PROJECT TITLE

Development of methods for maintenance of lettuce-infecting tospoviruses, effective germplasm screening, and identification of sources of resistance

PROJECT INVESTIGATORS

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ABSTRACT

Two tospoviruses have become problematic for California production of lettuce and leafy greens over the past few years; *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV). TSWV has a very wide host range encompassing a diverse array of crop and weed species that host both the virus and its thrips vector. INSV also has the ability to infect a substantial number of crops, and the presence of these viruses in perennial weeds, ornamentals and crops further complicates management. Management of tospoviruses in lettuce is quite difficult, as there is very little information on resistance to either TSWV or INSV in lettuce. This proposal focuses on development of effective methods to evaluate lettuce and related *Lactuca* germplasm for resistance to tospoviruses, leading to the development of breeding lines with high level of resistance to tospoviruses. Studies through this project demonstrated that without regular passage through thrips, even highly mechanically transmissible tospovirus isolates can lose their transmissibility to lettuce and other plants; whereas mechanical transmission to some other host plants is affected to much lesser degrees. This suggests that infection by tospoviruses requires virus-associated factors that can only be maintained with replication of the viruses in the insect vector as well as in the plant host. Related studies have examined transmission to lettuce from different virus host plants for each virus, and that *Datura stramonium* is a better source for virus and thrips maintenance as it accumulates higher levels of tospoviruses; whereas lettuce had the lowest mean titer among host plants examined in three experiments. Field evaluations had extremely low levels of infection preventing effective analysis; however, greenhouse evaluations using thrips have provided effective preliminary screening of lettuce and *Lactuca* germplasm in initial tests with INSV. We are still optimizing parameters for mechanical transmission, but using thrips transmission of INSV we have begun to evaluate lettuce for performance against INSV under greenhouse conditions. Additional studies are exploring resistance to TSWV using similar methods.

BACKGROUND

Two tospoviruses have become problematic for California production of lettuce and leafy greens over the past few years; *Impatiens necrotic spot virus* (INSV) and *Tomato spotted wilt virus* (TSWV). TSWV has a very wide host range encompassing a diverse array of crop and weed species hosting both virus and its thrips vectors. INSV also has the ability to infect a wide range of crops. The presence of these viruses in perennial weeds, ornamentals and crops further

complicates management. Dispersal of infectious thrips from these reservoirs to susceptible crops and weeds occurs during a short time in the spring. This problem has been exacerbated in recent years due to a dramatic increase in thrips populations that has resulted in severe thrips damage and more importantly, virus transmission by thrips to a wide range of crops, including not only lettuce, but also tomato, onion (another related virus) and numerous others.
More than 800 plant species, both dicots and monocots, in more than 80 plant families are susceptible to these tospoviruses. The *Solanaceae* and *Asteraceae* contain the largest numbers of susceptible species, both crops and weeds. Major crops susceptible to TSWV infection include lettuce, tomato, spinach, pepper, potato, papaya, peanut, tobacco and chrysanthemum. Over the past two years, INSV has been most prevalent in the Salinas Valley, based on samples tested by the Wintermantel lab, and reports from colleagues at UC Extension and UC Davis. However, TSWV has also been found to infect lettuce in coastal production regions and has been detected periodically over many years, particularly when lettuce or other susceptible crops are grown in close proximity with tomato. TSWV seems to be more prevalent in the San Joaquin Valley where tomato production is much greater than in coastal regions. It is likely that the prevalence of each virus varies, and may be influenced by cropping practices, availability of infected reservoir hosts, as well as vector population fluctuations. Both viruses are present throughout all other production regions in California as well, with the dominant tospovirus varying by region and adjacent cropping among other factors.

INSV and TSWV are transmitted from plant to plant by several species of thrips (Thysanoptera: *Thripidae)*. Two thrips species, *Frankliniella occidentalis* (Western flower thrips) and *F. fusca* (tobacco thrips) are the major vectors of these viruses in the U.S. although recently *Thrips tabaci* (onion thrips) appears to be increasing in importance. As with many insect vector/virus associations the thrips/TSWV relationship is very specific, with less than a dozen of the many known thrips species being able to acquire and transmit these viruses. Thrips can only transmit tospoviruses if they are acquired during their larval stages although both larval and adult thrips are able to transmit virus. Unlike most other insect-transmitted viruses, tospoviruses actually replicate inside the thrips vector, providing a steady supply of virus throughout the life of the insect.

Management of tospoviruses in lettuce is quite difficult, as there is very little information on resistance to either TSWV or INSV in lettuce. This proposal focuses on development of effective methods for testing lettuce and related *Lactuca* germplasm for resistance to tospoviruses. Transmission of these viruses can be accomplished by either thrips or mechanical transmission. The latter method is preferable as it does not require the production and management of large thrips populations. Previous studies using mechanical transmission have varied in success, with some experiments resulting in highly efficient transmission of both viruses to lettuce, whereas other experiments have had much lower rates of transmission using the same approach (Wintermantel and Simko, previous studies). In order to be successful, we need to either optimize transmission efficiency using mechanical transmission or focus on using thrips to deliver the virus. We hope that thrips transmission of virus will not be necessary due to a significant breakthrough in maintaining tosposviruses in alternate host plants, but are also exploring thrips transmission in case this will be necessary. This project aims to optimize mechanical transmission of tospoviruses to lettuce in order to facilitate more efficient long term studies aimed at selection of lettuce breeding lines with increased resistance to both INSV and

TSWV. If efficient mechanical transmission is unsuccessful, we will focus efforts on thrips-based transmission using an existing thrips colony maintained at the USDA-ARS, Salinas, CA. Loss of yield due to tospoviruses is the highest in the Huron area of the San Joaquin Valley, but both viruses have also been detected in other lettuce-producing areas of California. To limit loss of yield caused by tospoviruses the most viable option is development of lettuce cultivars with genetic resistance. Initially we are focused on identifying sources of increased resistance. Once accessions with high level of resistance are identified, they will be used in our breeding program. The crosses will be performed to introgress resistance genes into commonly grown types of lettuce (iceberg, Romaine, leaf, and butterhead). Breeding lines with improved resistance to tospoviruses will subsequently be released to the lettuce industry.

LONG-RANGE OBJECTIVE

Our goal with the proposed one year study is to determine specific parameters for routine greenhouse evaluation of lettuce for resistance to the tospoviruses, INSV and TSWV. Once completed, the project will contribute to the efforts of the USDA-ARS lettuce breeding program and those of the lettuce seed industry. The ultimate objective of the project is development of breeding lines with high level of resistance to tospoviruses.

OBJECTIVES

1. Determine reliability of TSWV and INSV infection on susceptible lettuce when each virus is transmitted mechanically (manual inoculation) from infected spinach, pepper, or *Emilia sonchifolia*. (leaf and iceberg)

2. Compare transmission to susceptible and limited known sources of resistant lettuce, as well as wild *Lactuca* species in order to observe differential symptom development and determine effective scoring system. (leaf and iceberg)

3. (*If Necessary*). Repeat Objective 2 using thrips vectors for transmission of INSV and TSWV. (leaf and iceberg)

4. Identify sources of resistance to tospoviruses in field conditions. (leaf and iceberg)

PROCEDURES

Objective 1: Determine reliability of TSWV and INSV infection on susceptible lettuce when each virus is transmitted mechanically (manual inoculation) from infected spinach, pepper, or *Emilia sonchifolia*

Preliminary studies demonstrated that INSV can be reliably maintained on spinach (*Spinacea oleracea*) for multiple passages. Studies by colleagues have demonstrated the TSWV can be maintained for multiple passages on pepper (*Capsicum annuum*) and tasselflower (*Emilia*

sonchifolia), as well as jimsonweed (Datura stramonium). Lettuce isolates of INSV and TSWV from the Salinas Valley, maintained by the USDA-ARS virology lab in Salinas as frozen stocks and in live plants, are used for transmission experiments. Each virus isolate is mechanically transmitted to numerous susceptible seedling (2 leaf stage) lettuce plants. Control plants of each propagation host (spinach, pepper, jimsonweed, tasselflower, and others), as well as *N. benthamiana* were inoculated in each experiment to confirm transmission efficiency. Infectivity was evaluated using two parameters; symptom development and ELISA (Enzyme-linked immunosorbent assay). Symptoms were evaluated visually, and plant extracts tested using the serological method, ELISA with commercial antiserum (Agdia Inc.). This was to determine the most effective and reliable propagative hosts for virus maintenance and transmission to lettuce.

Objective 2: Compare transmission to susceptible and limited known sources of resistant lettuce, as well as wild *Lactuca* species in order to observe differential symptom development and determine effective scoring system**..**

Studies involved inoculation of known resistant and susceptible lettuce varieties, as well as selected *Lactuca* species and germplasm resources, with TSWV and INSV. Lettuce and *Lactuca* germplasm were mechanically inoculated with INSV and TSWV from source plants from which reliable and consistent infection of lettuce was demonstrated in Objective 1. This was to determine performance of each host plant as a reliable inoculum source for establishing consistent infection of susceptible lettuces and differential infection among lettuce and other *Lactuca* resources. *Nicotiana benthamiana* and spinach were inoculated as a susceptible controls due to the ease at which they develop tospovirus infections.

In all experiments infection was confirmed by ELISA, using antiserum obtained from Agdia, Inc., Elkhart, IN. This antiserum is used routinely by our laboratories and is highly reliable for differentiation of INSV and TSWV..

Objective 3: Repeat Objective 2 using thrips vectors (*Frankliniella occidentalis*) for transmission of INSV and TSWV**..**

This objective was essentially a contingency plan. Based on literature and discussions with colleagues we remain hopeful that mechanical transmission can be reliable for inoculation of lettuce germplasm screenings. Because we have not been able to optimize mechanical transmission early, we began examining transmission using thrips populations. We prefer mechanical transmission as this will pose a lower risk of thrips oubreaks in other parts of the breeding program, and will be easier to perform for both public and private sector breeding programs than would development of a thrips-based transmission program, but it was necessary to begin evaluations using thrips based transmission.

Objective 4: Identify sources of resistance to tospoviruses in field conditions.

Testing lettuce for resistance to tospoviruses in field conditions is challenging due to irregular infection pressure. Therefore we performed field trials in the Huron area of the San Joaquin

Valley that regularly has very high disease pressure, as well as locations in the Salinas Valley. *Lactuca* accessions were planted for resistance evaluation in a replicated randomized complete block design. This experimental design allows replicated testing of all accessions in different areas of the field, thus minimizing the effect of uneven infection pressure. Unfortunately none of the field plots developed sufficient levels of infection for viable analysis during the 2013 growing season even though they were planted in areas known for previous virus outbreaks.

RESULTS AND DISCUSSION

Experiments were conducted by Drs. Simko and Wintermantel to determine the efficacy and availability of resistance within *Lactuca sativa* to both INSV and TSWV. Research to date has focused on separate screenings of broad-based germplasm with lettuce isolates of each virus. Initial experiments have used mechanical inoculation of these viruses, since transmission by thrips is labor intensive due to vector propagation, and can potentially result in dispersal of the virus throughout a greenhouse facility by escaped virus-carrying thrips vectors. Initial experiments involved propagation of each virus in either lettuce or the wild tobacco relatives, *Nicotiana benthamiana* or *N. clevelandii*, followed by transmission to lettuce seedlings. This did not result in efficient tospovirus infection of lettuce even though source plants were infected. Tospoviruses such as INSV and TSWV replicate in their thrips vector, as well as in host plants. Without passage through the thrips vector, infectivity is usually lost within approximately 2 or 3 plant passages (sequential inoculations).

In order to identify an appropriate host in which to propagate these viruses, we examined transmission of tospoviruses to lettuce from different host plants for each virus. This involved propagating INSV and TSWV in different host plants, and determining the amount of virus present using ELISA with virus-specific antiserum (Agdia Inc., Elkhart, IN) in order to identify the host plant with the highest virus titer. Results demonstrated *Datura stramonium* and *Nicotiana clevelandii* are both far superior as sources for transmission than lettuce and other host plants, and thrips will propagate on *Datura*, which is important for maintenance of tosposviruses because the viruses replicate in the insect itself and must be acquired during the larval stage. Although we are interested in both INSV and TSWV resistance, initial analyses focused on INSV, as the source of this virus was more easily increased due to an abundance of the virus in the local area, and therefore was used as a model for testing the system. Among several hosts studied for transmission of INSV, lettuce had the lowest mean titer among three experiments with an O.D. of 1.644 (**Table 1**). In contrast, *Datura stramonium* had the highest mean titer at 2.963. Other hosts, including *Nicotiana* species also had higher titers than lettuce, but *Nicotiana* species are not ideal if the host is to be maintained in the presence of thrips, as the vector will not propagate well on *Nicotiana*.

Datura stramonium, spinach, and lettuce were each used as host plants for inoculation of lettuce and other test hosts using standard methods for mechanical inoculation of plant viruses. Results provided infection of most control plants (*C. quinoa, N. benthamiana, N. clevelandii,* spinach), but resulted in low rates of lettuce infection with all three types of source plants. This demonstrated that simply choosing a high titer host for virus propagation and as a source of

inoculum for transmission to lettuce is insufficient to increase transmission to and infectivity on lettuce.

Table 1. Mean INSV titer among several host plants determined by ELISA analysis with INSV-specific antiserum.

Host Plant	**O.D. (405 nm)**
Nicotiana clevelandii	2.135
Nicotiana benthamiana.	1.952
Nicotiana glutinosa	2.019[b]
Datura stramononium.	2.963
Lettuce (*Lactuca sativa*)	1.644
Spinach (*Spinacea oleracea*)	2.487
Chenopodium quinoa	1.924
Chenopodium murale	2.245 [b]
Tomato (*Solanum lycopericum*)	2.428 [b]

It seemed possible part of our difficulty might be the need to maintain the virus by regularly passaging through thrips, since the virus must replicate in both virus and vector to remain viable. Consequently, a colony of INSV infested thrips was established using a USDA research station isolate in thrips-proof cages in a greenhouse. For comparison, a separate virus source was established that was propagated by mechanical transmission (rubbing infected sap on leaves). Results demonstrated that the virus isolate that was maintained with a colony of thrips, allowing the virus to replicate in both plant and insect, resulted in much higher rates of transmission to most host plants than the virus isolate that was maintained by mechanical inoculation alone in the absence of thrips (**Table 2**). The lone exception to this was spinach, in which transmission to spinach in the absence of thrips was better than when the isolate was propagated with thrips. It is believed this is simply and unusual artifact, but further analysis may be valuable if indeed spinach infections are more efficent without the thrips vector.

Studies also examined sequential inoculation of lettuce a few days apart to determine if this would increase efficiency of tospovirus infection; however, the primary result was increased damage to inoculated leaves and no significant increases in transmission of virus to lettuce (data not shown). Therefore sequential inoculation is not recommended as plants will be overly stressed, affecting growth, and evaluation for tospovirus resistance was marginal at best. Research in progress is examining the potential for using concentrated inoculum to enhance efficiency of infection on lettuce. This is done by increasing tospovirus inoculum in large numbers of infected plants, concentrating the inoculum by centrifugation, and using this to inoculate lettuce. These studies are continuing and results are pending.

Table 2. Rates of INSV transmission to severaly common host plants comparing a virus source propagated in the presence of thrips (Thrips (+) source) and a source maintained in the absence of the thrips vector (Thrips (-) source).

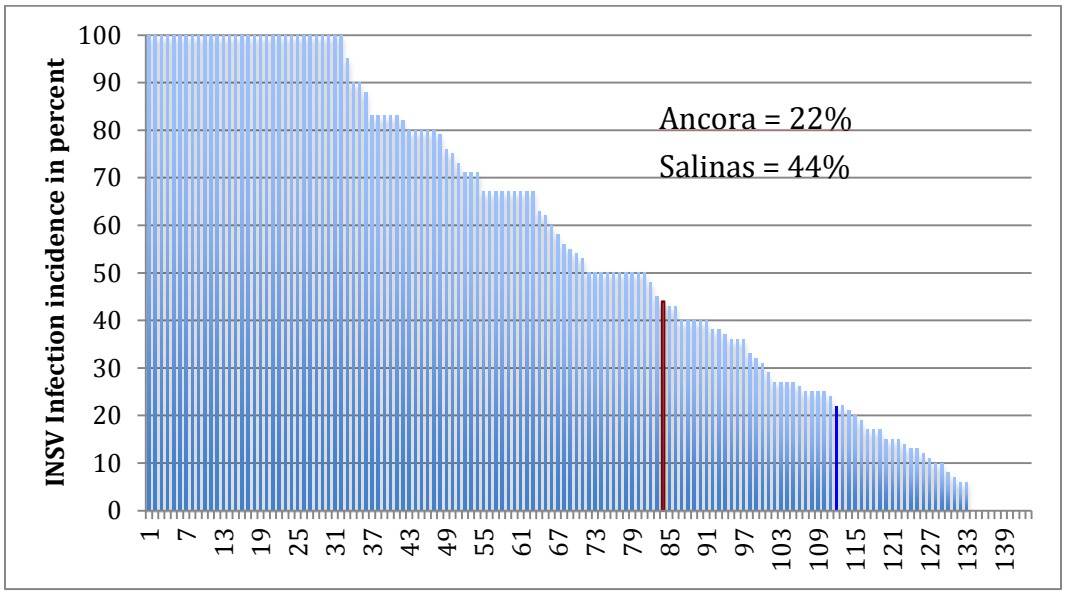
Host Plant	Thrips (+) Source	Thrips (–) Source
Lettuce	9/16 (56%)	2/8 (25%)
C. quinoa	8/8 (100%)	8/8 (0%)
N. benthamiana	13/13 (100%)	1/6 (17%)
N. clevelandii	13/15 (87%)	4/8 (50%)
Spinach	6/19 (32%)	6/10 (60%)
D. stramonium	14/16 (88%)	1/1* (100%)

Three experiments were performed in 2013 to evaluate lettuce resistance to Tospoviruses in the field conditions. Two of the experiments were carried out in the Salinas Valley (Salinas and Gonzales area) where INSV is predominant while one experiment was located in the Central Valley (Huron area) with prevalence of TSWV. The experiments were seeded on March 15 in Salinas, June 27 in Gonzales, and September 5 in Huron. The Salinas experiment was located at the USDA-ARS research station, whereas the Gonzales and Huron experiments were carried out in grower's field arranged through Steve Koike, Ed Mora, and Tom Turini. The disease pressure in all three experiments was very low to none. In Salinas we have tested 142 accessions in six replications. Only 12 plants out of 8,520 evaluated showed disease symptoms (0.14%). Laboratory testing confirmed presence of the INSV virus in symptomatic plants. The experiments in Gonzales and Huron included 44 identical accessions tested in three and two replications, respectively. In Gonzales none of the 3,276 evaluated plants showed disease symptoms. In Huron nine plants out of 2,992 evaluated (0.30%) showed disease symptoms. Laboratory tests using virus specific antiserum detected TSWV in symptomatic plants. We also tested for the presence of virus in symptomless plants that were located next to the symptomatic plants. Neither INSV nor TSWV were detected in plants without symptoms, indicating that the disease pressure in all three locations was very low. A very small number of infected plants does not allow statistical analyses of the experiments. Accessions that have had at least one infected plant in at least one of the experiments are: APP01-04A (romaine), Balady Bahera (stem type), PI 251246 (oil type), PP001-4C (romaine), two lines from a cross between cvs. Grand Rapids and Iceberg (leaf types), and four lines from a cross between cv. Salinas 88 and PI 251246 (not commercial types). These very limited results indicate that oilseed lettuce accession PI 251246 is highly susceptible to both INSV and TSWV and could be used as susceptible check in Tospovirus experiments. Extremely low to zero disease incidence was observed for INSV in 2013 in all monitored commercial fields in Salinas Valley. In the greater Fresno production area, TSWV was rather sporadic (<1%) in fall and spring planted lettuce fields (O. Batuman, N. McRoberts, and T. Turini; personal communication).

Although field evaluations had low levels of infection, greenhouse evaluations using thrips have provided effective preliminary screening of lettuce and *Lactuca* germplasm. We are still working out optimal parameters for mechanical transmission, but using thrips transmission of

INSV in the greenhouse we have begun to evaluate lettuce for performance of lettuce germplasm against INSV. Results produced a gradient of infection levels ranging from 100% of plants infected per breeding line, to very low percentages that may hold promise for resistance (**Fig. 1**). We plan to use the same approach with TSWV, although maintenance of the TSWV isolate has been more challenging. The difficulty with using thrips is that management of thrips is difficult, and there remains a significant risk that additional greenhouses could become infested with a viruliferous thrips population. Therefore this is a less than desired approach, but initial results indicate it can be an effective screening method if appropriate control measures are used. These methods will be optimized over the next year, even as we continue to explore the potential for mechanical tranmission in germlasm evaluations.

Figure 1. INSV infection of lettuce varieties and germplasm accessions through natural infestation in the greenhouse with virus-carrying thrips illustrates a gradient from 100% infection to low rates of infection[1].



[1] The standard susceptible variety Salinas (44% infection) and the putative semi-resistant variety Ancora (22% infection) are shown for perspective.

RELEVANT LITERATURE

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