POLLEN TRANSMISSION OF *CHERRY LEAFROLL VIRUS* IN SWEET CHERRY (*PRUNUS AVIUM* L.)

By

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To the Faculty of Washington State University:

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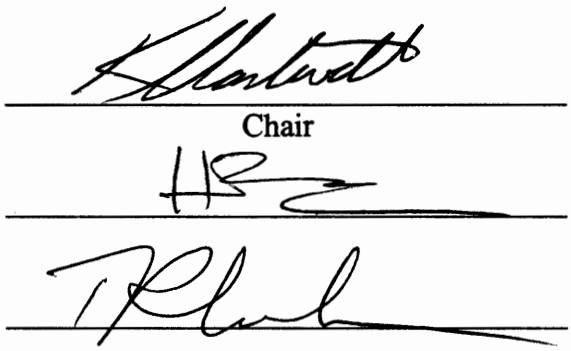

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(*PRUNUS AVIUM* L.)

Abstract

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This project examines pollen-mediated horizontal transmission of *Cherry leafroll virus* (CLRV) in sweet cherry. In a commercial orchard, three 'Van' trees were tested and found to be free of CLRV at the beginning of the study; these trees were adjacent to infected 'Bing' trees. At shuck fall, CLRV was detected by RT-PCR in extracts from ovaries and pedicels of the 'Van' trees, whereas at pit hardening and commercial harvest, all tissues including exocarp/mesocarp, seed and pedicel yielded detectable CLRV. Three weeks after commercial harvest, extracts of spur and leaf tissue of fruit bearing branches contained detectable CLRV. These results suggest that a pathway exists to transport CLRV from pollen through the pedicel, into the main plant.

Immunolocalization studies substantiated the RT-PCR results and revealed CLRV in ovary, endosperm, and pedicel tissues of developing fruit. Label was concentrated in and near vascular bundles of the ovary at shuck fall. When pedicels were examined at shuck fall and at pit hardening, label was primarily associated with the vascular bundles with additional label in sub-epidermal cells. At commercial harvest, label was only located within sub-epidermal cells. The occurrence of virus in vascular tissues of the

pedicel before pit hardening presents an opportunity for movement of CLRV through the pedicel from fruiting structures to the mother tree.

The mechanism(s) of pollen-mediated horizontal transmission of CLRV was also studied by hand pollination experiments at a Moxee research block. Four treatments were established to explore the role of fertilization and/or thrips involvement in horizontal transmission. At shuck fall, CLRV was detected by RT-PCR in ovary and pedicel samples from all treatments. The frequency with which virus was detected is not altered significantly by the presence or absence of added thrips, or whether the flower-infected pollen combination was a compatible or incompatible interaction. Immunolocalization revealed the presence of CLRV inside the ovary of flowers pollinated with incompatible infected pollen; the label was adjacent to cells of vascular tissue but not in the epidermal layer where thrips feed. These data suggest that CLRV is transported from pollen to pedicel without requiring fertilization or thrips activity.

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CHAPTER ONE

INTRODUCTION

Sweet cherry *(Prunus avium* L.*)* is a high value crop and the value of each ton was over $1,400 in 2003 (Washington Agricultural Statistics, 2004). In the United States, sweet cherry is mostly grown in the west and Washington State is the largest producer (NASS, 2003).

Cherry leafroll virus (CLRV, family *Comoviridae*, genus *Nepovirus*) was first documented in 1955 in England by Posnette and Cropley associated with a disease in sweet cherry (Cropley, 1961). In1998, CLRV was identified in sweet cherry in Washington for the first time (K. C. Eastwell, *communication*). Since that discovery, it has been found in many orchards in the Yakima Valley and the Columbia Basin areas of Washington (Watson, 2003). In recent years, CLRV has become a growing threat to Washington sweet cherry production. Alone, CLRV causes a slow decline of sweet cherry trees over a period of seven to ten years. However, in mixed infections with either *Prune dwarf virus* (PDV, genus *Ilarvirus*) or *Prunus necrotic ringspot virus* (PNRSV, genus *Ilarvirus*), CLRV is devastating, resulting in a much quicker and severe decline. Most diseased trees are removed, but it is presumed that the declining trees would eventually die. This disease seriously affects yield and quality of fruits. Currently, tree removal is the only effective method of controlling diseases caused by CLRV. Growers remove the infected trees to keep healthy trees from becoming infected. However, it is difficult to prevent the virus spread because CLRV newly infected trees may not display obvious symptoms for several years, providing a continuing source of inoculum to facilitate further spread. For example, CLRV is established in a commercial orchard

located south of Grandview, WA; new infections have been found every year over the past seven seasons even though infected trees are removed as they become evident by visual inspection and ELISA.

In addition to sweet cherry, CLRV commonly infects birch (*Betula pendula*), black elderberry (*Sambucus nigra*), golden elderberry (*Sambucus canadensis*) and English walnut (*Juglans regia*) (Rebenstorf *et al.*, 2006). The virus has been reported in Europe, North America, Chile, New Zealand, Australia, China and Japan (Rebenstorf *et al.*, 2006). CLRV is an isometric bipartite virus and its genome consists of two single-stranded positive-sense RNA molecules designated RNA-1 and RNA-2. CLRV appears to spread naturally through seeds and pollen in some hosts (Bandte & Buttner, 2001). Unlike other nepoviruses, CLRV is not considered to be transmitted by nematodes. CLRV was reported to be transmitted by some nematodes such as *Xiphinema* spp. and *Longidorus* spp. (Jones *et al*., 1981). However, those results could not be repeated and verified (Wang, *et. al*., 2002).

Plant viruses have been reported to be associated with pollen since the 1940's. Since that time, more than fifty different viruses have been identified in which their transmission is mediated by pollen (Cooper *et al.,* 1988). Nepoviruses and ilarviruses are two virus groups that are most often associated with pollen transmission. Two categories of pollen-borne viruses can be defined based on their mode of transmission. One is vertical transmission which is transmission of the virus directly from a mother plant to its offspring (Cooper, *et al.* 1988). Most viruses that have pollen transmission reported as one aspect of their epidemiology are transmitted vertically from pollen to seed. However, some viruses are spread via horizontal transmission from plant to plant with pollen being the carrier (Mink, 1993).

Nineteen pollen-borne viruses are documented to be vertically transmitted from pollen to seed (Mandahar & Gill, 1984). Several CLRV strains including elm (Callahan, 1957), elder (Schimanski & Schmelzer, 1972), walnut (Mircetich *et al.*, 1982) and birch (Copper *et al*., 1984) are transmitted in this way. In studies of ilarviruses, Gilmer and Way (1960) demonstrated that PNRSV and PDV could be vertically transmitted in sour cherry trees (*Prunus cerasus*) to seed by pollen. When healthy flowers were hand pollinated with PNRSV- and/or PDV-infected pollen, about 25% of the seeds that developed from these flowers were infected (Gilmer & Way, 1960).

The mechanism of vertical transmission of pollen-borne viruses is still unknown. There are two ways that virus can move from infected pollen to the seed: 1) mechanical infection of ovary through wounds caused by pollen tube growth or by insect behavior, or 2) virus-infected male gamete infects the female gamete during fertilization (Carroll, 1974). A large body of work has been done on vertical transmission of PNRSV. Kelly and Cameron (1986) used PNRSV-infected almond pollen mixed with virus-free cherry pollen to pollinate healthy cherry flowers; no seed from these hand pollinated trees was infected. However, the virus could be found in seeds from healthy cherry trees hand-pollinated with infected cherry pollen. This result indicated that PNRSV was transmitted from pollen to seed by fertilization since no infection occurred without fertilization since almond pollen cannot fertilize cherry ovules. In recent years, this mechanism has been studied at the molecular level (Aparicio *et al*., 1999; Amari *et al*., 2004). Using *in situ* hybridization, PNRSV was found in the cytoplasm of the vegetative cells but not in the generative cells of pollen in nectarine (Aparicio *et al*., 1999). This result suggests that fertilization may not be involved in vertical transmission in nectarine since no virus was detected in the generative cells from which the

sperm cells develop. On the other hand, viruses were located in the vegetative cytoplasm of pollen that moves to the embryo sac with pollen tube growth. This result suggests two possibilities: 1) the male gametes (sperm cells) are contaminated by these cytoplasmic virus particles before fertilization and are then transmitted to the ovule by fertilization, or 2) the virus is transmitted to the ovary during pollen germination. This redistribution of virus derived from pollen may be a critical step in the transmission from pollen to seed during the pollination or fertilization process.

Seven viruses have been demonstrated to spread horizontally by pollen including three nepoviruses [*Artichoke yellow ringspot virus* (AYRV), *Blueberry leaf mottle virus (*BBLMV), and CLRV], four ilarviruses *[Blueberry shock virus* (BlShV), PDV, PNRSV and *Tobacco streak virus* (TSV)], *Sowbane mosaic sobemovirus* (SoMV) and *Raspberry bushy dwarf ideaovirus* (RBDV) (Mink, 1993). Horizontal pollen-transmission of viruses plays a very important role in viral disease epidemiology (Mandahar, 1984). Pollen from one infected plant can transport the virus to many other healthy plants. These secondary infected plants produce more virus-infected pollen. This process will repeat each year during bloom season and cause destructive results in a short time (Mandahar, 1985). For example, CLRV walnut stain causes blackline disease in English walnut (*Juglans regia* L.) in the USA. This disease is dispersed rapidly from plant to plant through infected pollen in nature. Blackline disease can cause great loss and is thought to be the most important negative factor affecting walnut production in California (Mircetich *et al*., 1980).

The processes involved in vertical transmission of virus from plant to plant by pollen are complicated and, as with horizontal transmission, the mechanisms are not truly understood. Virus moving from infected-pollen to the mother plant occurs during pollination

and fertilization. However, no conclusive evidence convincingly demonstrates that pollen horizontal transmission of virus can occur only through fertilization (Mink, 1993). It is well known that a callose layer surrounding the embryo is formed before fertilization that would restrict virus movement from the embryo to the plant. It seems unlikely that horizontal transmission happens as a direct result of fertilization. Mandahar (1984) presented the "back-infect" hypothesis which suggests that flower parts might be mechanically infected by honeybee activity and then the virus could be introduced into the maternal plant via the plasmodesmata connecting flower tissues. Subsequently, additional evidence suggested that thrips or honeybees may also contribute to the pollen-mediated horizontal transmission of ilarviruses (Mink, 1992; Boylan-pett *et al*., 1991).

Sdoodee and Teakle (1987) first reported that TSV-contaminated pollen could be carried by *Thrips tabaci* and then transmitted to leaves of the experimental host plant *Chenopodium amaranticolor*, probably via wounds caused by thrips. TSV was found to infect tobacco crop plants in Queensland, Australia. Research showed that the high incidence of TSV in test plants resulted from the presence of both thrips and the weed pollen (Greber, *et al*., 1991). Thus the interaction of thrips *Microcephalothrips abdominalis* and pollen of the weed *Ageratum houstonianum*, the most common wild host of TSV in that area, probably caused the disease epidemic.

BBLMV is distributed randomly and spreads quickly in highbush blueberry fields. Honeybees were shown to mediate this horizontal transmission, transporting virus infected pollen from plant to plant during pollination (Childree & Ramsdell, 1987; Boylan-pett *et al*., 1991). The pattern of BlShV spread and distribution in the field is similar to BBLMV and

the combination of honeybee activity and virus-laden pollen is responsible for this transmission pattern (Bristow & Martin, 1999).

CLRV is known to be spread from plant to plant in sweet cherry orchards by root grafting (K. C. Eastwell, *personal communication*). But the mechanism(s) of transmission of CLRV over longer distances is still unknown. Evidence suggests that pollen probably plays an important role in the transmission of CLRV. CLRV of sweet cherry is a pollen-borne virus as are other CLRV strains. Honeybees can carry virus-laden pollen from one tree to another during pollination. Insects that feed in and around flowers such as western flower thrips (*Frankliniella occidentalis*) create wounds on flowering tissues. Therefore, thrips may be a vector of CLRV pollen transmission. In greenhouse experiments, thrips transferred the virus from infected cherry pollen to a herbaceous experimental host *Chenopodium quinoa* but not to young cherry seedlings (W. E. Howell, *personal communication*).

The control or management of a viral disease requires a firm understanding of its epidemiology. To improve our knowledge of the mechanisms of CLRV transmission, this research explores the following hypothesis: infected pollen plays a critical role in the horizontal transmission of CLRV in sweet cherry. This project sought to investigate the mechanism(s) of pollen-mediated horizontal transmission of CLRV in sweet cherry including the possible role of thrips in pollen-mediated transmission

CHAPTER TWO

MATERIALS AND METHODS

Description of field plots

The 'Grandview' plot is located south of Grandview, Washington. It is within a commercial orchard planted in the mid-1980's and managed with standard commercial practices. The trees are planted with 6.4 meters between trees in a row and 6.0 meters between rows. The irrigation system is an under-tree sprinkler system and there is a mix of grass and broadleaf weeds in the understory. Natural pollination is augmented with leased bee hives in spring. *Cherry leafroll virus* (CLRV) infection is widespread in this orchard and most diseased trees have been removed. A small area of infected trees was retained for research purposes (Figure 1). The trees in the orchard are tested annually for CLRV, *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) as part of a monitoring program. According to the CLRV survey data from recent years, four 'Van' trees were not infected with CLRV but were adjacent to CLRV-infected 'Bing' trees. In this orchard, 'Van' is the pollinizer for commercial production, which is cross compatible with 'Bing'.

The Moxee plot is located 12 mi east of Moxee, Washington in an experimental orchard of USDA-ARS, Wapato. The management of the Moxee orchard is similar to Grandview orchard except that honeybees from nature are responsible to pollination and no pesticides are used for insect control. A section of the orchard in one corner was selected for the field plot (Figure 2). All trees were tested for CLRV, PNRSV and PDV by ELISA and were negative in this block.

Pollen source

Sweet cherry trees (*Prunus avium* L.) cultivar 'Bing' and 'Van' in the research plot at the Grandview orchard were tested for CLRV by ELISA in spring, 2006. Anthers were collected from flowers of infected and healthy trees at the balloon stage. The anthers were air dried for 24 h at room temperature, and the mixture of anthers and released pollen were stored at 4°C in glass vials.

Thrips

Western flower thrips (*Frankliniella occidentalis*) were collected from feral Balsam root flowers (*Balsamorhiza sagittata)*. The species was identified by Dr. Tom Unruh, entomologist in Yakima Agricultural Research Laboratory, USDA. Thrips were dislodged from the flowers by lightly tapping flowers against a white counter top. Both adult and larval thrips were aspirated directly into clean vials in groups of 50 thrips per vial. These were stored at 4°C until used on the following day.

Experimental design at the Grandview site

During the 2005 growing season, preliminary experiments were conducted in this orchard. Fruits were collected from four healthy 'Van' trees V1 (3-3), V2 (5-5), V3 (7-5) and V4 (1-5) at pit hardening and at commercial harvest. Each fruit was separated into the exocarp and mesocarp (hereafter referred to as the mesocarp), seed and pedicel tissue. Mesocarp was tested for CLRV by RT-PCR and ELISA; seeds were tested by ELISA only. Pedicels were cut into two parts crossways and each half tested separately by RT-PCR.

In the 2006 growing season, samples were collected from three remaining healthy 'Van' trees V1 (3-3), V2 (5-5) and, V3 (7-5) at four different cherry growth stages: shuck fall, pit hardening, commercial harvest and 21 days post harvest. Extracts from each sample were

Row / Tree	10	9	8	7	6	5	4	3	2	1
ORCHARD ROAD - ROW NUMBER										
1	B[4]	B	V[4]	B	B	B	B	B	B	V
2	B	B	V	B[4]	B	V	B[1,3]	B	B	B
3	B[4]	B	B	B[2,3]	B	B	⊗[1,3]	V1[3]	B[1]	B
4	V	V	B[3]	B[3]	B[1,3]	B[3]	B[3]	B[1]	B	B[3]
5	B[4]	B	B[2,3,4]	V3[3]	B[1,3]	V2[3]	B[3]	B	⊗[1]	V4
6	B	V	B[2,3]	B[3, 4]	B[3]	B[3]	B[3]	B	B[1]	B[1]
7	B	B[4]	B[4]	B	B	B	B	V	B	B
8	B[4]	B[4]	B	V	B	B	B	B	B	B
9	B	B	B	B	B	V	B	B	B	V
10	B	V[4]	V[2,3]	B[2,3]	B[2,3]	B	B	B	B	B

V='Van' tree; B= 'Bing' tree

⊗= unknown variety naturally infected and previously removed

V1 to V4: 'Van' trees used in this experiment

1. The tree was infected with *Cherry leaf roll virus*
2. The tree was infected with *Prunus necrotic ringspot virus*
3. The tree was infected with *Prune dwarf virus*
4. The tree was about 35 years old whereas others are about 20 years old

Figure 1. Plot plan of the orchard in Grandview, WA.

N
↓

Row / Tree	U	T	S	R	Q	P	O	N
7	B	V[1]	B[1]	B	V[1]	B[1]	B	V
6	B	B	B	B	B	B	B	B
5	B	B	B	B	B	B	B	B
4	B	V	B	B	V[2]	B	B	V
3	B	B	B	B	B	B	B	B
2	B	B	B	B	B	B	B	B
1	B	V[1]	B[1]	B[1]	V[1]	B	B	V

V='Van' tree; B= 'Bing' tree;

1. Trees where hand pollination experiments were conducted
2. 'Van' Q4, negative control

Figure 2. The plan of the plot located in the Moxee, WA orchard.

tested for CLRV by RT-PCR. At the earliest developmental stage tested, samples consisted of the ovule and pedicel. At the next two stages, the mesocarp, embryo and pedicel were tested separately. Ten samples of each tissue type from each 'Van' tree were tested at each stage. At each stage, another ten ovules, embryos and pedicels were fixed and embedded for immunolocalization. At the post harvest stage, four pedicels and the spurs to which they were connected were collected from each of five different major limbs per tree. The 20 samples from each tree were tested individually. In addition, five leaves, ten fruits and embryos from each tree were tested. Positive control tissues were collected from a CLRV-infected 'Bing' tree (row 6 and tree 4 in the Grandview orchard, Figure 1). Samples for the negative control were from a virus-free 'Van' tree (row Q and tree 4 in the Moxee orchard, Figure 2).

Experimental design at the Moxee site

Four treatments were established in the Moxee orchard to test the mechanisms of pollen vertical transmission of CLRV in sweet cherry:

A. Pollination of healthy 'Van' flowers with CLRV-infected 'Bing' pollen in the presence of added thrips
B. Pollination of healthy 'Van' flowers with CLRV-infected 'Bing' pollen without added thrips
C. Pollination of healthy 'Bing' flowers with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen (5:1) in the presence of added thrips
D. Pollination of healthy 'Bing' flowers with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen (5:1) without added thrips

In this experiment, four 'Van' trees and four 'Bing' trees were used. The trees are located at the edge of the orchard as indicated in Figure 2; two 'Van' and two 'Bing' trees are located on the uphill (south) portion of the orchard and the same on the downhill (north) portion. Ten branches were selected on each tree and ten flowers were isolated on each branch. These flowers were enclosed in an organdy cloth cage on the branch. The ends of the cage were secured on the branch by wrapping the branch with steel wool and tying the end of the cage onto the steel wool securely with flagging tape; a different color tape was used to represent each treatment (Figure 3). In late April, one day before hand pollination, 100 thrips per cage were added to those cages requiring added thrips. Hand pollination was performed in two ways to increase the possibility of fertilization and to maximize interaction between thrips and pollen (hand pollination date: April 26 and April 27). Pollen was dusted onto the flowers by a hand atomizer pump; four squeezes of the pump provided about 1.2 mg pollen per cage. Additional pollen was applied to the stigma of each flower with a small brush. Pollination of the plots on the North and South locations were performed on two consecutive days.

Ten days after pollination, two hand-pollinated flowers were collected from each cage for a total of 20 flowers from each treatment. Each flower was divided into the ovule and pedicel. Ten samples of each tissue were tested for CLRV by RT-PCR and another ten samples were fixed and embedded for immunolocalization.

Surface contamination experiment

At pit hardening, six pedicels from negative control (Moxee plot row Q, tree 4) were collected and placed in a plastic bag. The mixed pollen used in the hand pollination experiments was dusted into the bag with the hand atomizer pump (four squeezes). These

Figure 3. The organdy cage used in the hand pollination experiment. Orange ribbon referred to 'Van' tree; blue ribbon referred to thrips treatment; the number of the treatment was recorded on the yellow ribbon.

samples were washed three times with PBS-Tween (20 mM Na_2HPO_4-12 H_2O, 130 mM NaCl, 1.5 mM KH_2PO_4, 3 mM KCl; 0.05% Tween-20, pH 7.4), each wash lasting one minute. Extracts from the pedicels were then tested for the presence of CLRV by RT-PCR.

Pollen grain germination *in vitro*

The test method was that described by Hicks *et al.* (2004). Dried pollen grains from healthy and CLRV-infected 'Bing' trees were incubated in liquid media (18% sucrose, 0.01% boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 1 mM $Ca(NO_3)_2$, pH 6.5) at room temperature for 24 h in darkness. Each germination test was performed in three replications. An Olympus A011 light microscope was used for observing pollen tube growth. Three areas were chosen randomly from each plate to count germinated pollen grains and the average germination rate was calculated.

ELISA procedures

CLRV: Mircotiter plates used for ELISA were 96-well polystyrene plates (Maxisorp: Nalge Nunc International, Denmark). Each well was coated with 100 µl rabbit IgG prepared against the cherry strain of CLRV (Cat. number: 150912: Bioreba, Switzerland) diluted 1:1000 in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Plates were placed in a humidified sealed container for 2 h at room temperature. Wells were washed three times with PBS-Tween using a 8-channel manual plate washer (Nunc-immuno^{TM} Wash 8, Nalge Nunc International, Denmark). After the final wash, excess buffer was gently tapped out of the inverted plate. Samples were ground in CEP (10 mM Na_2CO_3, 40 mM $NaHCO_3$, 0.5 mM polyvinyl–pyrrolidone, 2.0 g ovalbumin, pH 9.6) plus DIECA(0.45% (w/v) sodium diethyldithiocarbamate, trihydrate) at 1 g/10 ml for tissue and 1 g/50 ml for pollen. The sample (100 µl) was pipetted into each well and incubated overnight at 4°C. Wells were

washed again as described above and 100 µl antiserum conjugated to alkaline phosphatase (Cat. number: 150922, Bioreba) diluted 1:1000 in 3% milk block [(w/v), Carnation nonfat powdered milk in PBS-Tween)] was added to each well and incubated at room temperature for 2 h. Plates were washed again as described and 100 µl substrate solution (0.01 g / 10 ml *p*-nitrophenyl phosphate in 1 mM $MgCl_2$, 9.7% diethanolamine, pH 9.8) was added per well. Absorbance values at 405 nm wavelength light (A_{405}) were read 30 to 60 min after addition of substrate. Absorbance readings were performed using a microplate reader (Emax: Molecular Devices, Sunnyvale, CA).

PDV: Microtiter plates were coated with 100 µl per well PDV-E rabbit polyclonal antiserum (WSU-Prosser ELISA Service Center), and diluted 1:500 in carbonate coating buffer. Plates were placed in a humidified sealed container for 2 h at room temperature. Wells were washed three times with PBS-Tween. After the final wash, excess buffer was gently tapped out of the inverted plate and 100 µl 3% milk block was added to each well and tapped out after 30 min. Samples were ground in buffer CEP plus DIECA and 100 µl sample was put in each well and the plates incubated overnight at 4°C. Wells were washed again as described above and 100 µl tissue culture supernate from hybridoma PDV-A3C (Rampitsch *et al*., 1995), diluted 1:3 in 3% milk block, was added to each well and incubated at 37°C for one hour. Plates were washed again as described and 100 µl goat anti-mouse alkaline phosphatase conjugate (Cat. number: 075-1807, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) diluted 1:3000 in 3% milk block was added to each well and incubated at 37°C for 2 h. Plates were washed again as described and 100 µl substrate solution was added for per well. After 30 to 60 min, absorbance values were measured.

PNRSV: Plates were coated with 100 µl per well PNRSV coating antibody (Cat. number: SRA 30500/5000, Agdia, Elkhart, IN) diluted 1:200 in carbonate coating buffer. Plates were placed in a humidified sealed container for 2 h at room temperature. Wells were washed three times with PBS-Tween. After the final wash, excess buffer was gently tapped out of the inverted plate and 100 µl 3% milk block in PBS-Tween was added to each well and tapped out after half an hour. Samples were ground in CEP plus DIECA and 100 µl sample was put in each well and incubated overnight at 4°C. Wells were washed again as described above and 100 µl PNRSV detection antibody (Cat. number: SRA 30500/5000, Agdia,) diluted 1:200 in 3% milk block was added to each well and incubated at room temperature for one hour. Plates were washed again as described and 100 µl PNRSV conjugate (Cat. number: SRA 30500/5000, Agdia) diluted 1:200 in 3% milk block was added to each well and incubated at room temperature for 2 h. Plates were washed again as described and 100 µl substrate solution was added for per well. After 30 to 60 min, absorbance values were measured with the microplate reader.

Reverse transcription polymerase chain reaction (RT-PCR)

Detection of CLRV by RT-PCR was done by using total RNA extracts as template and primers CLRV1-L (5'-CGACCGTGTAACGGCAACAG-3', positions 1185-1204 on CLRV walnut stain genomic RNA) and CLRV2-R (5'-CACTGCCTGAGTCCGACACT-3', positions 1501-1520 on CLRV walnut stain genomic RNA) (Genbank, accession number: Z34265). This primer pair from the 3'-untranslated terminal regions of CLRV genomic RNA1 and RNA2 was a modification of a previously published primer pair (Werner *et al.,* 1997), and base on highly conserved sequences found in isolates from birch, rhubarb, walnut and beech.

Before extraction, all tissue samples were washed three times with PBS-Tween for one minute per wash. Total RNA was isolated by using RNeasy® Plant Mini Kit from (Qiagen, Valencia, CA). Approximately 300 mg fresh tissue was ground with 1,000 µl buffer RLT (Qiagen) in a grinding bag (Agdia). The lysate was transferred to a 1.7 ml microcentrifuge tube and incubated 3 min at 65°C. The remainder of the isolation procedure followed the manufacturer's recommendations as follows. The lysate was transferred to a QIAshredder spin column and centrifuged for 2 min at 16,000 × *g*. The cleared lysate (about 450 µl) was added to 225 µl 95% ethanol in a clean 1.7 ml microcentrifuge tube and mixed. The mixture was transferred to the RNeasy mini column and centrifuged for 15 second at 5,220 × *g*. The RNeasy column was put into a new collection tube (2 ml) and 700 µl buffer RW1 was added and centrifuged (5,220 × *g*, 15 second), then 500 µl buffer RPE was added and centrifuged (5,220 × *g*, 15 second), then another 500 µl buffer RPE was added and centrifuged (5,220 × *g*, 2 min). Finally, the column was set into a 1.5 ml collection tube, 40 µl RNase-free water was added and centrifuged for 1 min at 5,220 × *g*. The eluted RNA was stored at -70°C.

One-step RT-PCR was performed in a 25 µl reaction volume consisting of 1 µl total RNA template, 0.25 µl each primer (20 mM) , 1 µl SuperScript™ III RT with Platinum® *Taq* Mix (Invitrogen, Carlsbad,CA), 12.5 µl 2X Reaction Mix (supplied with enzyme and contains 0.4 mM of each dNTP, 3.2 mM $MgSO_4$), and 10 µl autoclaved distilled water.

The reaction was run at the following thermocycling conditions: hot start at 55°C, then 30 min at 55°C, 2 min at 94°C, 40 cycles of 15 s at 94°C, 30 s at 55°C, 1 min at 68°C and an elongation step of 5 min at 68°C, finally, temperature was reduced and held at 4°C. Products of amplification were analyzed by electrophoresis in 3 % Nusieve GTG® agarose

(Cambrex, Rockland, ME) using 1×TAE (40 mM Tris-acetate, pH 7.6; 1 mM EDTA) as electrophoresis buffer and 100 V for 1.5 h. Gels were stained in 0.5 µg/ml ethidium bromide. The standard molecular size marker was a 100 bp DNA ladder (Invitrogen). The results were observed and pictures recorded (Quantity One Biorad Geldoc System, Biorad, Hercules, CA).

Immunolocalization

Tissues were cut into small pieces (1mm x1mm) , and fixed with 1.25% glutaraldehyde, 2% paraformaldehyde in 50 mM Pipes buffer, pH 6.7 at 4°C overnight then rinsed in three changes of 50 mM Pipes buffer, 10 min per wash. The segments were dehydrated in a graded series of ethanol concentrations (30%, 50%, 60%, 70%, 80% and 95%) for 10 min each at each concentration. Tissue was finally dehydrated in 100% ethanol with three washes of 10 min each. The tissues were infiltrated in a series of LR White resin (London Resin Co., Basingstoke, UK) concentrations in ethanol (1:3, 1:2, 1:1 and 3:1) on a rotary table, each infiltration step was overnight. The final infiltration step was with 100% LR White resin 3 to 4 times with each step overnight. Finally, the tissues were cured at 60°C for 24 h in polypropylene cups (Cat.number: 25384-076 VWR International, West Chester, PA). Tissues embedded with resin were cut into sections (800 nm) with an ultramicrotome (Reichert-Jung, Germany). Ten sections were put on each "Snow coat" microscope slide (Cat. Number 00299, Surgipath, IL); three slides of each sample and two slides of positive and negative control samples were prepared for immunohistochemical staining.

All manipulations were performed at room temperature unless otherwise noted. Sections were incubated in TBST+BSA (10 mM Tris-HCl, pH 7.2-8.3; 250 mM NaCl; 0.3% Tween-20, 1% bovine serum albumin) for 1 h in a sealed, humidified chamber to block non-specific protein binding sites in tissues. Polyethylene sealing tape (Cat.number: 11211-932,

VWR International) was applied to the slides to keep solutions confined to the area around the sections. The primary antibody (IgG from rabbit, CLRV-ch, Bioreba) was diluted to 1:100 in a suspension prepared by grinding 2 g leaves of non-infected *Chenopodium quinoa* in TBST+BSA; this mixture was allowed to stand for approximately 30 min during which time the antibody reacts with the protein in *Chenopodium quinoa* leaf to reduce non-specific binding to cherry tissue and to reduce background labeling. The primary antibody solution was centrifuged and the supernatant layer was used for immunolocalization. After 1 h, the blocking solution was removed and cross-reacted primary antibody was added to the sections and incubated for 4 h. To remove unbound primary antibody Sections were washed 4x with TBST+BSA for 15 min for each wash. Then, the washed sections were incubated with secondary antibody (5 nm gold conjugated goat anti-rabbit IgG, British BioCell International) diluted with TBST+BSA (1: 100) for 1 hour, and washed with TBST+BSA two times, 15 min each time, to remove unbound secondary antibody. Sections were washed twice with TBST, 10 min each time, to remove TBST+BSA, and finally rinsed with distilled water three times, 5 min per rinse. To enhance the gold label, sections were incubated with 1:1 ratio of silver enhancing solutions (Silver enhancement kit for Light and Electron Microscopy, Ted Pella, Inc., Redding, CA) or10 min, then gently rinsed with distilled water and stained with 2% Safranin-O. Stained sections were observed and photographed with the confocal laser scanning microscope (BioRad MRC 1024); label appeared as a red color.

CHAPTER THREE

RESULTS

Surface contamination result

At pit hardening, six pedicels from the healthy tree 'Van' Q-4 at Moxee (Figure 2) were dusted with infected pollen, immediately washed three times with PBS-Tween, and their extracts were tested for *Cherry leafroll virus* (CLRV) by RT-PCR, two yielded positive results. This result indicates that surface contamination can not be ignored even after the sample is washed three times with PBS-Tween before extraction.

Virus status of subject trees

Trees used in this study were tested by ELISA to determine their status with regard to CLRV and viruses commonly found in orchards of the Pacific Northwest. The pollen borne ilarviruses are most frequently encountered. The ELISA results are summarized in Table 1. For trees at the Grandview site, the four 'Van' trees adjacent to CLRV-infected 'Bing' trees were CLRV-negative but all were infected with *Prune dwarf virus* (PDV). The positive control 'Bing' tree 6-4 was CLRV-infected and also PDV positive while positive control 'Bing' tree 3-4 was infected with CLRV only. The negative control 'Van' tree 13-13 was not infected with CLRV but was infected with both PDV and *Prunus necrotic ringspot virus* (PNRSV). None of the trees used in this study at the Moxee orchard were infected with any of these three viruses.

Season one: assessment of methodology

Initial analyses were done by RT-PCR and ELISA during summer 2005 to obtain general information about the distribution of CLRV in flowering tissues from healthy sweet

Table 1. Detection of *Cherry leafroll virus* (CLRV), *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) by ELISA in spring, 2006 in extracts of buds from trees used in experiments.

Tree identification	ELISA result[1]		
	CLRV	PDV	PNRSV
GRANDVIEW ORCHARD:			
'Van' 1(3-3)	-	+	-
'Van' 2 (5-5)	-	+	-
'Van' 3(7-5)	-	+	-
'Bing' (3-4)[2]	+	-	-
'Bing' (6-4)[2]	+	+	-
'Van' (13-13)[3]	-	+	+
MOXEE ORCHARD:			
'Van' (T-1)	-	-	-
'Van' (T-7)	-	-	-
'Van' (Q-1)	-	-	-
'Van' (Q-4)[3]	-	-	-
'Van' (Q-7)	-	-	-
'Bing' (S-1)	-	-	-
'Bing' (S-7)	-	-	-
'Bing' (R-1)	-	-	-
'Bing' (P-7)	-	-	-

1. An ELISA positive result is one in which the A_{405} is at least three times higher than that of negative control.
2. CLRV-infected positive control.
3. CLRV-free negative control.

cherry trees, and to verify methods for detecting it (Table 2). In the 2005 growing season, samples were obtained from the Grandview site only. CLRV was detected in extracts from pedicel and mesocarp tissues of cherries from the four uninfected 'Van' trees adjacent to infected 'Bing' trees. The virus was detected in the mesocarp by RT-PCR but not ELISA. Seed extracts from cherries collected from these trees were tested by ELISA only. At pit hardening, two of five seeds from 'Van'1 (3-3) were positive; one of five seeds from 'Van'2 (5-5) was positive and two of six seeds from 'Van'4 (1-5) yielded positive results at harvest. Therefore, at pit hardening and commercial harvest, CLRV could be detected in seeds (by ELISA), mesocarp, and pedicels (by RT-PCR) of CLRV-negative trees adjacent to virus-infected pollinators.

At commercial harvest, pedicel and leaf tissues from the tested trees were embedded for immunolocalization. Labeled particles were located primarily in the sub-epidermal layer of pedicels from 'Van'1 (3-3) but in both the vascular bundle and sub-epidermal layer of pedicels of positive control 'Bing' (3-4) (*data not shown*). Label was located in the cytoplasm in CLRV-infected leaves. However, non-specific labeling of some samples and of the negative control was apparent. To avoid this background problem, *Chenopodium quinoa* extracts were used to cross-absorb the antiserum in subsequent immunolocalization experiments.

Season two: the distribution of CLRV in sweet cherry at various developmental stages

In spring 2006, the extracts of buds from all trees in the Grandview and Moxee plots used in these studies were tested for the presence of CLRV by RT-PCR and ELISA (Table 1); the presence of PDV and PNRSV was tested by ELISA only (Table 1). Samples from

Table 2. Detection of *Cherry leafroll virus* (CLRV) in extracts from sweet cherry fruit tissues collected in the Grandview orchard during the summer, 2005.

Tested tissues	RT-PCR and ELISA assay results *number of positive / number of tested samples*					
	'Van' 1 (3-3)[3]	'Van' 2 (5-5)[3]	'Van' 3 (7-5)[3]	'Van' 4 (1-5)[3]	'Bing' (3-4)[4]	'Van' (13-13)[5]
PIT HARDENING						
Pedicel-distal[1]	0/4	0/5	4/5	0/5	5/5	0/5
Pedicel-proximal[1]	0/4	0/5	0/5	4/5	5/5	0/5
Mesocarp[1]	4/4	5/5	4/5	5/5	5/5	0/5
Seed[2]	2/5[6]	0/5	0/5	0/5	5/5	0/5
COMMERCIAL HARVEST						
Pedicel-distal[1]	0/5	0/5	0/5	5/5	5/5	0/5
Pedicel-proximal[1]	0/5	0/5	0/5	5/5	5/5	0/5
Mesocarp[1]	5/5	4/5	2/2	5/5	5/5	0/5
Seed[2]	0/5	1/5	0/5	2/6	5/5	0/5

1. Extracts from pedicel and mesocarp were tested by RT-PCR
2. Extracts from seeds were tested by ELISA. An ELISA positive result is one in which the A_{405} is at least three times higher than that of negative control.
3. 'Van' trees that tested negative for CLRV by ELISA but are situated adjacent to CLRV infected 'Bing' trees.
4. Positive control CLRV- infected 'Bing' tree.
5. Negative control CLRV free 'Van' tree.

'Van' 4 (1-5) were positive for CLRV by RT-PCR. Therefore, only the CLRV-free trees 'Van' 1 (3-3), Van' 2 (5-5), and Van' 3 (7-5) in the Grandview plots were used for experiments in 2006.

a) Shuck fall stage: CLRV was detected by RT-PCR in extracts from ovary and pedicel tissues from three uninfected 'Van' trees (Table 3). The results from individual flowers included all possible combinations: both ovary and pedicel were positive or negative; extracts of only the ovary were positive and extracts of the pedicel were negative, or the reverse.

b) Pit hardening stage: At pit hardening, extracts of mesocarp, seed and pedicel tissues separated from fruit yielded positive results for CLRV by RT-PCTR (Table 4). In these samples, the virus was detected in extracts of mesocarp tissue from all three 'Van' trees and was detected in the seed from 'Van' 1 (3-3) and in the pedicel from 'Van' 3 (7-5). In samples from one fruit, the mesocarp and seed were infected, and in another fruit, the mesocarp and pedicel were infected.

c) Commercial harvest stage: RT-PCR results from samples collected at commercial harvest time are presented in Table 5. CLRV was detected in extracts of all tissues associated with the fruit including mesocarp, seed and pedicel. Of the samples tested, extracts from seeds of all three 'Van' trees yielded positive amplification products (Figure 4), extracts of the pedicels from 'Van' trees V1 (3-3) and V3 (7-5) were positive, and the mesocarp from 'Van' V1 (3-3) only appeared to be positive. For those fruits in which CLRV was detected in the pedicel, the mesocarp and /or seed from the same fruit were also infected.

d) Post harvest stage: Approximately three weeks after commercial harvest, CLRV was detected by RT-PCR in extracts of mesocarp, seed and pedicel (Table 6) of samples collected from the virus free 'Van' trees exposed to CLRV-infected pollen.

Table 3. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in extracts from sweet cherry fruit tissues at shuck fall stage after natural pollination. Samples were collected on April 24, 2006 at the Grandview plot.

Tree label	RT-PCR assay results *number of positive / number of tested samples*	
	Ovary	Pedicel
'Van' 1(3-3)	3/10	7/10
'Van' 2 (5-5)	4/10	7/10
'Van' 3 (7-5)	5/10	7/10
'Bing' tree (6-4)[1]	5/5	5/5
'Van' tree (Q4)[2]	0/5	0/5

1. CLRV-infected positive control from the Grandview plot.
2. CLRV free negative control from the Moxee plot.

Table 4. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in extracts from sweet cherry fruit tissues at pit hardening stage after natural pollination. Samples were collected on May 31, 2006 from the Grandview plot.

Tree label[1]	RT-PCR assay results *number of positive / number of tested samples*		
	Mesocarp	Seed	Pedicel
'Van' 1(3-3)	4/10	1/10[2]	0/10
'Van' 2 (5-5)	8/10	0/10	0/10
'Van' 3 (7-5)	9/10	0/10	1/10[2]
'Bing' tree (6-4)	5/5	5/5	5/5
'Van' tree (Q4)	0/5	0/5	0/5

1. All the trees were the same as table 3.
2. Mesocarp from the same fruit was positive.

Table 5. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in extracts from sweet cherry fruit tissues at the commercial harvest stage after natural pollination. Samples were collected on July 3, 2006 from the Grandview plot.

Tree label[1]	RT-PCR assay results *number of positive / number of tested samples*		
	Mesocarp	Seed	Pedicel
'Van' 1(3-3)	2/10	10/10	2/10[2,3]
'Van' 2 (5-5)	0/10	10/10	0/10
'Van' 3 (7-5)	0/10	8/10	1/10[4]
'Bing' tree (6-4)	5/5	5/5	5/5
'Van' tree (Q4)	0/5	0/5	0/5

1. All the samples were from the same sources as in Table 3.
2. Seed and mesocarp from the same fruit were positive
3. Seed from the same fruit was infected
4. Seed from the same fruit was infected

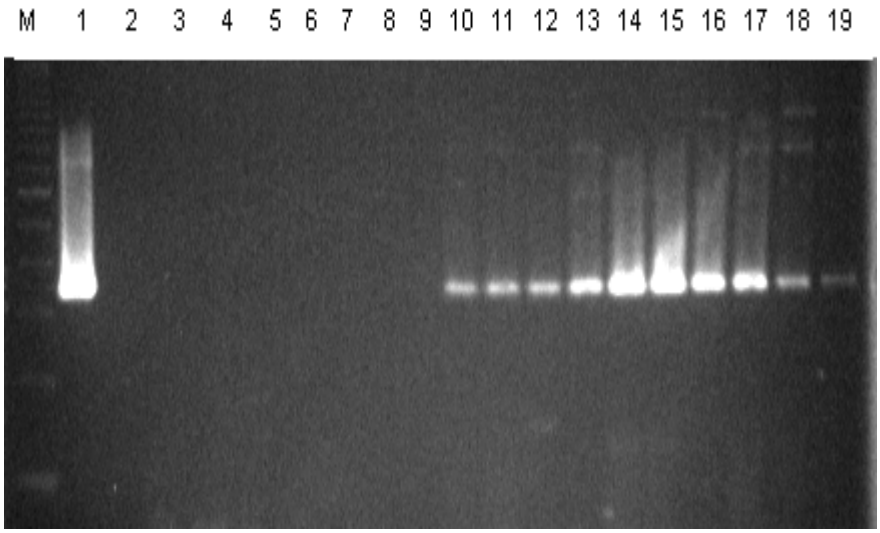


Figure 4. An example of gel analysis of *Cherry leafroll virus* amplification products after RT-PCR. Extracts of mesocarp tissues from 'Van' 2 (5-5) at commercial harvest following natural pollination were collected on July 3, 2006 from the Grandview plot. M: 100 bp Marker; lane 1: positive control (seed from 'Bing' tree 6-4) containing a product of the expected size 335 bp; lanes 2, 3: water control; lane 4: negative control (seed from 'Van' tree Q4); lanes 5-9: pedicel from 'Van' 2 (5-5); lanes10-19: seeds from 'Van' 2 (5-5).

Table 6. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in extracts from sweet cherry fruit tissues at 3 weeks post harvest stage after natural pollination. Samples were collected on July 24, 2006 from the Grandview plot.

Tree label[1]	RT-PCR assay results *number of positive / number of tested samples*		
	Mesocarp[2]	Seed[2]	Pedicel
'Van' 1(3-3)	2/10[3]	6/10	3/20
'Van' 2 (5-5)	2/10	2/10	0/20
'Van' 3 (7-5)	0/10	1/10	1/20
'Bing' tree (6-4)	5/5	5/5	5/5
'Van' tree (Q4)	0/5	0/5	0/5

1. All the samples were from the same sources as in Table 3.
2. One fruit was separated into mesocarp and seed
3. Seed from the same fruit was infected

Therefore, in 2006, at every developmental stages of sweet cherry, CLRV was detected by RT-PCR in all generative structures of uninfected 'Van' trees adjacent to infected 'Bing' trees. At shuck fall, the virus was detected in the ovary and pedicel. At pit hardening, commercial harvest and post harvest, these generative structures including the mesocarp contained detectable CLRV.

Potential movement of CLRV from fruit to the fruit-bearing tree

To test the hypothesis that CLRV is capable of entering the fruit-bearing tree from infected flowering and fruit structures, a pedicel and the fruiting spur to which it was attached were tested (Table 7). One pair of linked pedicel and spur were positive from 'Van' 1 (3-3) and another pair in 'Van' 3 (7-5). On the other hand, one spur was positive but a pedicel attached to it was negative in one case from 'Van' 1 (3-3), and the converse situation was also detected in this tree.

As part of this experiment, leaves were collected from each of the five branches from which pedicels and spurs had been collected. Interestingly, the data showed that some leaves were positive but some were negative in the same tree (Table8). To confirm the presence of CLRV in leaves, samples were collected just prior to leaf drop and extracts again tested by RT-PCR. The results were generally consistent with those obtained previously in that individual trees bore leaves with and without detectable virus (Table 8).At shuck fall, CLRV was found by immunolocalization inside the ovary and pedicel tissues from the uninfected 'Van' trees. Label was evident in the cells of the vascular tissues of the ovary (Figure 5A). The virus was localized in vascular and sub-epidermal cells of the pedicel (Figure 6A). The positive control had obvious label widely distributed in all cell types of the cross-sectional area of the pedicel (Figure 5B, Figure 6B) while the negative

Table 7. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in connective and vegetative tissues of sweet cherry at approximately 3 weeks post harvest. The trees sampled were CLRV-free 'Van' trees located adjacent to CLRV-infected pollinating cultivars. Tissue collection was performed on July 24, 2006 at the Grandview site.

Branch	RT-PCR assay results *number of positive / number of tested samples*					
	'Van' 1 (3-3)		'Van' 2 (5-5)		'Van' 3 (7-5)	
	Pedicel[1]	Spur	Pedicel	Spur	Pedicel	Spur
1	0/4	0/4	0/4	0/4	0/5	0/5
2	0/3	0/3	0/4	0/4	0/3	0/3
3	2/3[2]	1/1[2]	0/4	0/4	0/4	0/4
4	0/5	1/5	0/4	0/4	0/4	0/4
5	1/5	0/5	0/4	0/4	1/4[2]	1/4[2]
Total	3/20	2/18	0/20	0/20	1/20	1/20

1. The results reported here are for the same pedicels represented in Table 6.

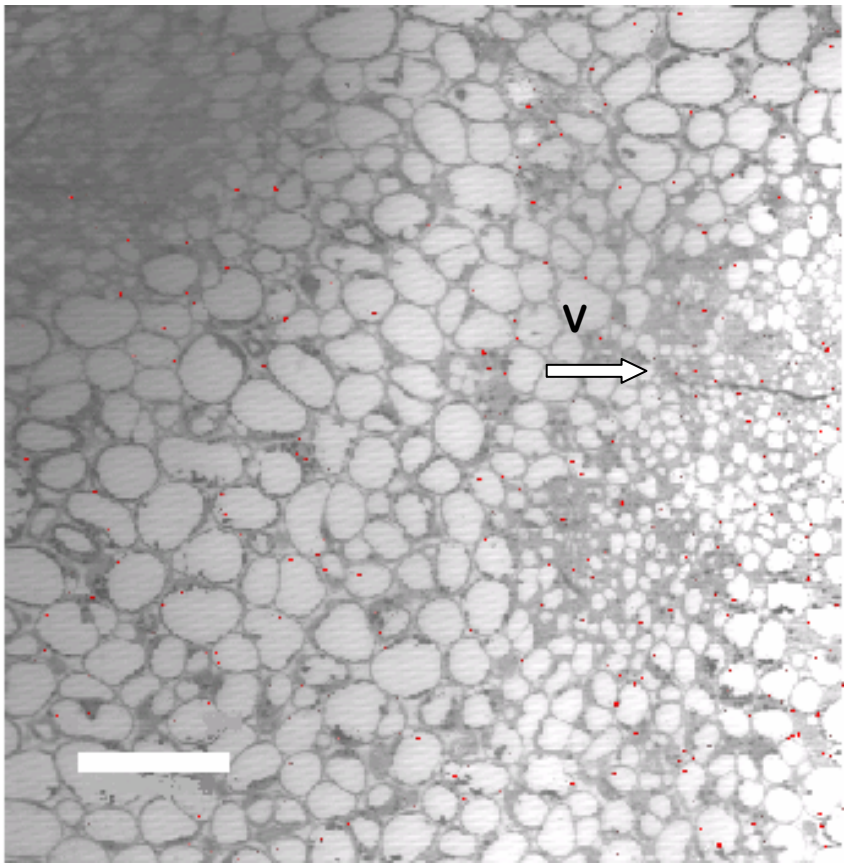
2. The infected spur was connected to an infected pedicel.

Table 8. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in leaves of sweet cherry after commercial harvest and prior to leaf drop. The trees sampled were CLRV-free 'Van' trees located adjacent to CLRV-infected pollinating cultivars. Tissue collection was performed 3 weeks after harvest on July 24 and on October 19, 2006 just prior to leaf drop at the Grandview plots.

Branch	'Van' 1 (3-3)		'Van' 2 (5-5)		'Van' 3 (7-5)	
	3 weeks post harvest	Before leaf drop	3 weeks post harvest	Before leaf drop	3 weeks post harvest	Before leaf drop
1	+	+	+	+	-	+
2	+	+	-	-	-	+
3	+	+	-	+	+	-
4	+	+	-	-	+	-
5	-	+	-	-	+	+

Figure 5. *Cherry leafroll virus* (CLRV), as indicated by immunolocalization, is distributed through the ovary at shuck fall after natural pollination. A. Cross-section of ovary from 'Van' tree V1 (3-3) which is CLRV–free and adjacent to infected 'Bing' trees. Labeled particles are localized near the vascular bundles; bar=50 μm. B. Cross-section of ovary from CLRV-infected 'Bing' tree 6-4. Labeled particles are distributed evenly through the section; bar=50 μm. C. Cross-section of ovary from CLRV-free 'Van' tree Q4; there is no obvious specific labeling; bar=50 μm. D. Enlarged image of A, label is evident in the cytoplasm of the vascular cells; bar=12.5 μm. The arrow labeled 'V' indicates the position of the vascular bundle and arrow 'C' indicates cytoplasm containing labeled virus particles.

A.



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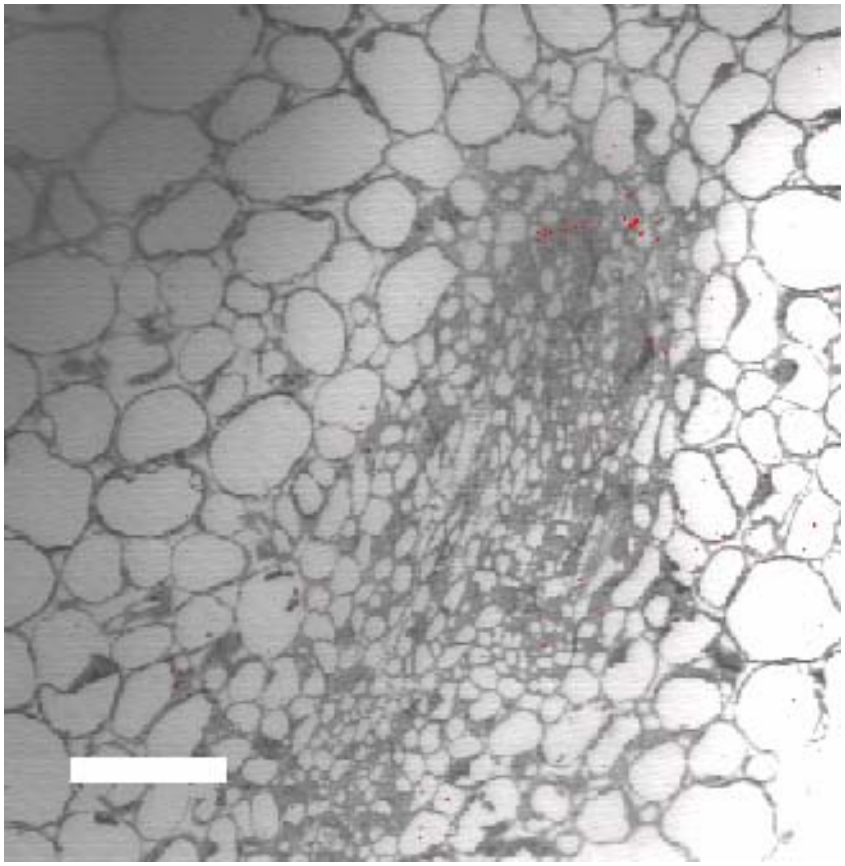

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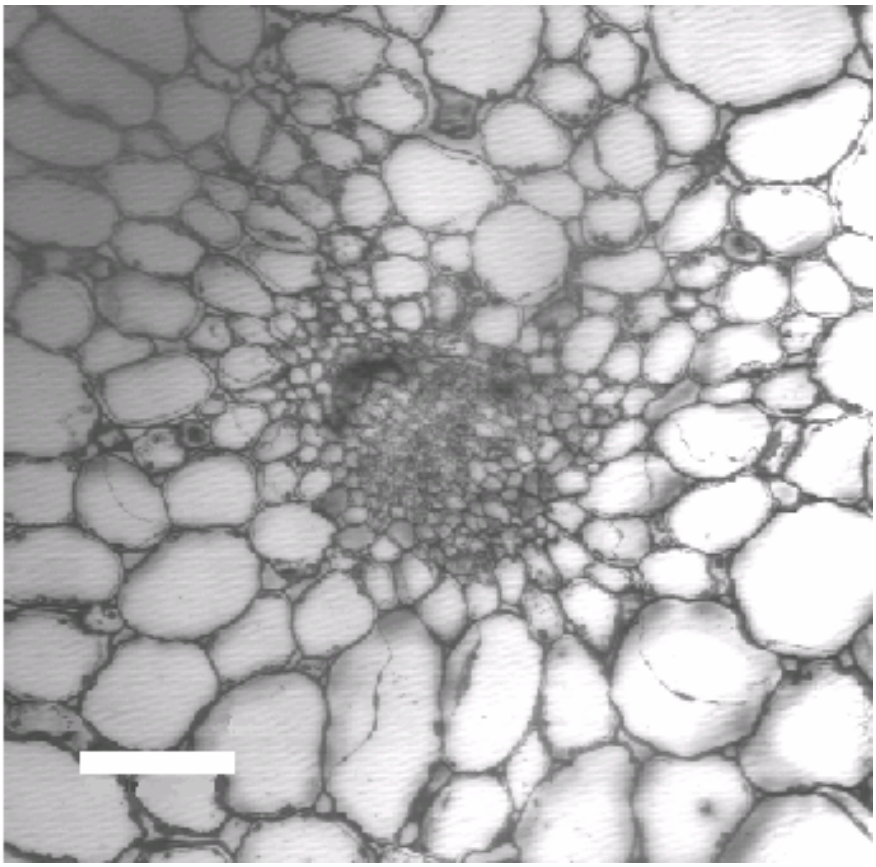


D.

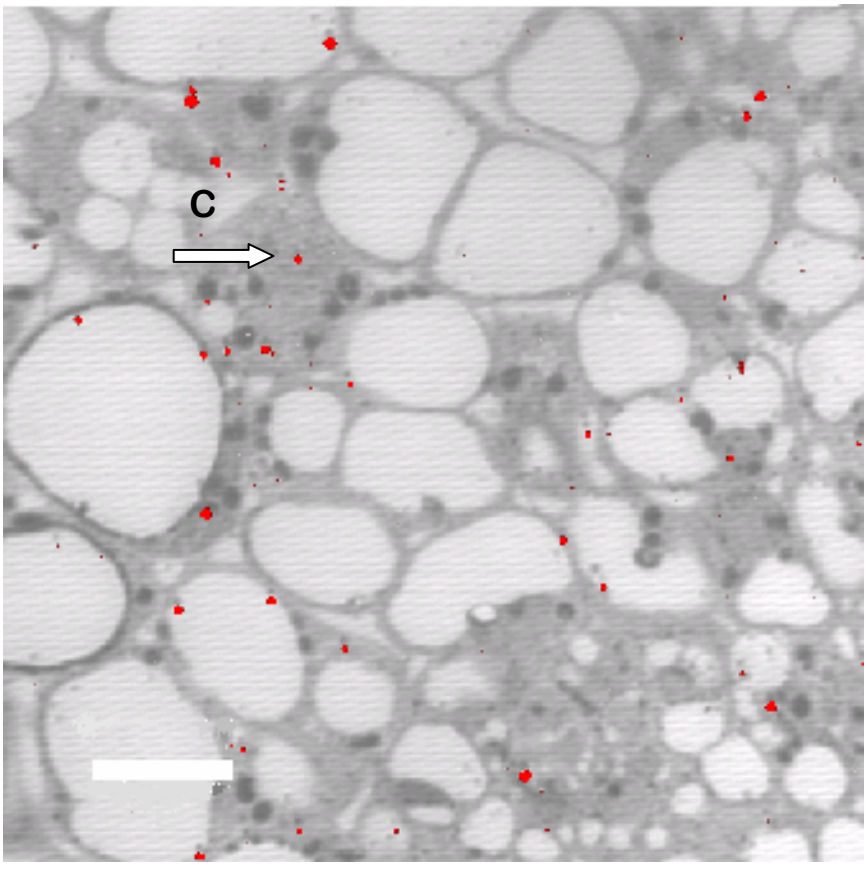
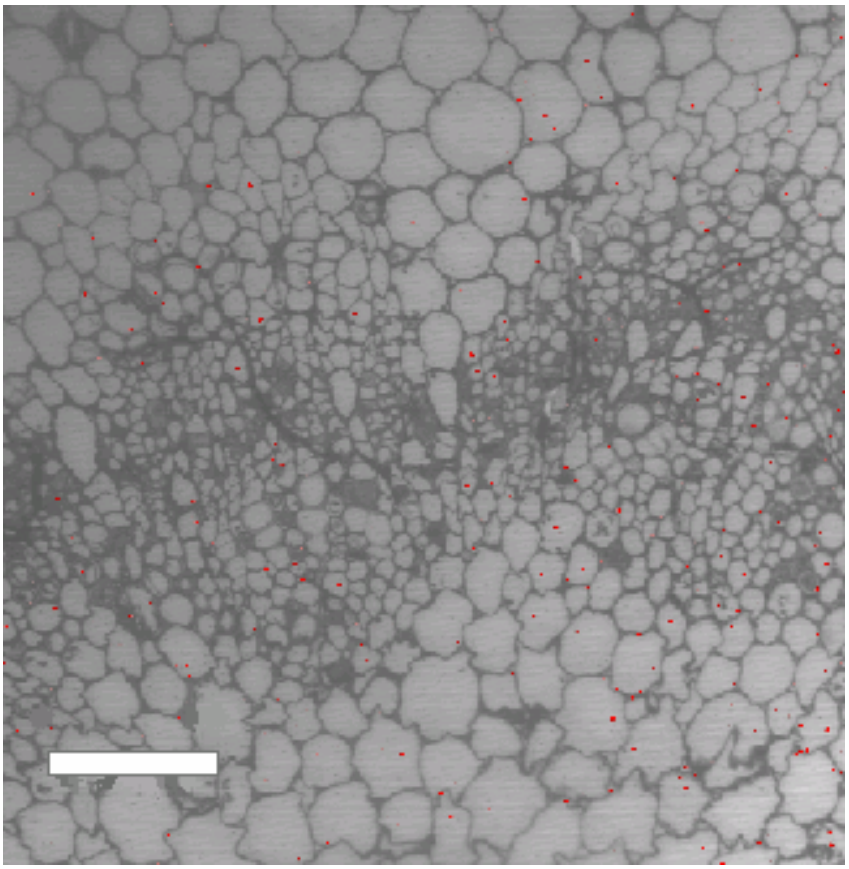
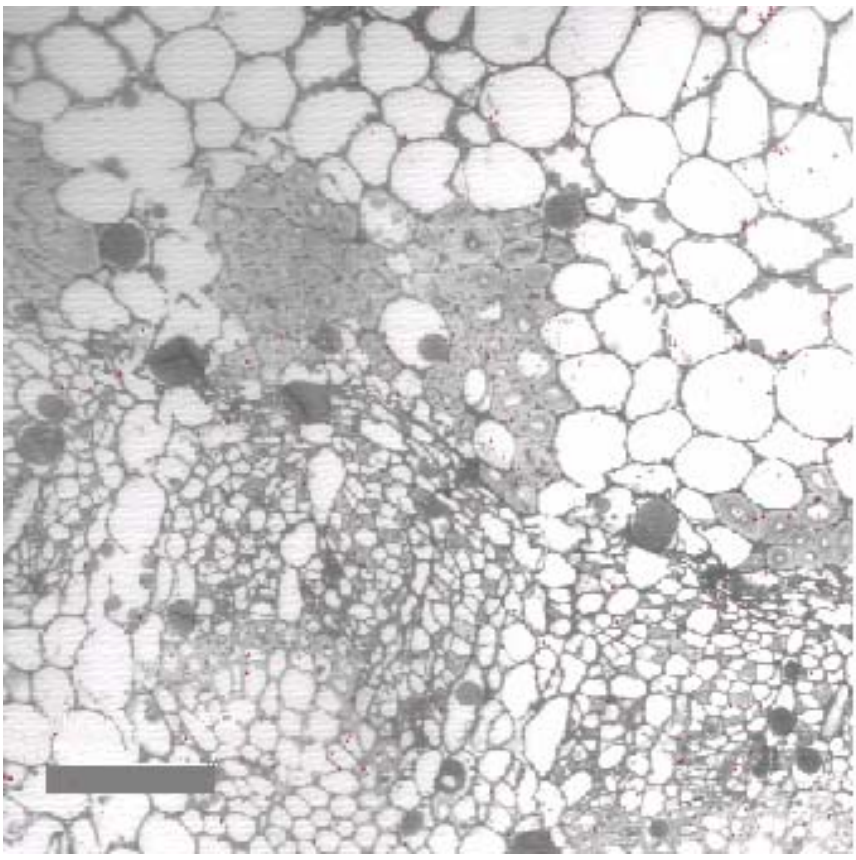


Figure 6. *Cherry leafroll virus* (CLRV) is distributed throughout the pedicel at shuck fall after natural pollination as indicated by immunolocalization. A. Cross-section of pedicel from CLRV–free 'Van' tree V1 (3-3) which was adjacent to a CLRV-infected 'Bing' tree. Labeled particles are located mostly in the vascular bundles with some additional label in sub-epidermal layers; bar=50 µm. B. Cross-section of pedicel from CLRV-infected 'Bing' tree 6-4. Labeled particles are distributed throughout; bar=50 µm. C. Cross-section of pedicel from CLRV-free 'Van' tree Q4. There is no obvious label retention; bar=50 µm. The arrow labeled 'V' indicates vascular bundle and the arrow 'E' indicates sub-epidermal layer.

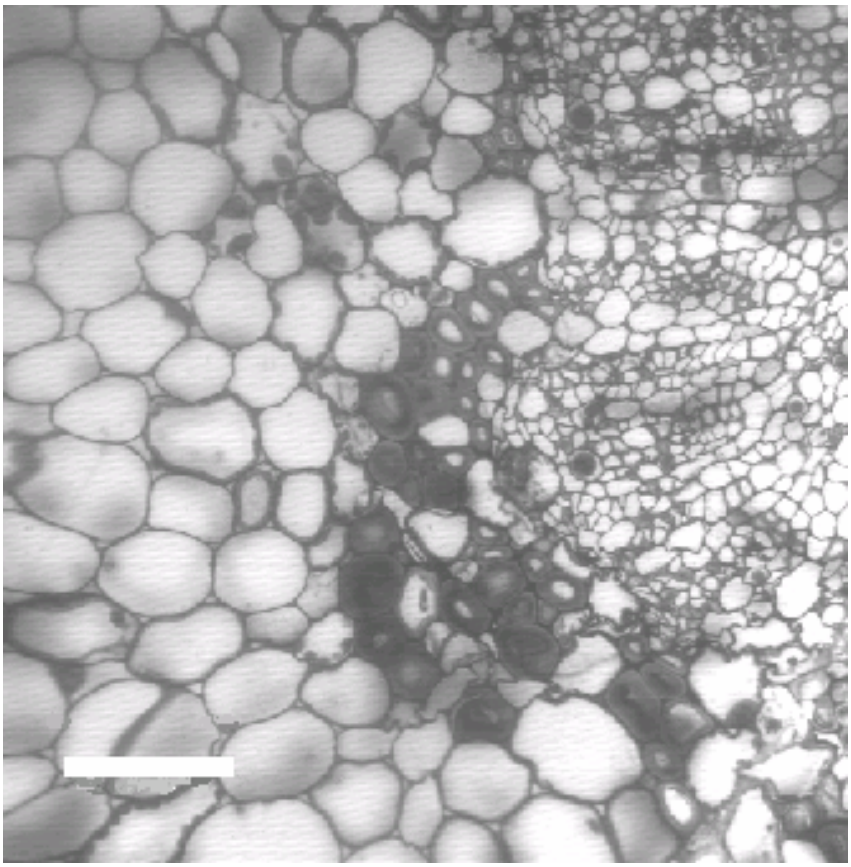
A.



B.



C.



control exhibited no labeling (Figure 5C, Figure 6C). At pit hardening, CLRV was also found in vascular and sub- epidermal cells of the pedicel of uninfected 'Van' trees. The virus was also localized inside endosperm (*data not shown*). At commercial harvest, label appeared only in the sub-epidermal cells of the pedicel (Figure 7A). Compared with samples from infected tree, label was found mostly in cells of the vascular bundles with a relatively small amount of label associated with sub-epidermal cells (Figure 7B). No obvious labeling was observed in the negative control samples (Figure 7C). CLRV was also found inside endosperm tissues (Figure 8A). The positive control had significant label throughout (Figure 8B) and the negative had no obvious label (Figure 8C).

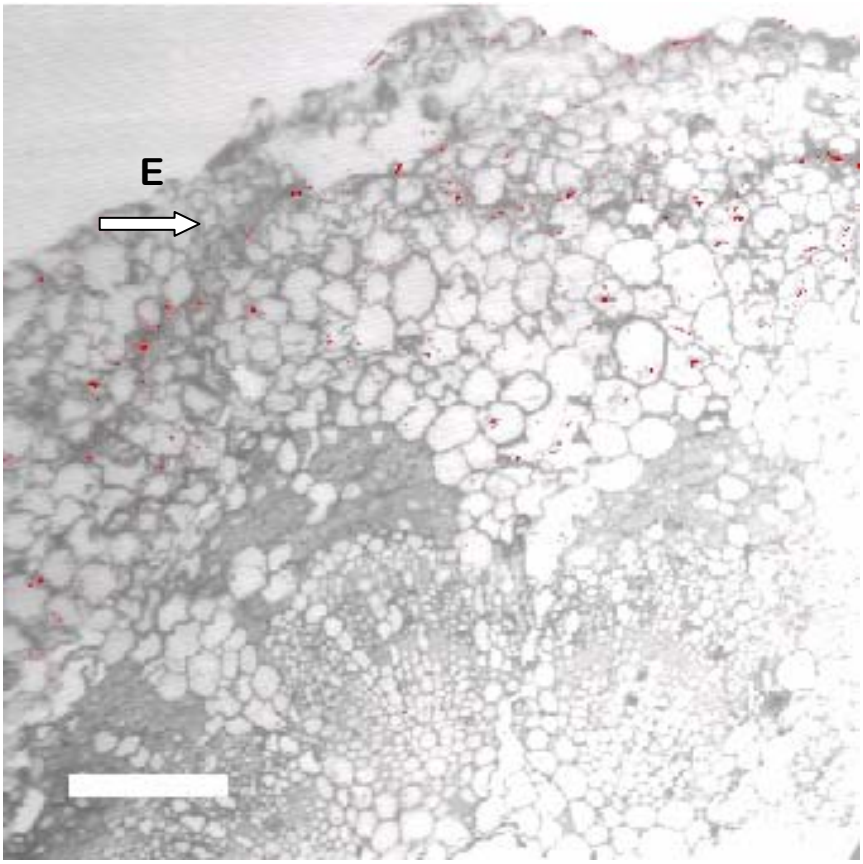
Experiments at the Moxee orchard

Ten days after hand pollination with virus-infected pollen, flower and fruit samples were collected from Moxee orchard and tested by RT-PCR for the presence of CLRV (Table 9). CLRV was found both in extracts of ovary and pedicel tissues. There were no significant differences between the results from each treatment. The distribution of CLRV in the ovary and pedicel tissues was determined by immunolocalization. One ovary sample from a healthy 'Bing' flower pollinated with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen (5:1) and in the presence of added thrips (treatment C), showed that label was localized in or near the vascular bundles and in ovule tissue, but no label was detected associated with the epidermal layer (Figure 9).

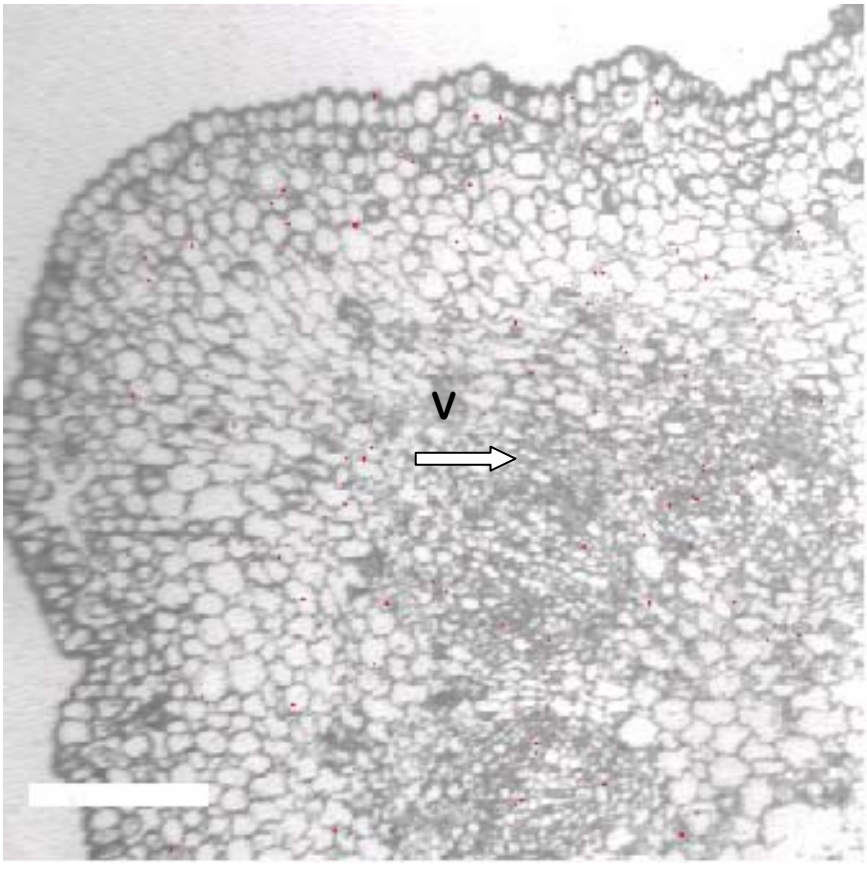
One month after hand pollination, only two fruits of 800 flowers were set; this indicated very inefficient fertilization. Temperature and wind data were collected from the Moxee station to indicate if weather conditions had contributed to the poor fruit set. According the weather records (Table 10), the temperatures of last three days were

Figure 7. *Cherry leafroll virus* (CLRV) is distributed through the pedicel at commercial harvest after natural pollination as indicated by immunolocalization. A. Cross-section of pedicel from 'Van' tree V1 (5-5), a CLRV–free tree adjacent to infected 'Bing' trees. Labeled particles are located only associated with the sub-epidermal layer; bar=100 µm. B. Cross-section of pedicel from 'Bing' tree 6-4 which is CLRV-infected. Labeled particles are distributed throughout the vascular bundles and sub-epidermal layer; bar=100 µm. C. Cross-section of pedicel from 'Van' tree Q4 which is CLRV-free. No specific labeling of any tissue is evident; bar=100 µm. The arrow 'E' indicates sub-epidermal layer and the arrow 'V' indicates vascular bundle.

A.



B.



C.

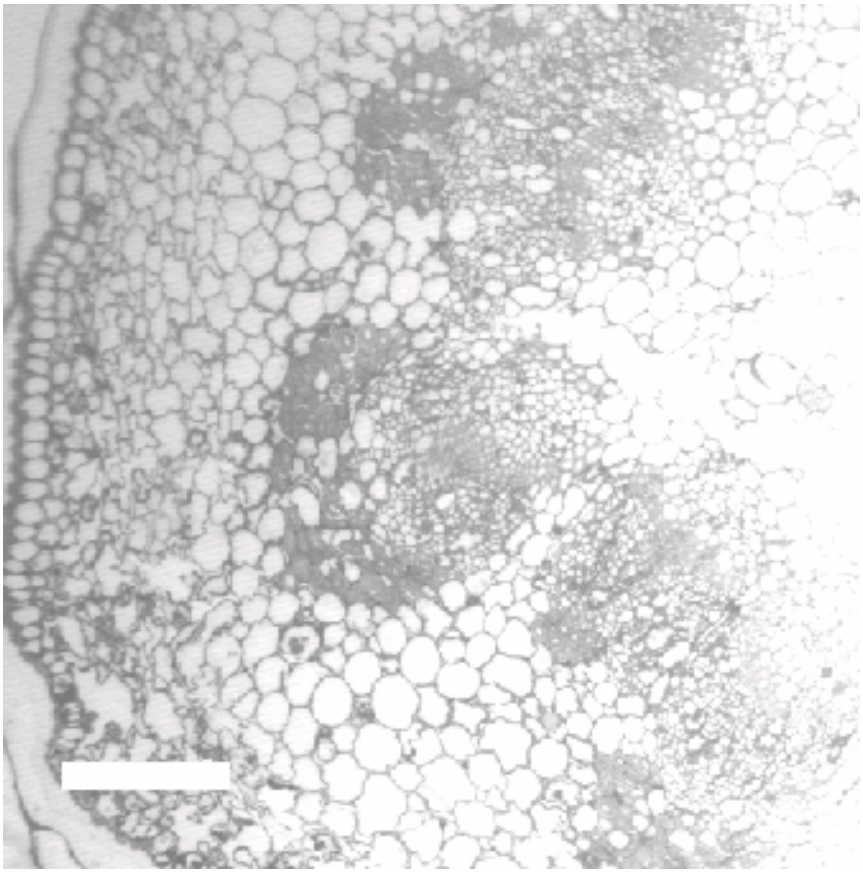
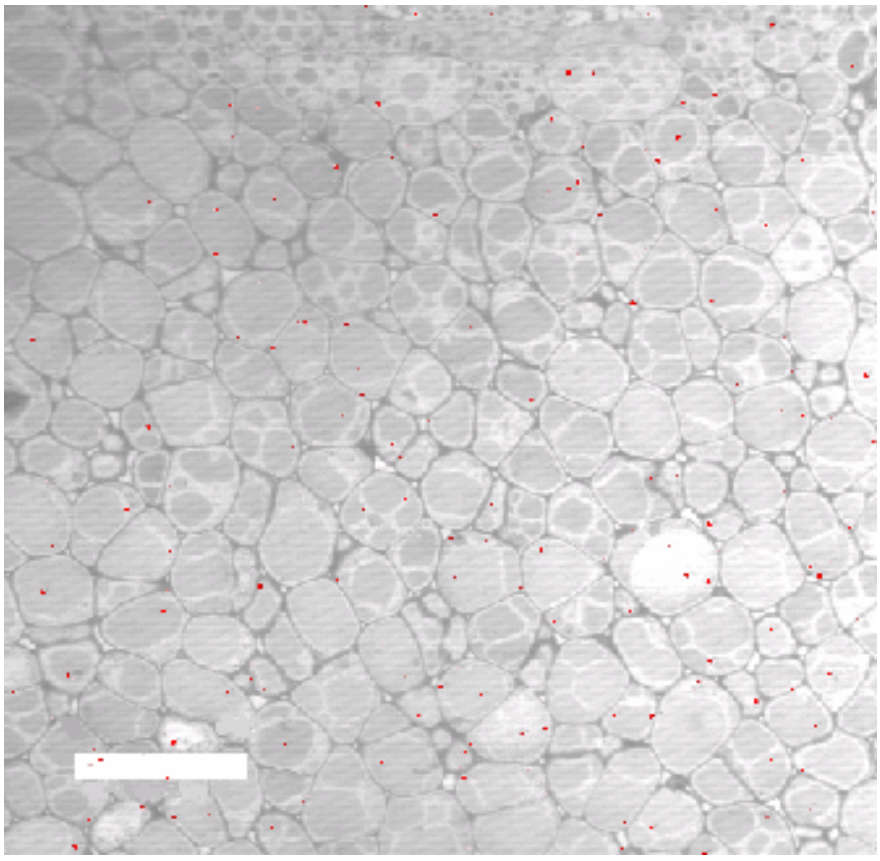
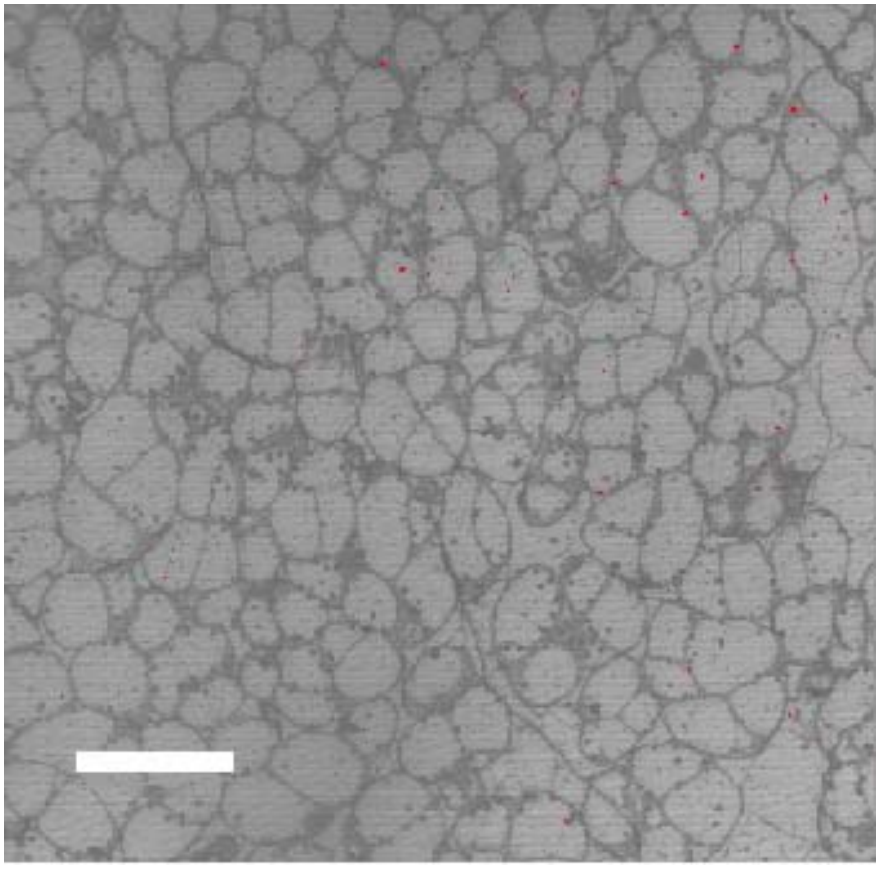


Figure 8. *Cherry leafroll virus* (CLRV) is distributed through the endosperm at commercial harvest after natural pollination as indicated by immunolocalization. A. Cross-section of endosperm from CLRV–free 'Van' tree V1 (5-5) adjacent to infected 'Bing' trees. Labeled particles are located in cells responsible for starch storage; bar=50 µm. B. Cross-section of endosperm from CLRV-infected 'Bing' tree 6-4 revealing the presence of labeled particles distributed in cells that store starch; bar=50 µm. C. Cross-section of endosperm from CLRV free 'Van' tree Q4 showing no obvious specific labeling; bar=50 µm. D. Enlarged portion of image A; bar=25 µm. The arrow labeled 'S' indicates starch body.

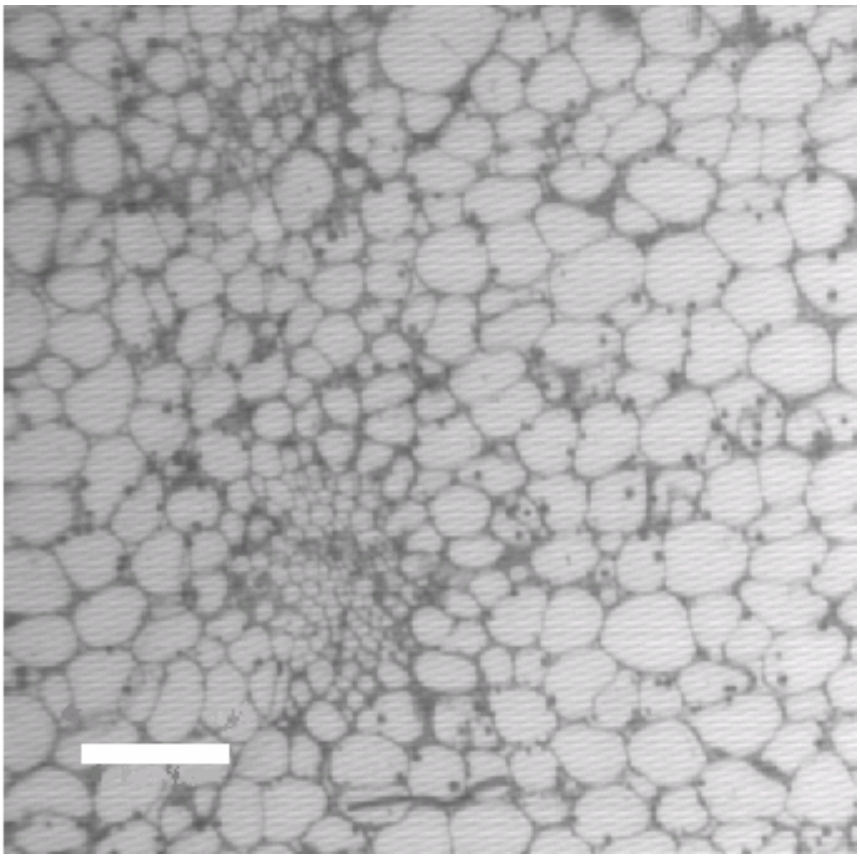
A.



B.



C.



D.

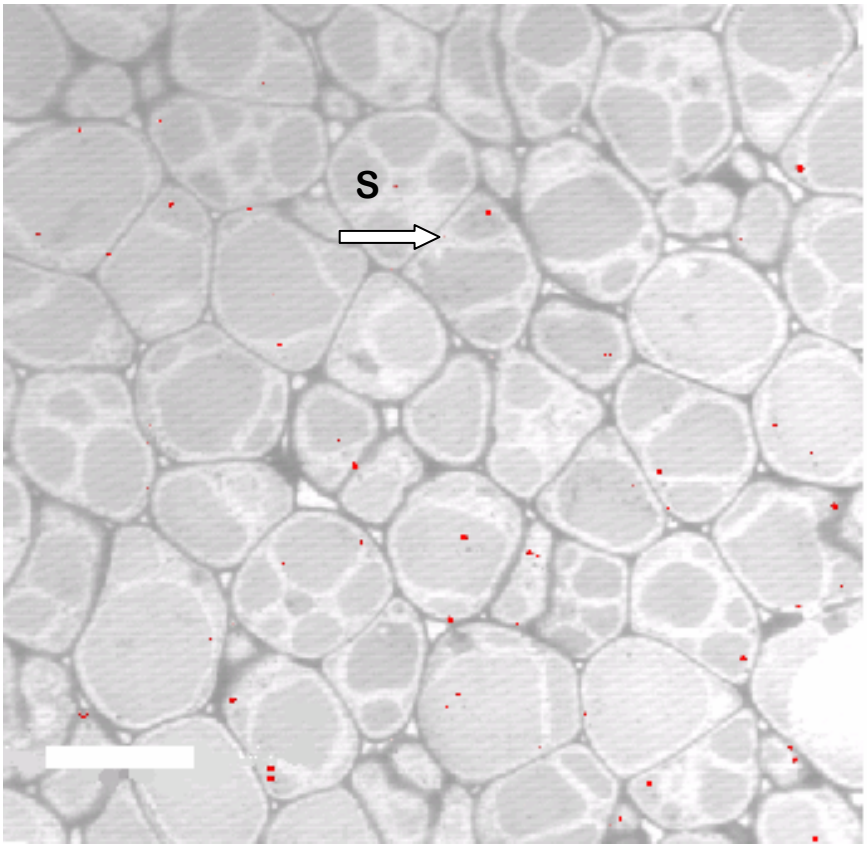
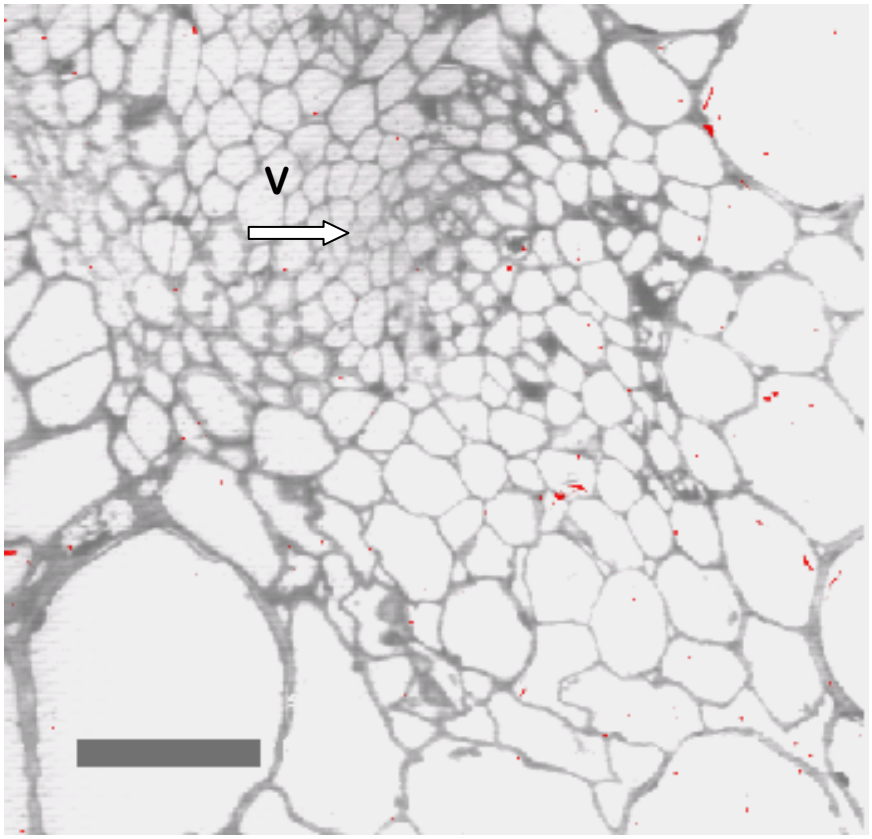


Table 9. Detection of *Cherry leafroll virus* (CLRV) by RT-PCR in sweet cherry fruit tissues at shuck fall stage after hand pollination. Samples were collected on May 9, 2006 from the Moxee orchard.

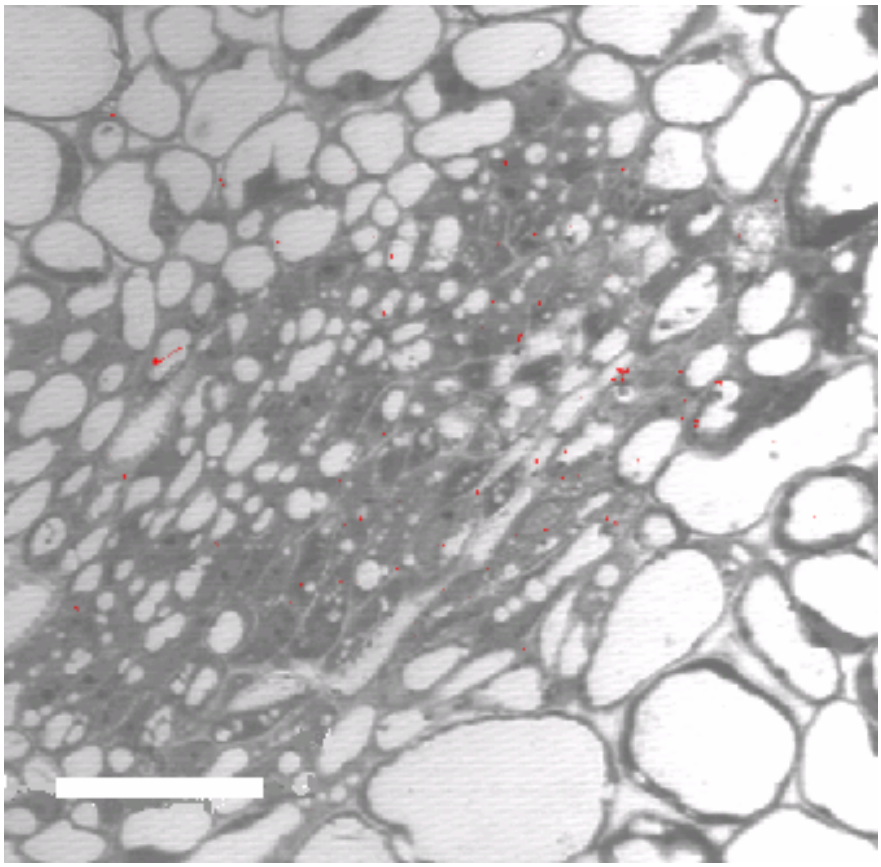
Sample	RT-PCR assay results *number positive / number tested*	
	Ovary	Pedicel
A. Uninfected 'Van' flower pollinated with CLRV-infected 'Bing' pollen plus added thrips	3/10	4/10
B. Uninfected 'Van' flower pollinated with CLRV-infected 'Bing' pollen with no added thrips	7/10	7/10
C. Uninfected 'Bing' flower pollinated with mix of CLRV-infected 'Bing' and healthy 'Van' pollen plus added thrips	6/10	5/10
D. Uninfected 'Bing' flower pollinated with mix of CLRV-infected 'Bing' and healthy 'Van' pollen with no added thrips	6/10	4/10
CLRV-infected 'Bing' tree (6-4)	5/5	5/5
CLRV-free 'Van' tree (Q4)	0/5	0/5

Figure 9. *Cherry leafroll virus* (CLRV) is distributed through the ovary at shuck fall after hand pollination as indicated by immunolocalization. A. Cross-section of ovary from treatment C, which was pollination of healthy 'Bing' flowers with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen (5:1) in the presence of added thrips; labeled particles are localized near vascular bundles; bar=25 μm. B. Cross-section of ovary from CLRV-infected 'Bing' tree 6-4 revealing labeled particles distributed in vascular cells; bar=25 μm. C. Cross-section of ovary from CLRV free 'Van' tree Q4 demonstrating no specific labeling; bar=25 μm. The arrow labeled 'V' indicates vascular bundles.

A.



B.



C.

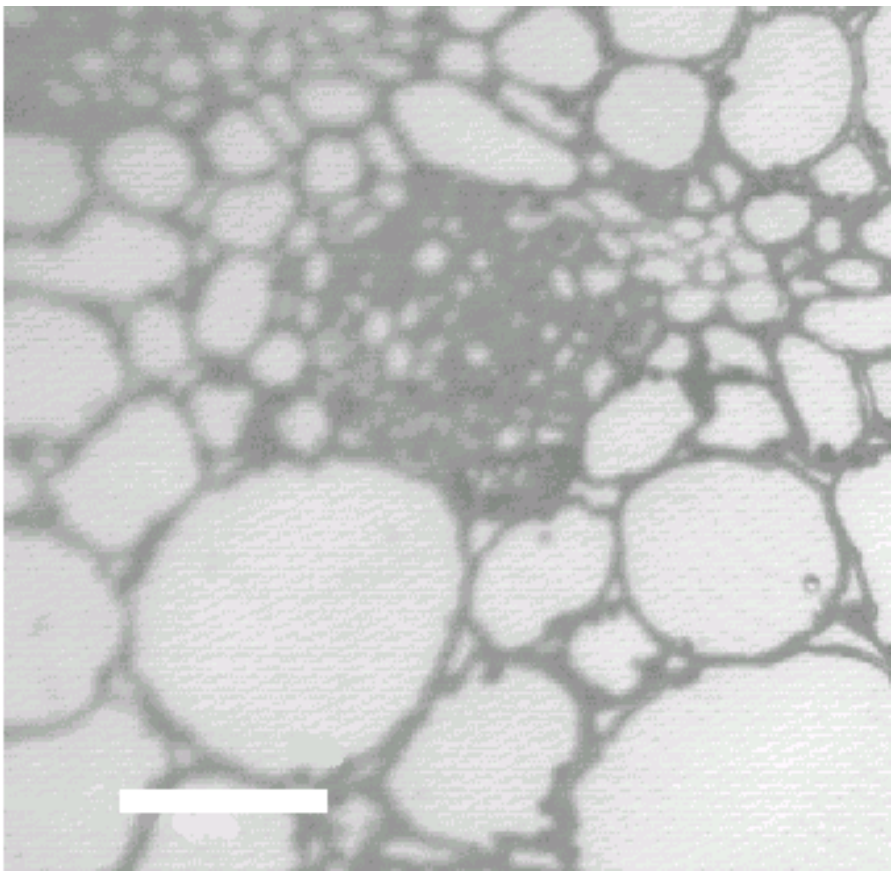


Table 10. Weather record from Moxee experimental station at the period of pollination

Date	Maximum temperature (°C)	Minimum temperature (°C)	Remark
4/26/06[1]	21	2	
4/27/06[1]	23	2	
4/28/06	25	5	
4/29/06	26	9	Windy evening
4/30/06	20	1	
5/01/06	15	0	Windy day
5/02/06	16	-1	
5/03/06	18	-0.5	Windy all day

1. The dates of hand pollination

significantly lower than the early time of pollination. Windy conditions prevailed on three days during this time.

Pollen grain germination *in vitro*

In order to assess the quality of sweet cherry pollen used in this experiment, germination rate of pollen was tested *in vitro* (Table 11). The pollen collected in 2006 has low germination rate. The mixed pollen was used to hand pollination experiment in Moxee and its germination rate was only 3%. However, a sample of CLRV-infected 'Bing' pollen collected in 2004 showed a much higher germination rate of 49.29%.

Table 11. Germination rate of sweet cherry pollen *in vitro.*

	Germination rate *number of germinated grains / number observed*		
	Healthy pollen[1]	CLRV-infected pollen[2]	Mixed pollen[3]
	6/24	10/21	0/22
Plate 1	2/40	15/26	0/15
	3/27	5/16	1/30
	10/45	15/32	1/28
Plate 2	3/28	19/27	0/25
	3/35	22/38	2/33
	1/14	31/58	2/27
Plate 3	3/48	12/30	1/17
	2/26	11/36	0/34
Average	11.50%	49.29%	3.0%

1. The pollen was collected from *Cherry leafroll virus* (CLRV) -free 'Bing' trees from Pear Acres orchard in spring, 2006
2. The pollen was collected from CLRV-infected 'Bing' tree from Grandview orchard in spring, 2004
3. The pollen was CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen (5:1), 'Bing' pollen was from Grandview orchard, 'Van' pollen from Pear acres orchard, and collected in spring, 2006

CHAPTER FOUR

DISCUSSION

The pathway of pollen-associated horizontal transmission of *Cherry leafroll virus* (CLRV) in sweet cherry was studied with naturally pollinated trees in a commercial production orchard. CLRV was detected by RT-PCR in extracts of sweet cherry fruiting tissues at all developmental stages of healthy 'Van' trees surrounded by CLRV-infected 'Bing' trees (Table 12).

Shuck fall occurs about 10 days after full bloom and is the stage of growth when expansion of the fruit breaks the shuck that is formed by the fused sepals. CLRV was found in extracts of ovary and pedicel parts by RT-PCR. Furthermore, ovary appears to be highly vascularized and CLRV was localized mostly in the cytoplasm of vascular cells in ovary and pedicel at this time by immunolocalization (Figure 5; Figure 6). These results indicated the movement of the virus along vascular bundle; the virus might transport from pollen to the ovary and enter the pedicel during pollination and fertilization.

Pollination of cherry trees is achieved through insect activity, and CLRV, a pollen-borne virus, adheres strongly to the surface of cherry pollen (Massalski & Copper, 1984). Virus contaminated pollen is carried throughout the orchard by honeybees. Leaves from healthy trees can be contaminated with CLRV and appear positive by ELISA at this stage (K. C. Eastwell, *personal communication*). Also, surface contamination experiments in this study demonstrated that pedicels from a healthy tree exposed to infected pollen could be positive by RT-PCR after washing three times with

Table 12. Summary of *Cherry leafroll virus* (CLRV) detection rates in extracts from fruit tissues at various developmental stages. Samples were collected in 2005 and 2006 from CLRV-free 'Van' trees exposed to CLRV infected pollen in the Grandview plot

Stage	Season	CLRV infection rate (%)[1]			
		Ovary	Mesocarp	Seed	Pedicel
Shuck fall	2005	--[2]	--	--	--
	2006	46	--	--	73
Pit hardening	2005	--	87	13[3]	26
	2006	--	70	3	3
Harvest	2005	--	92	7[3]	0
	2006	--	7	93	10
Post harvest	2005	--	--	--	--
	2006	--	13	30	7

1. Samples were collected from 'Van' 1 (3-3), 'Van' 2 (5-5) and 'Van' 3 (7-5)
2. -- = Not tested
3. Samples were tested by ELISA; all other results were determined by RT-PCR

PBS-Tween. With such high level of pollen- borne CLRV, it is difficult to avoid surface contamination early in the growing season. Therefore, some samples that were positive by RT-PCR might be positive because of surface contamination. On the other hand, CLRV was localized inside the cytoplasm of vascular cells of the ovary and pedicel at shuck fall by immunolocalization (Figure 5; Figure 6). This result confirmed the internal location of the virus in the ovary and pedicel at shuck fall stage.

Pit hardening refers to a period of seed development in which endocarp and seed coat becomes hard and the embryo is growing. During the current study, this occurred approximately 7 weeks after bloom so surface contamination of sample is unlikely at this time. CLRV was detected in mesocarp, seed and pedicel tissues by RT-PCR at this stage of development. Cells of the mesocarp originate from ovary tissues that surround the ovule. The virus was probably transmitted to these tissues from infected pollen during pollen tube growth or by insect behavior or other factors that cause mechanical injury to the tissues. Seed is a fertilized ovule so the virus detected in it could have been introduced during fertilization or pollen germination. In at least one fruit sample, both the mesocarp and pedicel were positive as revealed by RT-PCR. Two possibilities are suggested by this result: 1) the virus in the pedicel might be transported from the mesocarp since vascular connections between the mesocarp and pedicels exist before pit hardening (Stosser *et al*., 1969) and the virus was localized by immunolocalization in the vascular tissues of the pedicel at pit hardening; or 2) the pedicel became infected directly through mechanical injury and transmission.

At pit hardening, CLRV was detected in the seeds by RT-PCR and was detected inside the endosperm by immunolocalization. Endosperm is an important part of the seed

formed by fertilization and provides nutrition to the developing embryo. The pit of cherries also includes the seed coat and embryo which, if infected, could result in seed and seedling infection. Therefore, distribution of the virus in seed coat and embryo needs to be examined in a further study. CLRV-infected seed can result in an infected seedling which means the virus is vertically transmitted through seed (K. C. Eastwell, *personal communication*). This is indirect evidence of embryo infection because the virus infected seedlings frequently develop from infected embryos (Mink, 1993).

The fruit becomes ripe and seed is nearing full maturity at commercial harvest stage. CLRV was found in the mesocarp, seeds and pedicels of the healthy 'Van' trees by RT-PCR when samples were collected at this time. As indicated by RT-PCR results from 2006 (Table 12), there is a dramatic change in the distribution of CLRV in mesocarp and seed tissues between pit hardening and harvest. The reason for this variation is unclear and may be due to physiological changes of cherry fruit. By immunolocalization, CLRV was detected inside the endosperm at harvest (Figure 8). Examination of the pedicels at this stage of fruit development indicated that labeled particles were found mostly associated with sub-epidermal layers (Figure 7). This is in contrast to the label that was distributed mostly near vascular bundles with lesser amounts at sub-epidermal layer of pedicel at shuck fall and pit hardening (Figure 6). Before pit hardening, fruiting tissues and the mother tree exchange nutrition by the vascular connection provided by the pedicel. The virus may be transported through the phloem during the first two stages of development. After pit hardening, nutrition movement and vascular transport becomes unidirectional from the main tree toward the fruit. During this time, however, virus can still be translocated from the fruit to the

pedicel through plasmodesmata that connect the various tissues (Mandahar, 1984). Therefore, the detection of CLRV within interior cells is consistent with the introduction of CLRV through the flower rather than mechanical infection of the epidermal layers of the pedicel.

Additional evidence that CLRV moves from flower to the mother tree through pedicel was shown in this study. Three weeks after commercial harvest, CLRV was detected by RT-PCR in extracts of pedicels and the spurs to which they are connected in 'Van' trees V1 (3-3) and V3 (7-5) (Table 7), however; extracts from leaves of the same branch as the pedicel and spur also contained detectable CLRV (Table 8). Thus, the RT-PCR data did not indicated whether CLRV was moving toward the fruiting tissues from infection sites on the branch, or moving from infected fruiting tissues to the bearing tree. When fruit bearing trees are infected with CLRV, immunolocalization revealed CLRV in the mesophyll and vascular bundles of the pedicel, whereas when CLRV-free trees are exposed to CLRV-infected pollen, the virus is absent from the cells of the vascular tissue. That is, in the "healthy" trees exposed to CLRV-infected pollen, the virus was detected only in sub-epidermal cells indicating CLRV was not moving from the fruit bearing branch towards the fruiting structure. Despite the detection of CLRV in some leaves of these "healthy" trees, the distribution of CLRV in the pedicel is consistent with movement of CLRV from the flower towards the fruit bearing branch. The origin of the positive assay results for leaves of these trees is subject to speculation. The virus may progress from the flower through the pedicel and spur to the limb supporting the leaf. Alternatively, the virus detected in the leaves might result from naturally occurring mechanical inoculation and the virus moved from leaves to spurs and pedicels. CLRV

has been transmitted from infected cherry pollen to *Chenopodium quinoa* by western flower thrips in greenhouse studies. However, transmission from cherry pollen to cherry seedlings has not been successful (W. E. Howell, *personal communication*).

According to the research of Fuchs and Gruntzig (1994), virus distribution in woody host plants has three categories: sporadic, partly systemic and systemic distribution. CLRV is considered a virus with sporadic distribution virus and they confirmed that some limbs from CLRV-infected wild cherry (*Prunus avium*) were still virus-free (Fuchs & Gruntzig, 1994). This erratic distribution model of CLRV was also observed in sweet cherry (K. C. Eastwell, *personal communication*). Data from the current study (Table 8) showed that some leaves were infected but some were not in one tree. Furthermore, leaves were positive whereas pedicels and spurs from the same branch were negative (Table 7 & Table 8). This result indicated that the movement of CLRV is slow, the initial infection of CLRV may be limited to a smaller portion of a branch so that leaves from one branch are infected and pedicels and/or spurs from different shoots from this branch are not infected. Rowhani and Mircetich (1992) showed that the speed of CLRV walnut strain movement in a 'Bing' branch was about 15 cm per year. To test the movement rate of CLRV of sweet cherry, an experiment was initiated at Moxee orchard in August, 2006. Buds from a CLRV-infected tree was grafted to branches of healthy trees, four trees were used and two branches were inoculated in each tree. The new bud and leaf tissues growing from the inoculated branch and from the whole limb will be tested for CLRV in spring, 2007. Furthermore, the branches exposed to CLRV-infected pollen in this study will also be tested. Thus, the virus distribution in sweet cherry and the speed of the virus movement will be further understood.

Results from the experiments at the Grandview research site provides evidence that CLRV of sweet cherry may be transported from pollen through the pedicel and then to the fruit bearing tree. Support for this comes from three separate lines of evidence. CLRV was detected by RT-PCR in generative structures of fruits at various developmental stages after natural pollination. The tissues that contained detectable levels of CLRV included ovary, mesocarp, seed and pedicel. Pedicel is the structure that links the flower structure to the mother tree. The distribution of CLRV within the pedicel at different stage suggests movement of the virus along the pedicel. Lastly, spur and leaf from a 'healthy' tree contained detectable levels of CLRV three weeks after commercial harvest. This indicates that the mother tree had become inoculated and may be infected.

It was demonstrated that CLRV of sweet cherry moves from pollen to mother tree through the pedicel. To understand the mechanisms of pollen transmission in this horizontal transmission, hand pollination experiments were conducted at the Moxee site. Three hypotheses are suggested: 1) the ovary is infected by fertilization leading to infection of all of the reproductive tissues and subsequently the fruit bearing tree; 2) pollen tube growth provides mechanical transmission of CLRV to auxiliary tissues; or 3) thrips create wounds required for mechanical infection. Four treatments were designed to differentiate between these possibilities: A. pollination of healthy 'Van' flowers with CLRV-infected 'Bing' pollen in the presence of added thrips; B. pollination of healthy 'Van' flowers with CLRV-infected 'Bing' pollen without added thrips; C. pollination of healthy 'Bing' flowers with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen in the presence of added thrips; D. pollination of healthy 'Bing' flowers with CLRV-infected 'Bing' pollen mixed with healthy 'Van' pollen without added thrips. For

treatments A and B, CLRV-infected pollen tube growth could contaminate healthy ovule tissue during fertilization. In treatments C and D, the infected pollen tube would grow but not reach the healthy ovule since 'Bing' pollen is incompatible with 'Bing' flowers. However, fertilization would still occur because of the presence of 'Van' pollen. It was the intent of this study to allow the fruit to continue to develop. Of course, the healthy 'Van' pollen may be surface contaminated with CLRV by mixing with the pollen from infected trees which is a limitation of this experimental design. In addition, duplicate treatments in the presence or absence of added thrips would provide some insight as to whether thrips are involved in the virus transmission.

At shuck fall (ten days after hand pollination), CLRV was detected in extracts of ovary and pedicel tissue by RT-PCR (Table 9). The results from each treatment yielded no statistically difference. CLRV-infected pollen was involved in fertilization in treatment A and B but not in C and D. Increased thrips populations were present in treatment A and C but not B and D. Therefore, the results suggest that neither fertilization nor thrips activity were necessary for the virus to enter the reproductive tissues. However, surface contamination could also explain this result. During pollination, a hand-held aerosol pump was used to spread virus-infected pollen on the flowers. Some pollen may remain on the surface of the tested tissues. This same limitation applies to the natural pollination experiments conducted in the commercial orchard. Thus, RT-PCR data alone is insufficient to prove the hypothesis.

The distribution of CLRV in ovary and pedicel tissues from these treatments at shuck fall was examined by immunolocalization. One ovary sample from treatment C (Figure 9) showed that label was in cells of vascular tissues but not the epidermal layer.

In this treatment, the infected pollen was not involved in fertilization. Thus fertilization is not a prerequisite for virus transmission. Moreover, thrips were released into the sleeve cage of this treatment. The virus label was not limited to the epidermal cells where thrips feed indicating that CLRV transmission from pollen to the ovary did not require thrips activity. These conclusions are consistent with the RT-PCR result. Moreover, it demonstrates that the virus was internal, and not a surface contamination of the fruiting structures. Therefore, it appears that pollen tube germination is required for the mechanical injury required for horizontal transmission of CLRV. This result would be further confirmed by detection of fruits from these four treatments.

However, one month after pollination, most of the hand pollinated flowers abscised and not enough fruit remained to continue with detailed analysis. Sweet cherry belongs to the *Prunus* genus in which there is a strict requirement for fertilization in order for fruit development (Andrews, 2006). Three factors determine successful fertilization: stigma receptivity, ovule longevity and pollen germination rate (Andrews, 2006). The germination rate of the pollen used in this study was unknown before the hand pollination experiment. At the time that the field study was initiated, we had not found a reliable method to determine germination rate. Only after hand pollination experiment was the germination rate of the mixed pollen tested *in vitro* and its germination rate found to be only 3% (Table 11). All the pollen used in the hand pollination experiment was collected from flowers at the balloon stage of development of forced flowers. A recent study (Choi and Andersen, 2005) found that in three sweet cherry cultivars, germination percentage of pollen from balloon stage was much lower than pollen from fully open flowers. 'Bing' and 'Van' cultivars were not included in this study, but pollen quality could be the main

reason of the fertilization failure. Therefore, in future work, pollen should be collected at full bloom stage, and the pollen germination rate should be tested before hand pollination.

Climatic conditions have an important effect on fertilization success (Folta, 2006). For example, pollen survives longer at lower temperature with dry and low light conditions. However, low temperatures can reduce the rate of pollen tube growth. In this case, the ovule may no longer be receptive when the pollen tube reached it. High temperature will decrease stigma receptivity, ovule longevity and pollen viability. Wind is not good for fertilization since it can reduce stigma receptivity (Andrew, 2006).

The optimal temperature for cherry fertilization is 15-24°C. According to the weather records at the Moxee experimental station during pollination and fertilization (Table 10), the maximum temperature was 26°C and the lowest temperature was -0.5°C. The low temperature has a negative effect on pollen tube germination. However, the hand pollinated flowers were in cages where the temperature could be higher than ambient temperature. Windy conditions on some days would further reduce the rate of fertilization.

From the experiments in the Moxee plot, the mechanism of pollen horizontal transmission of CLRV could not be fully determined due to the failure of fertilization. However, the results from shuck fall indicate that the virus is transported from pollen to pedicel by pollen tube growth without fertilization and thrips. Unlike the commercial research block in Grandview, evidence suggests that this is the first exposure of these trees to CLRV, thus, the virus detected in the pedicels must originate from the CLRV-infected pollen rather than from potentially undetected pre-existing infections of CLRV. This result is consistent with the mode of CLRV horizontal transmission in nature. The

transmission of CLRV from plant to plant occurs primarily through pollen and seed which could explain the absence of inter-species transmission in nature. For example, in California, some sweet cherry orchards are next to walnut orchards containing CLRV-infected trees, but those sweet cherry trees have never been infected by the virus, whereas in the greenhouse, CLRV is easily transmitted to *Chenopodium* plants by mechanical inoculation, but infected plants of this genera have not been detected in orchards of infected cherry trees (K. C. Eastwell, *personal communication*). This host specific infection provides evidence that pollen germination or fertilization is required for natural CLRV transmission. A recent study (Rebenstorf *et al*., 2006) indicates that this mode of pollen and transmission in CLRV may decide the population structure of the virus, causing the serological and molecular diversity of CLRV strains based on host plant. Asynchrony of flowering times may help maintain separtion of virus isolates in different hosts.

The exact mechanism of virus transmission from plant to plant via pollen remains unknown. Mink (1993) suggested that occurrence of horizontal transmission requires interaction of pollen with other biological factors: 1) virus-infected pollen can be moved to healthy flowers by vectors such as honeybees; 2) wounds required for mechanical inoculation are caused by insects such as thrips or honeybees; and 3) virus is located in pollen in such a way as to allow mechanical transmission. Our experiment indicates that pollen may play an important role on CLRV horizontal spread in sweet cherry. Interactions with other biological agents such honeybees and thrips have to be studied.

In many instances, the pattern of the virus spread in an orchard is consistent with horizontal transmission through root grafting. CLRV of sweet cherry can spread from

tree to tree in an orchard by root grafting (K. C. Eastwell, *personal communication*). If one tree is infected, newly infected trees tend to be located around this original diseased tree and they form a small infected area. However, this pattern is also consistent with honeybee pollinating patterns. It has been reported that honeybees prefer working a certain area and moving from the hive to this area back and forward (Winston, 1987). Therefore, it is possible that honeybees transport infected pollen from an infected tree to the adjacent one within a confined area.

CLRV can also spread across an orchard and from one orchard to another; this long distance transmission cannot be attributed to root grafting. Honeybees and hives with virus-infected pollen can move between orchards. Therefore, it is possible that pollen can spread the virus horizontally in sweet cherry mediated by honeybees. Honeybees have been shown to be involved in horizontal transmission of other pollen-borne viruses including a member of the genus *Nepovirus* (*Blueberry leaf mottle virus*) (Boylan-Pett *et a*l., 1991) and viruses of the genus *Ilarvirus* [*Prunus necrotic ringspot virus* (PNRSV); *Prune dwarf virus* (PDV) and *Blueberry shock virus*] (Mink, 1983; Hamilton *et al*., 1984; Bristow & Martin, 1999).

Some species of thrips are flower feeding insects which are also considered potential vectors of pollen transmission of viruses. *Thrips tabaci* with *Tomato ringspot virus* (TSV)-infected pollen transmitted TSV to *Chenopodium amaranticolor* seedlings (Sdoodee & Teakle, 1987); Western flower thrips (*Frankliniella occidentalis*) mediated the transmission of PDV and PNRSV from cherry pollen to cucumber seedlings (Greber, Teakle & Mink, 1992). CLRV-infected cherry pollen with western flower thrips could infect *Chenopodium quinoa* but not young cherry seedlings (W. E. Howell, *personal*

communication). Most of this work was done in greenhouse and is not conclusive evidence of thrips involvement in pollen transmission of virus from cherry trees in the field. Our experiments in the Grandview plot did not examine the role of thrips in horizontal transmission of CLRV in sweet cherry. Therefore, the possibility that CLRV is transmitted from the pollen to superficial wounds caused by thrips, leading to infection of the mother tree cannot be ignored. On the other hand, our experiments in the Moxee plot indicated that supplementing blossoms with thrips is not necessary for CLRV movement from pollen to pedicel.

CLRV of sweet cherry also can be transmitted from pollen to seed and to seedlings (K. C. Eastwell, *personal communication*). Generally, vertical pollen transmission has little importance for viral disease spread in field situations (Mandahar, 1985). For example, CLRV in walnut can be vertically transmitted from pollen to seed but horizontal transmission is the main factor that threatens the walnut production in California (Mircetich *et al.*, 1980). Vertical transmission can produce infected seeds but most of the host perennial plants are not propagated by seed. However, it is different in the sweet cherry industry. The rootstocks used for sweet cherry propagation include both seedling and clonally propagated rootstock. Vertical transmission of virus is a critical threat to seedling rootstock production. If seeds are infected with CLRV, the resulting seedling rootstocks may also be infected. Mazzard seedling rootstock is currently used in most sweet cherry orchards in the Pacific Northwest, although the use of clonally propagated rootstock is increasing. CLRV was identified in at least one tree in a Mazzard orchard producing seeds for rootstock production in 2006 (L. J. Guerra, *personal*

communication). Therefore, pollen-associated vertical transmission of CLRV can be significant, as is horizontal transmission to sweet cherry production.

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