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 because 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 a lesser degree. 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 host plants for each virus, and that non-lettuce host plants are better sources for virus and thrips maintenance than lettuce itself. For example, jimsonweed (*Datura stramonium*) accumulates much higher levels of INSV than lettuce (although it does not survive long), whereas tasselflower (*Emilia sonchifolia),* can maintain relatively stable infections for longer periods of time. Field evaluations had extremely low levels of infection preventing effective analysis due to inconsistency in where thrips and virus outbreaks occur from crop-to-crop; however, greenhouse evaluations using mechanical transmission supplemented by thrips that carry virus provided effective preliminary screening of lettuce and *Lactuca* germplasm in initial tests with INSV. Similar results are expected with TSWV. A proposed greenhouse evaluation strategy is provided.

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) (Fig. 1). 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 several 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. Some studies have suggested differences in virus transmission efficiency may even exist among individual populations of the thrips vector. The viruses are present throughout 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. F. occidentalis is the predominant vector in California. 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 the viruses. 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 project 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, it is necessary to either optimize transmission efficiency using mechanical transmission or focus on using thrips to deliver the virus. It is optimal to avoid using thrips for transmission due to the difficulty in keeping the insects out of other greenhouses and preventing tospovirus transmission to nontarget plants. This project was focused on methods 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.

LONG-RANGE OBJECTIVE

The ultimate objective of the project is optimization of methods for evaluation of breeding lines to identify resistance to tospoviruses.

OBJECTIVES

1. Determine optimal parameters for mechanical inoculation of lettuce with TSWV and INSV using virus isolates maintained with regular propagation in both host plants and thrips vectors.

2. Compare transmission to susceptible and limited known sources of resistant lettuce, as well as wild *Lactuca* species.

3. Identify sources of resistance to tospoviruses in field conditions.

PROCEDURES

The source of all INSV isolates used has been lettuce, whereas TSWV isolates have come from either lettuce or tomato. The isolates used as sources for inoculation of lettuce and other test plants were maintained in separate greenhouses and within thrips-proof (or at least thrips resistant) cages to reduce the risk of cross contamination by free thrips populations that are common in the region and often carry INSV. Each virus isolate was initially introduced to source plants by mechanical transmission after which it was maintained within source plants and transmitted to lettuce by one of three methods:

1. Mechanical transmission to lettuce from virus infected source plants in which virus was passaged from source plant-to-source plant in the absence of thrips.

2. Mechanical transmission to lettuce from virus infected source plants in which virus was introduced to source plants by mechanical inoculation, and maintained

by mechanical transmission to new source plants in the presence of a thrips population.

3. Mechanical transmission to lettuce from virus infected source plants in which virus was initially introduced to source plants by mechanical inoculation, but maintained exclusively by thrips transmission to new source plants.

Mechanical transmission to lettuce was performed when lettuce seedlings were showing two to four true leaves. Source plant tissue was collected and tested for the presence of the correct tospovirus using immunostrips. Each sample was tested for both INSV and TSWV to assure that inoculum was derived from a source plant infected with the appropriate virus. Control plants of each propagation host (spinach, pepper, jimsonweed, tasselflower, and lettuce), as well as *N. benthamiana* were inoculated in each experiment to confirm transmission efficiency. Infectivity was evaluated using two parameters; symptom development based on visual observation of typical foliar necrosis symptoms characteristic of infection by INSV and TSWV (Figure 1) and ELISA (Enzyme-linked immunosorbent assay) and/or immunostrip detection using commercial antiserum, and in some cases immunostrips (Agdia Inc.).

A field experiment was conducted at Spence Field at the USDA-ARS in Salinas involving 159 accessions, set up using standard parameters. Virus transmission was conducted using natural thrips populations and natural infection from wild sources in order to minimize the risk to surrounding experimental fields.

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. Although this project is ending, we will continue the work with funding through an additional grant through the California Specialty Crop Block Grant Program. The research herein provided preparatory information that was helpful in obtaining the additional funding. Initial experiments used mechanical inoculation of the two tospoviruses, 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 during the first year of the project involved propagation of each virus in either lettuce or the wild tobacco relatives, *Nicotiana benthamiana* or *N. clevelandii*, followed by transmission to lettuce seedlings using mechanical transmission (infecting plants by rubbing leaves with sap of virus infected plants). This did not result in efficient tospovirus infection of lettuce even though some alternate host plants were consistently infected. Follow-up studies examined the efficiency with which INSV and TSWV can be maintained in a number of host plants through mechanical transmission and how effective those host plants are as sources for mechanical transmission of INSV and TSWV to

lettuce. Studies by colleagues previously demonstrated the TSWV can be maintained on pepper (*Capsicum annuum*) and tasselflower (*Emilia sonchifolia*), as well as jimsonweed (*Datura stramonium*); however, very few studies had been performed with INSV, which is the most common tospovirus found in coastal production regions. Therefore we focused our efforts for development of inoculation methods on this virus. It was important to identify effective propagative hosts for INSV. In addition to examining plants for infection, a secondary aspect of those studies involved evaluation of virus accumulation in the different host plants used for virus propagation because studies on other viruses have shown this to be an important factor in efficiency of plants serving as sources for virus transmission. Through those experiments, it was demonstrated that neither INSV nor TSWV transmits very effectively with serial passaging using mechanical transmission regardless of source plant. In fact, once either virus is no longer exposed to the thrips vector, efficiency of serial mechanical transmission drops off very quickly, with most mechanical transmissions failing to produce infected plants after only two sequential transmissions following removal from the thrips source (data not shown). Interestingly, this is generally true regardless of virus titer in host plants. In studies completed during the previous year evaluating the relationship between virus titer and transmission of INSV from several hosts, lettuce had the lowest mean titer among three experiments with an O.D. of 1.644 (Table 1). In contrast, jimsonweed (*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* due to its elevated nicotine content. Additional studies demonstrated that INSV can be reliably maintained on spinach (*Spinacea oleracea*) for multiple passages and accumulates relatively high levels of virus (Table 1); however, virus infectivity on lettuce following transmission from spinach declines rapidly. What this means is that even though INSV can be transmitted to spinach quite readily by mechanical inoculation, similar transmission to lettuce is ineffective from the same source. This is largely true with other host plants as well. *Datura stramonium* (jimsonweed) accumulates INSV quite well, but when transmissions are performed from this virus source in the absence of thrips, mechanical transmission to lettuce is also quite low. 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 rates to and infectivity on lettuce.

It should be noted that in all of the studies discussed above, the INSV source was maintained by mechanical transmission, and was no more than two generations removed from a thrips source. This may have impacted the results, because later studies have shown that if the virus isolate used for transmission has not recently replicated in the thrips vector, transmission can be greatly diminished (details follow). Performance of these alternate host plants might improve considerably if the source of the virus were passaged by thrips rather than mechanical inoculation.

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

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]

[1] This data was presented in the 2013 report, but is included here as it is relevant to the present year's study.

Tospoviruses such as INSV and TSWV replicate in their thrips vector, as well as in host plants. Without passage through the thrips vector, infectivity was usually lost within approximately 2 or 3 plant passages (sequential inoculations). 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). It should be noted that direct comparisons of visual scoring compared with ELISA demonstrated 86% consistency between the two scoring methods. Interestingly, when ELISA results did not match visual scoring this was because visual score was positive, but ELISA was negative. Regardless the visual scoring method is relatively accurate.

The lone exception to the need for exposure to thrips for consistent serial mechanical transmission was spinach, in which transmission to spinach in the absence of thrips was better than when the isolate was propagated with thrips. Originally this was believed to be an unusual artifact, but the consistency with which we have been able to perform serial mechanical transmissions from spinach-to-spinach-to-spinach in routine propagation studies suggests the virus requirements for replication and accumulation in spinach are minimally impacted by the whatever components of the virus particle may be contributed through replication in thrips. Although to date we have not heard of spinach experiencing losses due to tospovirus infections, if INSV or even TSWV infections become problematic in spinach, these studies have shown that mechanical transmission

experiments may be ideally suited for identification of virus resistance sources in spinach.

Table 2. Rates of INSV transmission to several 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%)

During the past year we began propagating TSWV and INSV separately, in the presence of separate thrips populations. This has greatly improved performance of mechanical inoculations, most likely because the virus isolates can replicate in the thrips as well as in the lettuce and other source plants, maintaining all aspects necessary for continued infectivity of each virus. Due to the prevalence of INSV in the Salinas Valley it has been relatively easy to maintain fresh sources of this virus for new experiments. When problems have developed with some of our isolates due to excessive mechanical tranmission, it has not been difficult to find a replacement source. In contrast, TSWV is far less common in the Salinas Valley, and obtaining replacement isolates for TSWV is much more difficult.

The decline of TSWV transmission efficiency due to frequent mechanical transmission was one of the factors that led us to the conclusion that steady exposure to thrips was required for effective transmission. When we initally began working with thrips the approach was to mechanically transmit the virus within virus propagation cages where we housed a population of thrips (transmission method #2 described above). We anticipated this would improve transmission of tospoviruses; however, we still observed low levels of transmission to lettuce during mechanical transmission from the TSWV source propagated in the presence of thrips. Two different experiments with inoculation of susceptible lettuce from this source yielded 9/32 (28%) and 8/50 (16%) infection of lettuce. Over the course of several experiments there was a steady decline in TSWV mechanical transmission rates even using the source exposed to the thrips population. The result was that we nearly lost infectivity of our TSWV isolate. A new isolate is now in use. The decline was not nearly as apparent with INSV, most likely because there was a large influx of INSV from external sources in most USDA greenhouses this spring. Similar experiments with INSV resulted in 31/48 (65%) and 23/38 (61%) transmission. This was most likely due to the prevalence of viruliferous thrips carrying INSV in the experiments. The presence of a thrips population likely contributed to virus spread among plants that might not have happened with simple inoculation. Two subsequent experients

in which very few thrips were present in the greenhouse where the tests were conducted had very low levels of transmission, confirming the important role of thrips for plant-to-plant spread.

In spite of the improvements, consistent infection is still best with live thrips. Consequently we are now using an approach involving propagation of virus and thrips together in isolation greenhouses, but also adding thrips to plants following mechanical transmission in order to enhance uniformity of infections. This method should optimize infectivity and allow larger scale evaluation of resistance in lettuce; the goal of this project. A proposed method based on our experience through this project is listed below. Please contact Dr. Wintermantel (bill.wintermantel@ars.usda.gov) for further details as needed.

Source plants for virus propagation:

Tasselflower, (*Emilia sonchifolia*) appears to be a valuable host plant for propagation of TSWV and INSV. Leaves of this plant, once infected, can be frozen at -80 C and used as a fresh inoculation source. Additionally, tasselflower can survive with INSV or TSWV infection much longer than most host plants. *D. stramonium* is also a good host plant for virus propagation and transmission, but plants do not survive as long once infected compared with tasselflower plants.

Standard propagation method for evaluation of tospovirus resistance in lettuce:

1. TSWV and INSV are each propagated separately on host plants (lettuce, tasselflower, or jimsonweed) in thrips-proof mesh cages in the presence of an active thrips colony. A "back-up" source of each should be stored at -80 C.
2. After initial transmission, all propagation of TSWV and INSV source plants are performed by natural infestation with viruliferous thrips. This is done by adding new plants to the cage and removing old plants on a weekly basis.
3. Every two weeks, and prior to use for inoculation, all source plants should be evaluated for infection of both INSV and TSWV to assure that only the desired tospovirus is present. This can be performed with ELISA, immunostrips, or RT-PCR.
4. Seedling lettuce plants at the two to four true leaf growth stage are inoculated mechanically by rub inoculation. (details available upon request).
5. Sources of viruliferous thrips carrying the desired virus should be placed strategically among test plants to facilitate virus spread throughout the greenhouses (Fig. 1).
6. After two weeks, test plants should be sprayed with a thripicide or treated with a systemic insecticide to prevent buildup of excessive numbers of thrips. NOTE: The use of an active thrips colony is still under study, and we cannot guarantee these methods will be effective to prevent accidental transmission to non-target experients due to the ease at which thrips move.

Figure 1. Strategically place a thrips/virus source plant among test plants following mechanical inculation of test plants. This will facilitate further transmission of virus within experiments.