Research Abstract for the
California Leafy Greens Research Board
April 2008 to March 2009

Project Title: Investigation of Tospovirus Outbreaks in California Lettuce

Project Investigators: Steven Koike, Bob Gilbertson, Yen-Wen Kuo, Tom Turini, and Richard Smith

Summary

Tospoviruses are plant-infecting viruses that are vectored by thrips. These viruses are extremely important because they are widespread throughout the world, are notoriously difficult to manage, and can cause significant losses for many crops. Two common tospoviruses in California are *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV). Historically, TSWV has been found occasionally in California lettuce but was never a significant economic factor; INSV had not been reported on lettuce in California. For the past few years, however, Koike and other researchers have noted an increasing incidence of TSWV in coastal California lettuce. In late 2006 and 2007, numerous coastal plantings of romaine, greenleaf, butter, and iceberg lettuce experienced some damage from TSWV. Beginning in 2006 and continuing through 2008, significant and damaging cases of INSV were found on numerous romaine, greenleaf, redleaf, butter, and iceberg plantings in Monterey and San Benito counties. To our knowledge, this is the first time INSV has been documented in California lettuce. In the San Joaquin Valley, thus far only TSWV infection has been confirmed on lettuce.

Molecular evidence indicates that the coastal INSV outbreaks are caused by a typical strain of INSV that does not appear to differ significantly from INSV strains previously characterized from ornamental or other hosts. Therefore, the lettuce INSV problem is not caused by a novel INSV strain or new tospovirus. The vast majority of thrips present in diseased lettuce fields are western flower thrips (WFT). WFT populations were monitored in lettuce fields affected by INSV. Thrips numbers varied but at times reached high levels. An RT-PCR assay has been developed that allows for detection of INSV in thrips. This assay could be a useful tool for monitoring for the virus. Field surveys failed to reveal a widespread weed or alternate host candidate that could act as a reservoir and source of INSV. Therefore the source of the recent INSV outbreaks remains unknown. Because INSV is unlikely to be seedborne in lettuce, identifying the reservoir or source of INSV will be a high priority for subsequent research. The reason or mechanism for the recent INSV problem on lettuce is also not known, but may be related to overall elevated populations of the thrips vector in Monterey County and elsewhere.

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Introduction:

Tospoviruses are plant-infecting viruses that are vectored by thrips. These viruses are extremely important because they are widespread throughout the world, are notoriously difficult to manage, and can cause significant losses for many crops. Perhaps the two most familiar tospoviruses encountered in California are *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV). TSWV is common throughout California, can infect a great number of plants (several hundred vegetables, ornamentals, and weeds), and is found throughout the state. INSV also infects many plants but is more often found infecting ornamental and not vegetable crops. Historically, TSWV has been found occasionally in California lettuce but was never a significant economic factor; INSV had not been reported on lettuce in California. For the past few years, however, Koike and other researchers have noted an increasing incidence of TSWV in coastal California lettuce. In late 2006 and 2007, some important developments occurred regarding tospovirus diseases on coastal lettuce:

> In 2007, numerous coastal plantings of romaine, greenleaf, butter, and iceberg lettuce experienced significant damage from TSWV. Other leafy vegetables (such as radicchio) also suffered significant disease caused by TSWV, both in coastal and inland counties.
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> Beginning in fall 2006 and continuing through 2008, significant and damaging cases of INSV were found on numerous romaine, greenleaf, redleaf, butter, and iceberg plantings

in Monterey and San Benito counties. To our knowledge, this is the first time INSV has been documented in California lettuce.

In a number of cases these TSWV and INSV pathogens resulted in very high disease incidence. A number of plantings had well over 50% infection. Due to the nature of tospovirus symptoms on lettuce, all diseased plants are unsuitable for harvest.

In October and November of 2007, TSWV was often detected in Fresno County lettuce fields, though incidence within fields was below 1%.

Objectives:

1. Monitor and survey lettuce fields to follow disease development.

2. Confirm causal agent of the INSV outbreak in coastal California.

3. Monitor thrips vector populations and identify thrips species involved.

4. Conduct surveys for weed and other alternate virus hosts.

Procedures:

1. Survey lettuce fields: In 2008 we selected six fields in the Salinas Valley in which tospovirus-infected lettuce was found. For each field, we surveyed disease incidence by assessing the number of symptomatic plants in 5 locations: the four corners and the center of the field. At each location, the number of plants with tospovirus symptoms was visually determined in randomly selected planted strips of 200 feet long by 5 beds wide. Overall incidence of tospovirus symptoms was then calculated. Selected plants with tospovirus symptoms were tested for INSV and TSWV by first using immunostrip quick tests and later RT-PCR (Gilbertson lab). Throughout the season, we also provided diagnostic services to growers and pest control advisors by testing submitted samples for INSV and TSWV; these diagnostic samples provided additional information on the extent of the tospovirus outbreak in the coastal area.

In the San Joaquin Valley, Tom Turini conducted surveys of lettuce in five fields in fall 2008 and three in spring 2009. In four sites, in each field, lettuce in a 100 ft long by one bed wide area was carefully inspected weekly. Any tospovirus-like symptoms were tested for TSWV and INSV with immunostrip quick tests. In addition, yellow sticky cards were placed in the fields in each corner and collected weekly.

2. Confirm causal agent: In 2008, most tospovirus outbreak fields were infected with INSV and not TSWV. Diseased lettuce and thrips obtained from symptomatic plants were sent to the Gilbertson lab for confirmation of INSV. Selected virus isolates from lettuce were characterized at the molecular level by sequencing part of the virus genome (usually the N gene). For one

lettuce isolate, the complete genetic sequence is being determined. The lettuce samples were first tested with INSV immunostrips and, for selected positive samples, total RNA was extracted for RT-PCR tests using INSV N gene primers. In addition, other plants testing positive for INSV infection also were tested by RT-PCR; the N gene fragment was amplified and sequenced to compare isolates from other hosts with those from lettuce. All samples that were positive for INSV were then stored in -80 C.

3. Monitor thrips: Following methods developed by Bill Chaney and others, we monitored thrips populations with a leaf washing method (Chaney. 2006). For each field, 25 symptomatic and 25 healthy plants (5 replications of 5 plants each) were cut, bagged, and taken back to the lab. Each plant was cut apart and every leaf held under running water to dislodge all thrips. The thrips were recovered by filtering the wash water through a fine mesh screen. Thrips were removed from the mesh screen and stored in alcohol.

Collected thrips were sorted into adults and juveniles and counted. Representative adult thrips (75 thrips each from healthy and diseased plants) for each field were removed, cleared by soaking overnight in sodium hydroxide, and mounted in Hoyer's solution for examination with a compound microscope. Thrips prepared in this way were identified to species using standard taxonomic features (Hoddle et al. 2004; Hoddle. 2008). Other thrips were maintained in alcohol and sent to the Gilbertson lab for testing for tospoviruses. The thrips were vacuum dried and then the RNA was extracted from the dried thrips with a Qiagen RNAeasy kit. The extracted RNA was used for RT-PCR tests using the INSV N gene-specific primers.

4. Surveys for weed and other alternate hosts: Weeds and other plants growing near lettuce fields were surveyed for INSV and TSWV at six sites in the summer of 2008. Sites were selected based on the presence of an outbreak of INSV in an adjacent or nearby lettuce field. Surveys included weed species found in the diseased lettuce field as well as weeds and other plants from surrounding fields, roadsides, reservoirs, and other weedy sites. Emphasis was placed on sites upwind from the infected field. Each sample consisted of tissue from five plants of one species. Samples were placed in a cooler and transported to the UCCE lab in Salinas. These bulked plant samples were tested using immunostrip quick tests for INSV and TSWV. Representative samples were also sent to the Gilbertson lab for examination. Besides RT-PCR test, a dot blot method with a cloned INSV probe was used to test the weed samples for INSV infection. However, the results from dot blot were similar to those from RT-PCR test. Most of the weed samples were not positive for INSV infection.

Fresno County weed surveys: In two uncultivated fields, 100 plants each of prickly lettuce and sowthistle were examined for tospovirus-like symptoms and each weed that had symptoms were tested separately for INSV and TSWV.

Results:

1. Survey lettuce fields: In contrast to 2007 when INSV was not reported to us until mid-summer, in 2008 infected lettuce was observed as early as April. Disease caused by INSV was present throughout the growing season, and our selected fields were sampled in April, June, July,

and August. For our six survey fields, disease incidence ranged from 0.5 to 27% (Table 1). Plants in these survey fields tested positive for INSV using both immunostrip quick tests and lab-based methods. TSWV was not detected in these fields.

With the help of growers and PCAs, we confirmed INSV in over 15 fields in the Salinas Valley from April through October. Only 2 samples tested positive for TSWV during this period. In commercial fields that were not a part of our formal survey, disease incidence ranged from less than 1 to over 50%; however, most of these field situations had disease levels between 1 and 10%.

In the San Joaquin Valley, TSWV incidence in the fields monitored in the fall ranged from 0 to 1.25%. Twenty-eight lettuce samples were tested from the monitored fields in fall and all tested negative for INSV and positive for TSWV. In the spring, no tospovirus-like symptoms were detected in the monitored areas. In the Huron-area site, one plant was found that tested positive for TSWV, though it was outside the designated area being evaluated. In the area west of Five Points, one lettuce plant in a field adjacent to the monitored location tested positive for TSWV. The one Huron and the one Five Points plants were the only two plants in these areas that expressed any tospovirus-like symptoms. In the San Joaquin Valley, no lettuce plants tested positive for INSV.

Table 1. Field survey for INSV disease incidence and thrips populations in the Salinas Valley

	Lettuce	INSV	Mean number of thrips per 5 lettuce plants			
Site / Month	type	incidence	adults	juveniles	total thrips	% juveniles
S7N (Gonzales)	romaine	0.0%	56	34	90	36
April		2.3%	34	28	62	46
ShR (Gonzales)	romaine	0.0%	27	7	34	21
June		1.0%	45	13	58	19
SpEB (Salinas)	romaine	0.0%	177	139	316	44
June		3.0%	168	144	312	47
S10 (Gonzales)	greenleaf	0.0%	68	214	282	76
June		0.5%	20	191	211	90
D7A (Soledad)	romaine	0.0%	328	42	370	11
July		27.0%	559	52	611	9
CGD (Soledad)	romaine	0.0%	60	6	66	9
August		14.8%	207	29	236	12

2. Confirm causal agent: The DNA fragments that were amplified with the RT-PCR test were sent for sequence analysis, and results confirmed that the causal agent was INSV. The N gene sequences were 98-99% identical (Table 2) with sequences of INSV isolates from the U.S.A., Italy, Japan, and The Netherlands. Furthermore, this level of sequence identity was the same from isolates from different host plant species, indicating that these are all isolates of the same INSV species.

Table 2. Sequence similarity of the N gene of INSV isolates from different hosts in Monterey County with previously characterized INSV isolates,

Host	Sequence identity with a known INSV isolate
Lettuce (romaine)	99%
Lettuce (greenleaf)	99%
Lettuce (redleaf)	99%
Fava bean	98%
Orchids	99%
Radicchio	98%
Snapdragon	99%

3. Monitor thrips: For the six surveyed fields in the Salinas Valley, thrips populations varied greatly (Table 3). For lettuce plants showing symptoms of INSV infection, total thrips recovered ranged from 58 to 611 per 5-plant sample. For healthy plants collected from the same fields the recovered thrips ranged from 34 to 370 per 5-plant sample. For most of the six fields, however, the numbers of thrips recovered from healthy and INSV-infected plants did not differ widely.

Because only the juvenile stage of thrips can acquire tospoviruses, we separated all collected thrips into juvenile and adult stages (Table 3). For lettuce plants showing symptoms of INSV infection, percent of juveniles recovered ranged from 9 to 90% per 5-plant sample. For healthy plants collected from the same fields the percent of juveniles recovered ranged from 9 to 76% per 5-plant sample. Again, for most of the six fields the percentages of recovered juvenile thrips from healthy and INSV-infected plants were similar.

For each field, approximately 75 thrips from healthy and 75 thrips from INSV-infected lettuce plants were examined microscopically to determine species. The majority of the thrips, in all fields and from both healthy and diseased plants, was western flower thrips (WFT) (*Frankliniella occidentalis*) (Table 3). This species made up approximately 90% or more of the recovered thrips. A small number of onion thrips (*Thrips tabaci*) and a few individuals of other species were identified from lettuce (Table 3). There were no significant differences between species identified from healthy or INSV-infected lettuce plants.

We are able to detect the presence of INSV in the thrips vector using the RT-PCR test (Table 4). Overall, INSV was detected in more thrips collected from lettuce plants with symptoms of INSV infection than from lettuce plants without symptoms. However, some thrips collected from healthy plants were positive, indicating that juvenile thrips are acquiring virus from diseased plants and moving onto healthy plants where they are likely spreading the virus. These results indicate that this method can be used to monitor for the presence of the virus during different times of the year by testing thrips for the virus.

Table 3. Thrips species from healthy or INSV-infected lettuce in the Salinas Valley

	Lettuce source:	Species of thrips recovered from lettuce*		
Site / Month	healthy/diseased	West. Flower	Onion	Other**
S7N (Gonzales)	healthy	93%	7%	0%
April	diseased	86%	11%	3%
ShR (Gonzales)	healthy	100%	0%	0%
June	diseased	97%	0%	3%
SpEB (Salinas)	healthy	94%	3%	3%
June	diseased	93%	4%	3%
S10 (Gonzales)	healthy	93%	4%	3%
June	diseased	94%	4%	2%
D7A (Soledad)	healty	99%	1%	0%
July	diseased	98%	0%	2%
CGD (Soledad)	healthy	99%	1%	0%
August	diseased	98%	2%	0%

*Western flower thrips = Frankliniella occidentalis; onion thrips = Thrips tabaci.
** Thrips australis, Frankliniella insularis, Frankliniella minuta, Ankothrips sp., Chirothrips sp., Aeolothrips sp.

Table 4. Detection of INSV in thrips using an RT-PCR test

Site/Month	Type of plant that thrips were collected from	Disease symptoms observed in the plants	Number of INSV-positive thrips/total vials of thrips tested
S7N / April	Romaine lettuce	Symptomless	3/5
		INSV symptoms	4/5
S10 / June	Greenleaf lettuce	Symptomless	0/0
		INSV symptoms	5/5
ShR / July	Romaine lettuce	Symptomless	0/0
		INSV symptoms	4/5
	Alyssum	Symptomless	2/5

4. Surveys for weeds and other alternate hosts: In the Salinas Valley weed and alternate host survey, 156 total samples were analyzed (Table 5). Only one sample (the weed shepherdspurse collected at site 3) was positive for INSV; four sites had at least one sample that tested positive for TSWV (Table 5). However, at all sites the number of tospovirus-infected weeds was very low.

Fresno County weed surveys: On 25 March, 6% of the sampled sowthistle was positive for TSWV; one plant was positive for INSV. Flowers from the sowthistle were examined; both juvenile and adult thrips were present. On 22 April, weeds in a fallowed field in the Five Points area were evaluated; 2% of the sowthistle and 7% of the prickly lettuce plants tested positive for TSWV, but all weeds tested negative for INSV.

Table 5. Salinas Valley weed and alternate host survey for tospoviruses

		Plants testing positive for a tospovirus (number positive/number tested)	
Site	Total number of samples	INSV	TSWV
1	21	None	Malva (1/2)
2	17	None	Malva (1/1)
3	31	Shepherdspurse (1/3)	Shepherdspurse (1/3)
4	48	None	Malva (1/4), Sowthistle (1/3)
5	12	None	None
6	27	None	None
Total	156	1 sample	5 samples

Discussion:

For the past several years, outbreaks of disease caused by tospoviruses have been occurring in California. The coastal region has been affected by both INSV and TSWV. In the San Joaquin Valley, thus far only TSWV infection has been confirmed on lettuce. Molecular evidence indicates that the coastal INSV outbreaks are caused by a typical strain of INSV that does not appear to differ significantly from INSV strains previously characterized from ornamental or other hosts. Therefore, the lettuce INSV problem is not caused by a novel INSV strain or new tospovirus. The vast majority of thrips present in diseased lettuce fields are western flower thrips, which is consistent with current research that indicates WFT is the main vector of INSV. An RT-PCR assay has been developed that allows for detection of INSV in thrips and this assay could be a useful tool for monitoring for the virus. Field surveys failed to reveal a widespread weed or alternate host candidate that could act as a reservoir and source of INSV. Therefore the source of the recent INSV outbreaks remains unknown. Because INSV is unlikely to be seedborne in lettuce, identifying the reservoir or source of INSV will be a high priority for subsequent research. The reason or mechanism for the recent INSV problem on lettuce is also not known, but may be related to overall elevated populations of the thrips vector in Monterey County and elsewhere.

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