection of the BL area. In preliminary examinations, no fungal hyphae were observed in the BL tissue. However, the possibility that the BL area may be colonised by fungi, resulting in the formation of calcium oxalate druse crystals cannot be ruled out.

A number of functions have been attributed to the formation and presence of calcium oxalate crystals in various parts of the plant, one of which is storage. The calcium or oxalate reserves in the plant are stored as calcium oxalate crystals, which can be broken down whenever needed (Francheschi and Horner, 1980). Defense processes in plants involve a dedifferentiation and redifferentiation of cells and the formation of secondary meristems. It is possible that the large number of the druse crystals seen in the BL region serve a storage function, providing the stores of calcium necessary, for instance, in the formation of the necrophylactic periderm.

Immunolabeling

Tomato ringspot virus was immuno-localised in the bark of the BL region of '70031'/Marianna 4001 and 'Valor'/St.Julian 655-2. The virus was also immuno-localised in Marianna 4001, St.Julian 655-2 and Myrobalan 29C rootstocks, and, in the bark tissue of scion '70031'. A major portion of the label in the bark was seen in the secondary phloem parenchyma, with least labeling in the periderm layers. Heavier labeling was seen in the axial phloem than in the radial phloem. Immunolabeling of the rootstock sucker leaf showed labeling in the mesophyll, epidermis, phloem of the vascular bundles and the parenchyma cells of the bundle sheath. The silver enhanced gold particles, in both bark and leaf, were seen on the cell wall and in the cytoplasm. However, a major portion of the label was seen on the cell wall of the different tissue types. Transmission electron microscopy studies have localized nepoviruses, of which TmRSV is a member, in tubules inside the plasmodesmata of virus infected cells (deZoeten and Gaard, 1969). Short distance cell to cell movement of the virus is believed to occur in these tubules, through structurally modified plasmodesmata (Lucas and Gilbertson, 1994). The presence of the major portion of the label on the cell wall may be explained in the light of the above findings. The plasmodesmata are intercellular cytoplasmic bridges that run through the cell wall, in the plant. The label seen on the cell wall may be interpreted as the labeling of the plasmodesmata that contain tubules with virions. In an immunosorbent electron microscopy study by Wieczorek and Sanfacon (1993), it was found that the antibodies to intact TmRSV virions failed to specifically label the virus-like particles within the tubules, in TmRSV infected tobacco plants. However,

cytoplasmic labeling was obtained in this case. They suggested that either the epitopes were masked or the particles in the tubules were a modified form of virus particles.

The difference in the amount of label seen on the axial phloem component as compared to the radial phloem component of the secondary phloem (Figure 39) suggests that the long distance movement of the virus particles occurs through the phloem. In case of the rootstock sucker leaf samples, a major portion of the label was seen in the bundle sheath parenchyma surrounding the vascular bundles. This finding also suggests that the virus is transported long distance through the phloem. From the phloem tissue, the virus then spreads into the surrounding bundle sheath first and then into the mesophyll of the leaf. Similarly, in the bark, the virus is transported from the axial secondary phloem cells into the radial components of the secondary phloem.

Three common sites of false positives, in immunosorbent electron microscopy on plant tissue, are the plant cell wall, nucleus and vacuolar inclusions (Herman, 1989). In the early attempts at the immunolabeling of the bark tissue, non-specific labeling was seen on the cell wall of the bark samples taken from a healthy plum tree. The TmRSV antibodies used in this attempt were made by injecting the rabbit with an emulsion made of the purified virus and Freund's complete adjuvant. In view of the problem of nonspecific labeling discussed above, new antibodies were produced by injecting into the rabbit, an emulsion made of purified virus and Freund's incomplete adjuvant. This new set of antibodies helped in reducing the nonspecific labeling considerably, but not entirely. There was a fair amount of labeling seen on the cell wall and cytoplasm of the bark from a healthy plum tree. This may be due to the unevenness of the face of a semi-

thin bark section. A fair amount of sample 'pull out' may occur during the sectioning of bark samples, due to the size and volume of the cells and, partly due to the composition of the tissue. In a bark sample there are the periderm cells with thick walls and collapsed cell contents, there are also the parenchyma cells of the secondary phloem, and the phloic fibers. A considerable amount of the labeling was being trapped in the depths of the uneven bark sample. To overcome this problem, the sections were floated on the buffer, rather than soaking the sections during incubations with the various buffers. Another modification in the labeling protocol was to use a jet spray of the wash solutions, to thoroughly dislodge any nonspecific antibodies and gold label that was stuck on the sample. Although jet washing of LR White embedded sections is not recommended during immunolabeling, it had become a necessity in this case, to reduce the nonspecific labeling. A high concentration of BSA (10%, w/v) was required to sufficiently block the non-specific antigenic sites on the sample. The above mentioned modifications in the immunolabeling protocol were necessary to obtain satisfactory labeling with a minimum of non-specific labeling on the cell wall and the cytoplasm.

An hypothesis for the PBL disease

Hoy and Mircetich (1984) did not detect TmRSV with ELISA in the scion tissue of PBL-affected plum trees on Myrobalan or peach rootstocks. They were also unable to transmit TmRSV and induce PBL in plum trees on Marianna 2624 rootstock. They reported that PBL results from an hypersensitive reaction of a resistant scion to TmRSV infection in a susceptible rootstock and, that Marianna 2624 is immune to TmRSV

infection. Cummins and Gonsalves (1986*a*) reported that PBL is analogous to Apple union necrosis and decline (AUND) disease, a hypersensitive reaction of resistant apple scions to TmRSV infection in a susceptible but tolerant rootstock. Tuttle and Gotleib (1985), in a study of AUND-affected 'Delicious'/Malling Merton 106 trees, reported that the histopathological changes in the trees began abruptly, after 8 yr of compatible growth. The authors concluded that the histological changes did not appear to be a result of a hypersensitive reaction of cultivar Delicious to TmRSV in the rootstock tissue. Instead they were of the opinion that AUND is caused due to a delayed graft incompatibility of the scion and rootstock.

An hypersensitive response of a scion is a type of resistance, that the scion shows to the presence of TmRSV, by reacting to such a degree, that the necrosis of the tissue in the reaction zone takes place. Therefore, for an hypersensitive response from the scion, there must be some interplay between the scion tissue and the incitant, namely TmRSV, in this case. Tomato ringspot virus has been detected in the scion tissue of two out of 32 'Stanley' propagated on TmRSV infected rootstocks (Cummins and Gonsalves, 1986*a*). 'Flame reaction' of the dead tissue in 'Stanley', extending up to 2.5 cm above the union (Cummins and Gonsalves, 1986*a*), suggests that there may be an interplay between TmRSV and the scion. In the present study, wound tissue surrounded by wound periderm was noticed in the BL region, with necrophylactic periderm strips progressing into the scion, apparently from the rootstock at the graft union. This observation suggests that, indeed the BL is a result of an hypersensitive reaction. However, it is not clear that the BL is the result of an hypersensitive response from the scion. The anatomical changes

suggest that it is more likely a result of an hypersensitive response from the rootstock rather than the scion.

In the present study, TmRSV has been immuno-localised in the scion tissue of '70031'/Marianna 4001 plum. Marianna 2624 rootstock tissue was also found to be TmRSV-positive with ELISA and hence, susceptible to TmRSV infection. A BL would then be expected to develop at the union of '70031'/Marianna 2624 as per the hypothesis of Cummins and Gonsalves (1986*a*). However, no BL has been observed as of the writing of this thesis, neither in '70031'/Marianna 2624, nor, in any of the other scion combinations with Marianna 2624.

If one can think of PBL as a situation of rootstock tolerance to TmRSV and an inherent ability of the rootstock to prevent the virus from replicating to high titer levels, then perhaps one can explain why plum trees on Marianna 2624 do not develop a BL. Marianna 2624 may have an ability or a quality that does not promote viral replication, hence, such low virus titers may exist in this rootstock, that it is not possible to elicit a defense reaction against the virus.

The histopathological observations made on the graft union bark tissue of 'Stanley'/Myrobalan 29C in this study, suggest that the BL symptoms may start in the rootstock region of the graft union. The elliptical BL regions seen in '70031'/Marianna 4001 also suggest that the BL starts in the rootstock or at the graft union (scion/stock) interface, but not in the scion. However, since it is not absolutely certain if the symptoms of the BL, in the samples observed, started at the scion/stock interface, it cannot be stated absolutely whether the hypersensitive response is being elicited from the scion or the

rootstock. It is quite likely, that the BL may be a result of the activities in both the scion and the rootstock. Extensive sampling of the scion tissue at the graft union of TmRSV-positive trees, and an anatomical study of such samples, would give an idea of any necrosis that may occur in the scion tissue. The results of this study suggest an hypothesis that, PBL is a result of an hypersensitive response to TmRSV infection in a rootstock. Hypersensitive response in the woody plants may be very different from the hypersensitive response in herbaceous plants. In woody plants the changes associated with hypersensitive response may not take place as rapidly as in herbaceous plants, where cell death occurs within hours of infection. Marianna 2624 is either tolerant to TmRSV infection or, the hypersensitive response is not turned on in this rootstock through an unknown mechanism. The cultivars appear to have a role in the various symptoms that are associated with PBL, as evidenced by the overgrowth of the scion at the graft union (Brase and Parker, 1955). Statistical analysis comparing the scions '70031' , 'Valor' and 'Stanley' shows that there are significant differences between '70031' and the other two scions in predisposing the rootstock to PBL disease. Further results from the study in Traverse City, MI., may help elucidate the role played by the scion in PBL development.

Chapter II - Summary

Bark at the BL region of PBL-affected plum trees from a field plot in Traverse City, MI., revealed areas suggestive of wound response activity. The anatomical changes seen may be grouped into two stages of wound response activity. The early stage, where a pinkish purple necrophylactic periderm is formed around the wound area and an advanced stage where, an exophylactic periderm is formed internal to the necrophylactic periderm, followed by sloughing off of the wound tissue. Several layers of necrophylactic periderm were formed before an exophylactic periderm formed. The pigmentation of the exophylactic periderm was similar to that of the original periderm of a healthy plum bark sample. Similar wound response areas were seen in bark samples from a severely infected orchard in Paw-Paw, MI.

Immunolabeling of the bark at the BL, rootstock and scion was seen on the cell wall and in the cytoplasm. When the axial and radial components of the bark were compared, relatively heavier labeling was seen on the axial components. The silver enhanced protein-A-gold labeling of the rootstock sucker leaves showed labeling on all the tissue types of the leaf. Relatively heavier labeling was obtained on the bundle sheath cells. The pattern of labeling in the bark and leaf suggests that the long distance transport of TmRSV may be through the phloem. Statistically non-significant labeling was obtained on bark and sucker leaf samples from a healthy rootstock and rootstock sucker leaf. Statistically non-significant labeling was seen on bark of the BL and a healthy rootstock when labeled with pre-immune serum.

Conclusions

An anatomical study of the bark at the BL region of PBL-affected trees showed the presence of necrophylactic periderms that were distinguishable from the exophylactic periderm. The necrophylactic periderm formation around the wound tissue in plum is comparable to the wound response activity seen in conifers. The similarity of the exophylactic periderm to the original periderm of the plant is revealed by the similar pigmentation in the two types of periderm. The pigmented nature of the wound periderm is not revealed when the bark samples are processed for paraffin or LR White embedding. The early stages in the formation of a BL revealed necrophylactic periderm formation in the rootstock and not in the scion of the graft union region. This finding is unexpected as the earlier reports on PBL have suggested that the BL is a result of an hypersensitive response of the scion to the presence of TmRSV in the susceptible rootstock. Immunolocalization of TmRSV in the scion immediately above the BL, suggests that the scion is susceptible to TmRSV infection. The spread of the BL inward suggests that the hypersensitive response in the rootstock is slow, leading to a systemic infection of TmRSV and subsequent spread of the BL around the circumference of the plant. Marianna 2624 rootstock has been shown to be susceptible to TmRSV infection as shown by the ELISA and northern hybridization results on the root and rootstock sucker leaf samples. Whether plum cultivars propagated on this rootstock develop a BL remains to be seen.

Statistically non-significant results were obtained with ELISA and northern hybridization when root samples were tested for TmRSV infection. The northern hybridization assay should be used in detection of TmRSV in plum sucker leaf.

Root, bark and sucker leaf samples should be tested during the season of increased growth that follows the dormancy period of the plum tree.

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