FINAL PROJECT REPORT
WTFRC Project Number: CH-04-404

Project Title: Virus control strategies to assist cherry production in the PNW

PI:	Ken Eastwell	**Co-PI(2):**	William Howell
Organization:	WSU-IAREC	**Organization:**	NRSP-5/WSU-IAREC
Telephone/email:	509-786-9385	**Telephone/email:**	509-786-251
Address:	24106 N Bunn Rd	**Address:**	24106 N Bunn Rd
City:	Prosser	**City:**	Prosser
State/Province/Zip:	WA 99350	**State/Province/Zip:**	WA 99350
Co-PI(3):	Eileen Perry	**Co-PI(4):**	
Organization:	CPAS, WSU-Prosser	**Organization:**	
Telephone/email:	509-786-9257	**Telephone/email:**	
Address:	24106 N Bunn Rd	**Address:**	
Address 2:		**Address 2:**	
City:	Prosser	**City:**	
State/Province/Zip:	WA 99350	**State/Province/Zip:**	

Cooperators: Narceo Bajet, USDA-ARS, Wapato
Lauri Guerra, WSDA, Prosser
Hui Hou, WSU, Prosser
Fran Pierce, Director, CPAS, WSU-Prosser
Tom Unruh, USDA-ARS, Wapato
Various growers

Budget History:

Item	**Year 1:** $26,616	**Year 2:** $31,655	**Year 3:** $36,973
Salaries	14,084	14,648	17,946
Benefits	4,084	5,292	7,282
Wages	1,960	4,000	0
Benefits	313	640	0
Equipment	0	0	0
Supplies	6,106	6,800	11,200
Travel			
- to plots/growers	24	0	0
Miscellaneous	45	45	45
Total	$26,616	$31,655	$36,973

Objectives of this three year project were:

1. Develop progressive strategies to control virus diseases that contribute to the decline of sweet cherry productivity in the Pacific Northwest.
2. Develop laboratory tests that will make virus testing more accessible to growers.
3. Monitor commercial sweet cherry orchards for emerging virus diseases.
4. Evaluate the use of remote sensing to identify areas of declining cherry production that may be associated with virus infections.

Significant findings:

- *Cherry leafroll virus* is detected in the pedicels of fruit and in fruiting spurs collected from trees that were not previously infected with the virus. This has significant implications for understanding natural spread of this virus and strategies for its control.
- Root grafting is a major route of tree-to-tree spread of several important diseases of cherry.
- *Cherry raspleaf virus* infects several agronomic crops and we demonstrated that the sequence of their coat proteins differ significantly across sources (host plant and geographical region). Furthermore, the sequence of local isolates of *Cherry raspleaf virus* is consistent with its classification as a member of the genus *Cheravirus* rather than as a *Nepovirus* as previously thought.
- The protein shell of *Cherry raspleaf virus* consists of three different peptides. We identified CP2 as the one most likely involved in soliciting and reacting with antibodies. This information is used to develop serological reagents for virus detection.
- The virus that causes little cherry disease in Europe (*Little cherry virus-1*) is widely distributed in the PNW. This is a particularly difficult virus disease to control because there are no readily discernible symptoms other than poor fruit production (low yields and smaller fruit than is currently profitable).
- The incidence of Western X disease is showing an alarming resurgence throughout WA.
- Viruses of the genus *Foveavirus* are associated with diseased and declining trees in many orchards west of the continental divide. An enzyme-linked immunosorbent assay (ELISA) was developed for Montmorency stem pitting foveavirus. This proved effective in discriminating between samples infected with this damaging virus from those infected with related viruses whose long term consequences are less significant.
- Virus infection may be detected using light reflectance. The silicon detectors required for this technology are inexpensive and can be incorporated into one of several different formats.

Results and discussion:

Objective 1: Develop progressive strategies to control virus diseases that contribute to the decline of sweet cherry productivity in the Pacific Northwest.

The identification of *Cherry leafroll virus* in sweet cherry orchards of the USA is still a relatively recent event being identified in the PNW for the first time in 1999. We have elucidated many key factors in disease epidemiology associated with this new virus disease. *Cherry leafroll virus* is unique in that, experimentally, it infects a wide range of host plants from many different plant families, but each host is associated with a distinct virus strain. In nature, infection of a host plant with a strain from a different host genus has not been reported. Thus, virus isolates from each host present unique biology and challenges.

Our program demonstrated that pollen of infected sweet cherry trees contains very high concentrations of infectious *Cherry leafroll virus* particles. Moreover, these infectious particles can be transported by bees. This presented two questions that required response: Does virus-laden pollen play a role in the transmission of *Cherry leafroll virus*? Do other factors play critical roles in pollen-mediated transmission?

During the first two weeks of April, 57 bees were collected from orchard trees and tested to determine their capacity to be carriers of *Cherry leafroll virus*; two bees contained detectable *Cherry leafroll virus* in their pollen sacs and on their bodies as indicated by ELISA. Virus in the pollen sacs was infectious. On April 22, approximately two weeks after peak cherry bloom in the vicinity, 58 bees were collected and tested. Although no virus was detected by ELISA, the more sensitive reverse transcription polymerase chain reaction (RT-PCR) assay revealed three bees still bearing trace amounts of *Cherry leafroll virus*. These experiments indicate that bees are able to transport *Cherry leafroll virus*. Substantial amounts of virus-laden pollen are moved as the bees forage. Based on bee feeding behavior, the greatest potential for tree-to-tree spread of pollen is to nearby trees. However, the potential for long distance dissemination of virus by this method is relatively small but significant.

Using ELISA, we demonstrated that *Cherry leafroll virus* can infect cherry pits of fruit on healthy sweet cherry trees growing adjacent to an infected pollinator variety. The *Cherry leafroll virus*-free status of subject trees was determined by ELISA performed on five leaves collected randomly from each tree each year. Obviously, the most likely source of *Cherry leafroll virus* detected in the pits was virus-infected pollen. To examine the role of flowers in pollen epidemiology in greater detail, pits, mesocarp (fruit flesh), and pedicels from subject trees were extracted separately and tested for *Cherry leafroll virus*. No virus was detectable in the fruit flesh. Since the mesocarp is derived solely from the tree bearing the fruit, this provided confirmation that the parent tree was not infected with *Cherry leafroll virus*. Virus was detected in up to 22.5% of the pits by ELISA, and none was found in the pedicels (Table 1).

Table 1: Fruit was harvested from trees that had previously tested negative for *Cherry leafroll virus*, but were located adjacent to an infected pollinator. Fruit flesh, pits and pedicels were tested separately by ELISA.

Tree identification	**ELISA results** *number positive/number tested (percentage positive)*		
	Pedicel	Pits	Mesocarp
'Bing' R1T5	0/200 (0.0%)	45/200 (22.5%)	0/200 (0.0%)
'Van' R15T10	0/400 (0.0%)	7/400 (1.8%)	0/400 (0.0%)

The inability of ELISA to detect *Cherry leafroll virus* in pedicels suggested that although the virus is entering the pit from infected pollen grains, the virus is not moving from the flower/fruit structures into the recipient tree. However, when RT-PCR is used to examine the same question, results differed significantly. With the increased sensitivity offered by RT-PCR (100- to 1,000-fold increase in sensitivity relative to ELISA), *Cherry leafroll virus* was detected in a significant number of fruit pedicels (Table 2). This result was confirmed in two growing seasons. One concern was that the increased sensitivity of RT-PCR would detect residual virus from pollen contaminating the surface of the pedicel, thus giving positive results in the RT-PCR assay even though the surface contamination would not be biologically significant. This issue was addressed by two strategies. The test of fruit pedicels was repeated throughout the growing season until two weeks past commercial harvest. *Cherry leafroll virus* was consistently detected in some of the pedicels at each sampling time. During this period, the virus in potentially contaminating virus-laden pollen is dissipated below the limits of detection by RT-PCR. This suggests that the virus that was detected by RT-PCR was internal to the pedicels. In a second approach, immunolocalization of virus particles in tissue sections from fruit and flower parts was used to differentiate between virus particles on the pedicel surface from those within cells. Tissues were collected from subject trees as well as from known infected and non-infected trees for comparison. Tissues collected at various times during fruit development were embedded, sectioned, and labeled with gold via virus-specific antibodies. Gold-label was silver enhanced and observed with a confocal microscope. Examination of sections reveal *Cherry leafroll virus* particles

localized within the cytoplasm of sub-epidermal cells of the pedicels. This confirms that the virus detected by RT-PCR is not surface contamination, but is within the cells of the pedicel. Interestingly, when the fruit-bearing tree is infected with *Cherry leafroll virus,* the virus is again localized in the cytoplasm of cells in sub-epidermal layers of the pedicel, but also associated with vascular bundles. This is consistent with the concept that during later stages of fruit development, the movement of

Table 2. *Cherry leafroll virus* is detected by RT-PCR in pedicels of fruit collected from 'Van' cherry trees adjacent to *Cherry leafroll virus*-infected 'Bing' trees.

Tree identification	**RT-PCR results** *number positive/number tested*			
	2005	2006		
	Pedicel	Pedicel	Pit	Fruit flesh
'Van' 1	5/5	†	†	†
'Van' 2	0/5	2/10	10/10	2/10
'Van' 3	0/5	0/10	10/10	0/10
'Van' 4	0/5	1/10	8/10	0/10
Positive control	5/5	5/5	5/5	5/5
Negative control	0/5	0/5	0/5	0/5

† In winter 2006, *Cherry leafroll virus* was detected in tree 'Van' 1 by ELISA, indicating that this tree had become systemically infected. The tree was not used for further pollination studies in 2006.

solutes through vascular tissue is unidirectional, toward the developing fruit, and no virus would move contrary to this source-sink flow. Cell-to-cell movement through parenchyma cells beneath the epidermis is still possible as demonstrated by this study. These experiments confirm that virus particles are entering the flower tissues from infected pollen and are capable of migrating into the structure that connects the flower tissue to the maternal tree.

We next examined the possibility that *Cherry leafroll virus* is capable of entering the vegetative portions of trees from the flowers. To explore the possibility that the virus is able to breach the abscission layer between the pedicel and the tree, samples were collected three weeks after commercial harvest and the flower spurs and associated pedicels were tested by RT-PCR. The results are summarized in Table 3. As in the previous experiments, *Cherry leafroll virus* RNA was detected in the pedicels. Moreover, in a significant number of instances, the viral RNA was detectable in the fruiting spurs of the maternal tree. Of the 58 spurs tested, three contained detectable virus. This strongly suggests that virus is being translocated from the reproductive tissues into the tree. That is, the virus is likely entering the tree through the flower. Further analysis is needed to confirm this phenomenon.

Pollen appears to play a key role in the epidemiology of *Cherry leafroll virus*. Based on our results, we established protocols to detect *Cherry leafroll virus* in commercial pollen sources. This protocol is being utilized by some commercial pollen companies to insure that their pollen is a virus-free product. In some instances, this procedure has alerted growers to the presence of *Cherry leafroll virus* in an orchard that had gone undetected before the pollen test. Consequently, the infected tree was identified and removed. Monitoring of cherry seedling rootstock production has also been initiated. This will help minimize the distribution of *Cherry leafroll virus* into new areas through infected propagation material. Both of these practices adopted by sectors of the industry are the direct result of our studies in the transmission of *Cherry leafroll virus*.

Table 3. *Cherry leafroll virus* is detected in tissues at three weeks post harvest by RT-PCR.

Branch number	**Assay results** *number of positive samples/ number of samples tested samples*					
	'Van' 2		'Van' 3		'Van' 4	
	Pedicel	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	1/1	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	1/4
Total	3/20	2/18	0/20	0/20	1/20	1/20

Transmission through infected pollen is likely the major route of infrequent long distance movement of *Cherry leafroll virus*, and also a factor in transmission to neighboring trees. In the latter case, there is a second mode of transmission that we demonstrated. Within an orchard, root grafting plays a significant role in tree-to-tree spread. This was illustrated by the number of trees reacting to herbicide treatment after cutting a nearby diseased tree and treating the resultant trunk with herbicide. Using herbicide damage as a guide, in the orchards that were studied, typically one in eight of the neighboring trees were root grafted to the virus-infected tree. These orchards were 10- to 18-years old at the time. Thus, our research on the epidemiology of *Cherry leafroll virus* demonstrated that transmission through root grafts is an important route of tree-to-tree spread in the orchard. To explore this further, a small pilot project was established in two separate commercial orchards. Cherry trees planted on 'Colt' rootstocks in the *Cherry leafroll virus* infested orchards did not become infected with the virus, whereas two-thirds of those on Mazzard did become infected. This occurred in both orchard settings. The difference was not related to bloom because any flowers that developed during the time of the study were removed manually. This suggests that 'Colt' offers some resistance to *Cherry leafroll virus* in the field setting. We are now beginning to explore other rootstocks that may offer protection against root grafting of *Cherry leafroll virus*.

Field studies revealed that different cherry cultivars respond to virus infections with different severity of symptoms. Of those tested, 'Tieton' is the mostly severely affected cultivar and shoot tip death was common in plants inoculated with *Cherry leafroll virus*, whereas the majority of cultivars such as 'Bing' display such symptoms only when the tree is infected with *Cherry leafroll virus* plus one of the ilarviruses. In our tests, 'Chelan' was a symptomless carrier of *Cherry leafroll virus* and hence, it may facilitate unintentional distribution through propagation material. Infected 'Chelan' trees could also be sources of infected pollen in the orchard that will be very difficult to identify because there are no outward symptoms of virus infection.

Objective 2: Develop laboratory tests that will make virus testing accessible to growers.
Substantial progress was made in developing diagnostic procedures for viruses associated with little cherry disease. Using these techniques, we demonstrated that the virus associated with the disease in Europe is also well established in western North America. This is in addition to the little cherry virus that had been previously identified in British Columbia and Washington.

The virus characterized in Europe, *Little cherry virus-1*, exhibits extreme sequence variability. This thwarted early efforts to develop reliable molecular assays. However, we successfully identified and characterized isolates obtained from many locations including Washington, Oregon, California, Pennsylvania, British Columbia and Europe. The result of our analysis is a molecular assay that provides a much greater level of confidence in identifying *Little cherry virus-1*. These advancements are coupled to our previous studies on *Little cherry virus-2* to develop a multiplex RT-PCR assay that

will detect both viruses in a single reaction, thus providing an opportunity for enhanced identification and management of little cherry disease.

Serological reagents are desired for the viruses associated with little cherry disease because they would reduce the cost and increase the availability of routine testing to growers, fieldmen and researchers. Substantial progress was made in the development of these diagnostic reagents. We characterized the gene encoding the two coat proteins of local strains of *Little cherry virus-2*. These were expressed in bacteria and monoclonal antibodies produced in response to the expressed proteins. We identified two hybridomas producing antibodies that recognize the major coat protein of the virus particle and have the potential to work well in ELISA for the detection of *Little cherry virus-2*. Further development of the assay is required and the use of these antibodies in routine assays will be validated through ongoing research.

In a major advancement this year, we also identified the major coat protein gene of *Little cherry virus-1* and using a strategy similar to that described above, we sought to develop antibodies suitable for the detection of this virus. After screening approximately 4,000 hybridomas for antibody production, we identified approximately 150 hybridomas producing antibodies that recognize the coat protein of *Little cherry virus-1* expressed in bacteria, and one of these was demonstrated to detect by ELISA virus particles in crude leaf extracts. During the course of the coming season, we will evaluate the ability of this antibody to detect virus at all seasonal stages of tree growth.

Studies by others identified *Green ring mottle virus* and *Cherry necrotic rusty mottle virus* as members of the *Foveavirus* genus. Our research has now shown that the viruses associated with cherry rusty mottle, cherry twisted leaf, and Montmorency stem pitting are also caused by closely related foveaviruses. As we accumulate information, it is apparent that there is extensive sequence variability between the different viruses associated with these diseases in cherry, but there are also areas of sequence conservation. This is allowing us to develop both broad spectrum and virus-specific molecular assays. Furthermore, we have produced antibodies against the Montmorency stem pitting virus that are very effective as a diagnostic aid. The antibodies also react with a number of other foveaviruses of cherry, albeit with lower avidity. This enables a quick response to growers for determining if a foveavirus might be associated with their diseased trees. *Green ring mottle virus*, which is generally regarded as latent in most sweet cherry varieties, does not react with the antiserum that we developed, thus, its presence does not interfere with efforts to detect disease-causing viruses.

We are developing molecular and serological methods to detect *Cherry raspleaf virus*. Although the distribution of this virus through the PNW is quite limited, where it does occur, it is devastating. The virus is transmitted by nematodes and also infects a wide range of broad-leaf weeds. Once it is introduced into orchard land, there are few options available to the grower. The virus also causes flat apple disease so converting to apple production is not an appropriate response. In on-going research, we are exploring the ability of certain rootstocks to offer resistance against *Cherry raspleaf virus*. In order to execute these studies, refined diagnostic tools are required. Towards this objective, we characterized the three peptides that make up the coat of the virus particles. We determined that CP2 is most likely involved in serological reactions, and hence, the best candidate to solicit antibodies for detection. The gene sequence for CP2 was expressed in bacteria and antibodies solicited in response to this peptide. Initially, as a preliminary trial, a small amount of polyclonal antibodies were produced. We are currently developing monoclonal antibodies that should provide a more specific and more reliable reagents to be integrated into an ELISA.

Objective 3: Monitor commercial sweet cherry orchards for emerging virus diseases.

Little cherry disease has re-emerged as a potentially serious virus. The disease that was recognized and so destructive from 1940 to 1960 was associated with *Little cherry virus-2*. *Little cherry virus-1* is now emerging as a serious problem. It can be very damaging because the leaf symptoms are non-existent or mild. On the surface, this statement seems contradictory. *Little cherry virus-1* causes a

reduction in fruit size and quality but the reduction is not as great as that observed in response to *Little cherry virus-2*. Therefore, the involvement of a virus is not immediately suspected. The reduced fruit size in orchards with trees infected by *Little cherry virus-1* is usually thought to be the result of other causes such as water and nutrition management. Valuable resources are inevitably used in efforts to improve fruit size with no success. Early and correct determination of *Little cherry virus-1* status of trees will permit correct response to these conditions; that is, tree roguing in the case of virus diseases or altered horticultural practices if viruses are not detected. There is growing concern world-wide that *Little cherry virus-1* may not yield a sufficiently strong response by the traditional biological indicator to be reliably identified. The development of laboratory tests is therefore very timely and critical. We have identified a growing number of orchards where poor production is associated with the presence of *Little cherry virus-1*. These orchards are located in Yakima, Grant and Chelan counties in Washington. Continued monitoring is required to establish the level to which this virus has penetrated cherry production in the PNW.

Foveaviruses emerged as an important group of viruses in cherry production over the past few years. Many different molecular forms of these viruses were detected. We have developed the means to discriminate one virus from another by their molecular properties and by the degree and nature of symptoms that they cause. Availability of detection strategies is greatly enhancing the ability to identify and react to virus infections. A general molecular assay for the foveaviruses of cherry was developed that greatly enhanced our ability to detect and characterize foveaviruses from a number of orchards and disease situations. A complex pattern that has arisen from the data is still being resolved.

Continued surveillance of cherry production areas over the past five years revealed a dramatic increase in the frequency with which Western X disease is encountered. This disease severely impacted WA cherry production in the 1950's and 1960's; careful management of blocks in which the disease occurs is necessary to minimize further impact. Recent Western X infections occur in all cherry production regions of WA State.

Objective 4: Evaluate the use of remote sensing to identify areas of declining cherry production that may be associated with virus infections.

Trees in a commercial orchard were analyzed for the presence of *Cherry leafroll virus* (CLRV), *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV). Based on the results, eight trees were selected for analysis representing trees that were not infected, infected with either *Prune dwarf virus* OR *Cherry leafroll virus,* or with both *Prune dwarf virus* and *Cherry leafroll virus*. Three spur leaves from each tree were collected and two spectral measurements per leaf were obtained using an ASD field spectrometer. It generates a reflectance curve from 400-2500nm at 2nm intervals, and the curve represents the reflectance of a single point of a leaf. To emphasize wavelengths where the virus-infected plants exhibit the greatest differences, the average spectrum for each infection type is normalized by the average of the healthy leaves (Figure 1). The further the spectrum deviates from 1.00, the greater the difference in reflectance. Spectra from *Prune dwarf virus*- and *Prune dwarf virus* plus *Cherry leafroll virus*-infected trees look similar, but different from trees infected with *Cherry leafroll virus* alone. Reflectance values of 582nm, 697nm, 1458nm and 1975nm, as well as derived stress indices of normalized difference vegetation index (NDVI), modified chlorophyll absorption in reflectance index (MCARI), photosynthetic response index (PRI), water band index (WBI), and red edge vegetation stress index (RVSI) were evaluated. Analysis of Variance (ANOVA) was used to quantify the differences among plants with and without *Cherry leafroll virus*. Only RVSI significantly differentiated non-infected leaves from infected leaves with an F value of 7.89 (> 0.001) and at a 95% confidence limit. When only the healthy and *Cherry leafroll virus* infected leaves were used in the analysis, RVSI again produced the most significant contrast between the infected and non-infected leaf measurements with an F value of 18.55 (> 0.001).

Thus a hand-held device could distinguish leaves from healthy trees from leaves from virus infected trees.

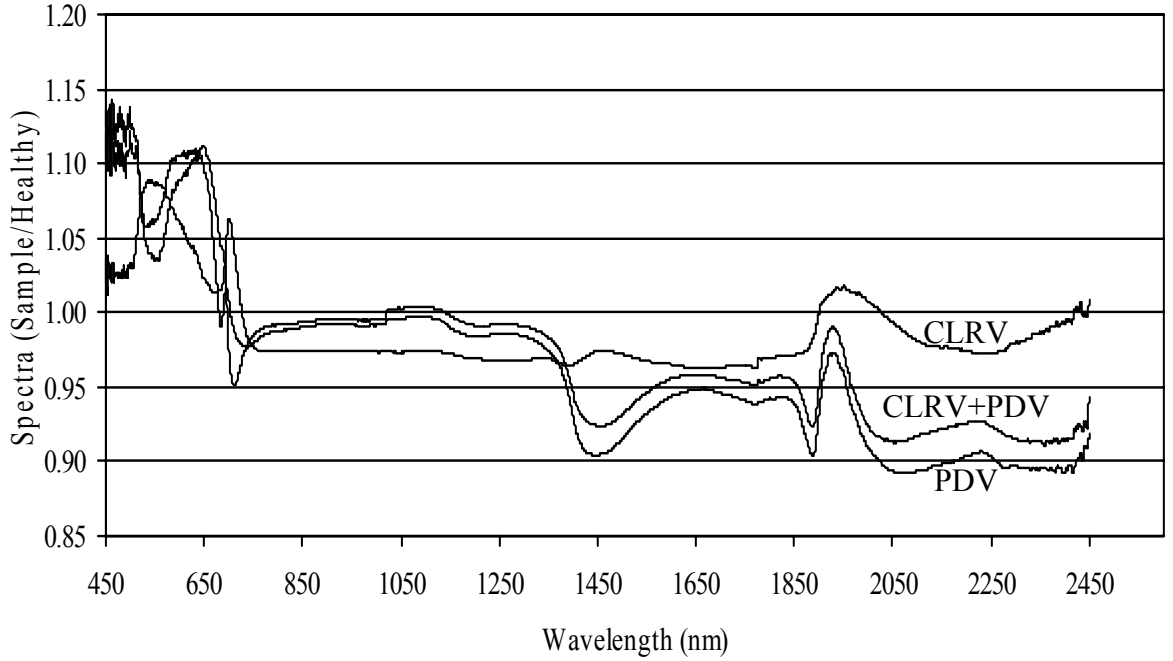

Figure 1. Averaged spectra from *Cherry leafroll virus*, *Prune dwarf virus* and *Cherry leafroll virus* plus *Prune dwarf virus* infected trees in a commercial orchard. The spectra were normalized using the averaged spectrum from healthy trees. Therefore, the graph represents deviation from readings from healthy trees which have the value of 1.00.

The RVSI and other indices that characterize the shape and location of the actual chlorophyll red-edge utilize narrow spectral bands measured with a portable spectrometer that makes point measurements. To gain an overview of an orchard disease status, a multispectral camera was used to view trees in a commercial orchard. The multispectral camera is based on broad spectral bands (green, red, and near IR) that cannot characterize the red-edge, thus image processing analogous to RVSI is not available, however, the image bands can be used to determine NDVI. The NDVI images use the red and near infrared bands in which infected trees appear as dark red and healthier canopy appears bright. Thus, the difference between *Cherry leafroll virus*-infected and non-infected trees becomes more noticeable. These results suggest that a small, portable, lightweight video system sensitive to the red and near infrared bands to produce a real-time NDVI would be of value in locating stressed trees in the orchard. This system could be worn by someone, or attached to a PDA to generate NDVI images. All of this preliminary data was obtained with rather generic interpretations of vegetation images. With the detailed spectral information obtained from individual leaf reflectance measurements, a dedicated imaging system based on this information could be developed. Perhaps in the future a method will become available to make narrow spectral band measurements with an imaging (camera) system.