

PROJECT TITLE: Development of a method for conducting tests for resistance to tobusviruses and lettuce dieback in the greenhouse.

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ABSTRACT: Lettuce dieback causes necrosis, stunting and death of lettuce plants throughout all western lettuce production regions in California and Arizona, and is caused by either of two tobusviruses, *Tomato bushy stunt virus* (TBSV) and the closely related and *Lettuce necrotic stunt virus* (LNSV). Symptoms include yellowing, necrosis, stunting and death of affected plants. Losses from this disease can range from a few plants to complete loss of crop. The movement and stability of the virus in irrigation water, along with its soil-borne nature, causes this disease to persist in soils for many years. Due to the significant economic threat lettuce dieback poses to the industry, it is important that the industry have the ability to screen plant material for resistance to TBSV and LNSV, the latter of which is now reclassified as *Moroccan pepper virus* (discussed in report). While field testing for resistance does occur, it is not always possible to obtain fields with a substantially broad array of virus isolates that can cause disease, and there are advantages to a greenhouse or controlled environment resistance testing program. Our goal with this proposal is to develop a cost effective and reliable method to test lettuce and *Lactuca* germplasm resources for resistance to lettuce dieback under greenhouse or controlled environment conditions. During the first year of this project we demonstrated effective differentiation of resistant and susceptible reactions using these methods through replicated experiments under what we believed were specific, known conditions. Both resistant and susceptible plants developed lesions on the inoculated leaves, but only susceptible plants became infected systemically when held at 24 hour light and high temperature (29°C). However, following these highly successful early results (three replicates) we began having difficulty obtaining sufficient numbers of systemic infections. We have not been able to completely restore the high levels of infection we achieved in early experiments, and believe there were additional, yet to be determined environmental factors that are influencing tobusvirus infection of lettuce. To date we have confirmed that the plant stress-inducing conditions of longer day length and higher temperatures both influence infection, but these are clearly not the only factors necessary. Studies have also examined the role of soil salinity and soil moisture in restoring the high rates of transmission achieved in early experiments. At present we are still observing relatively low rates of infection compared to early studies, indicating further clarification of environmental effects on infection is necessary. In order to optimize infection and avoid emergence of defective interfering RNAs (incomplete viral nucleic acids that affect replication and other virus functions that can occur during tobusvirus infections), we are using cloned versions of TBSV and LNSV to inoculate source plants; however, this approach alone was not sufficient to restore high rates of infection seen in early tests. Although we have learned a great deal in the course of these studies on environmental factors influencing disease development, we have not yet achieved consistent, reliable rates of infection. It is clear this project will require considerable in depth study that will likely take some time; research will continue.

BACKGROUND AND PREVIOUS ACCOMPLISHMENTS:

Lettuce dieback causes necrosis, stunting and death of lettuce plants throughout all western lettuce production regions in California and Arizona. The disease is particularly important to the lettuce industry because of its impact on all non-crisphead types of lettuce, which account for approximately 60% of all lettuce production in Monterey County. Two tombusviruses, *Tomato bushy stunt virus* (TBSV) and the closely related and newly described *Lettuce necrotic stunt virus* (LNSV), have been proven to cause this necrosis-inducing disease. Symptoms include yellowing, necrosis, stunting and dieback of affected plants. Losses from this disease can range from a few plants to complete loss of crop. Symptoms of the disease are frequently found in low lying areas with poor drainage, in areas near rivers, on recently flooded land, and in areas where soil has been dredged from a river or ditch and spread onto adjacent fields. Earlier studies of a disease causing necrosis of tomato showed that TBSV was responsible for that disease as well, and the virus originated in irrigation water from the Colorado River. TBSV and LNSV have been documented to survive in water and soil for long periods of time (Koenig and Lesemann, 1985). Studies conducted at the USDA in Salinas demonstrated that LNSV and TBSV virions suspended in water, remained infectious even after being stored for two months on a lab bench (Wintermantel and Anchieta, 2012). This is indicative of a highly stable and durable virus particle. Lettuce dieback has been a chronic problem for many growers, and some cannot plant non-crisphead lettuces in their fields due to the severity of the problem. The movement and stability of the virus in irrigation water, along with its soil-borne nature, poses a real threat of increased incidence of this disease through movement to additional areas and long-term persistence in the soil. This threat is borne out by recent developments. Both the Wintermantel lab (USDA, Salinas) and the UC extension diagnostic lab in Salinas (Koike) have been monitoring lettuce dieback occurrence. Over the past several seasons, growers continue to report problems with lettuce dieback. In addition, the labs confirmed that new, previously uninfested fields are becoming affected by this soilborne virus. Infested lettuce plants have also been obtained with some regularity from San Luis Obispo County, Santa Barbara County, and southern desert regions.

Previously, the USDA-ARS virology lab demonstrated that tombusviruses were responsible for what was originally known as “river disease,” now known as “lettuce dieback,” and that the disease could be caused by either TBSV or a closely related and previously undescribed tombusvirus called LNSV (Obermeier et al., 2001). The USDA-ARS lettuce genetics group also identified a dominant resistance gene from ‘Salinas’ lettuce (Grube et al., 2005). The gene, *Tvr1*, was mapped in an intraspecific *L. sativa* population to a location that corresponds to linkage group 2 on the consensus map of *Lactuca*. The largest cluster of resistance genes in lettuce, the *Dm1/Dm3* cluster, is found on this linkage group. Continuing efforts by Ivan Simko at the USDA-ARS in Salinas and collaborators from UC Davis and USDA-ARS in Stoneville, MS led to mapping of the precise position of *Tvr1* relative to this cluster (Simko et al., 2009; 2010). *Tvr1* was the first tombusvirus resistance gene identified for any plant host. The team of collaborators also developed molecular markers that are used for marker-assisted selection for resistance to dieback in lettuce-breeding programs in the USA and Europe. Variety trials continue to be conducted in fields infested with lettuce dieback in order to advance sources of resistance.

Due to the significant economic threat lettuce dieback poses to the industry, it is critical that the industry has the ability to screen plant material for resistance to the viruses responsible for lettuce dieback, TBSV and LNSV. While field testing does occur, it is not always possible to obtain fields with a substantially broad array of virus isolates that can cause disease. Consequently USDA-ARS virology and genetics labs in Salinas are developing a cost effective and reliable greenhouse and/or growth chamber method to test lettuce and *Lactuca* germplasm resources for resistance to lettuce dieback under greenhouse conditions.

The USDA-ARS Virology lab has developed a number of methods for detection of viruses causing lettuce dieback. Diagnostic assays developed include RT-PCR, immunocapture-RT-PCR, Enzyme-linked immunosorbent assay (ELISA), western blot analyses, and immunospecific electron microscopy. For several years there were difficulties with test reliability in some cases due to variability of tombusvirus isolates from the field. Although antisera have been developed to the virus coat protein (CP), the protein that covers the outer shell of the virus particle, for both TBSV and LNSV, these are very specific to the type of protein from which they originate, since the coat protein gene is the most variable region of the tombusvirus genome among species within the genus, *Tombusvirus*. TBSV antiserum will not detect LNSV and *vice versa*. Similarly, although most detection methods for tombusviruses have focused on the P19 gene located near the “end” of the virus genome for general detection of tombusviruses, we have found that molecular detection methods focused on this region are not always reliable. They work well much of the time, but not all of the time, leading to recent efforts by our lab to develop a new, more efficient method. Associated with the early stages of this project, we developed new primers to a region near the “front” of the virus genome, within the gene encoding the P33 virus replication protein. These primers reliably detect both TBSV and LNSV isolates, and based on our results, are specific to tombusviruses causing lettuce dieback. We have also developed PCR primers to the gene encoding the virus coat protein, a region that is genetically diverse among lettuce dieback-causing tombusviruses. These primers have worked well over the past three years and consistently differentiate LNSV and TBSV isolates from both field and laboratory.

OBJECTIVES:

Long Range Objective: Develop a more cost-effective and reliable method for greenhouse-based testing for tombusvirus (lettuce dieback disease) resistance in lettuce.

Specific Objectives for 2012-2013:

Test new methods for rapid greenhouse pre-screening of lettuce cultivars/lines for tombusvirus resistance.

1. Complete analysis of specific parameters for day-length and temperature based induction of lettuce dieback symptoms and differentiation of resistant and susceptible lettuce.
2. Develop and “field-test” methodology for induction of lettuce dieback symptoms in growth chambers for release to industry.

PROCEDURES:

Objective 1. Complete analysis of specific parameters for day-length and temperature based induction of lettuce dieback symptoms and differentiation of resistant and susceptible lettuce.

Experiments were conducted in growth chambers that allow precise control of lighting and temperature, to clarify which environmental factors allow full systemic infection (whole plant infected) of susceptible lettuce plants with tombusviruses. Prior to this year we knew that 24 hour day length and high temperatures of 30°C facilitated infection, whereas 16 hour day length and 22°C did not. However, we had also determined over several experiments that additional factors contributed to development of the systemic symptoms, and were still working to clarify what those factors might be. Full light for 24 hours is not a 'normal' situation. Therefore one aspect of these studies was to ascertain whether slightly shorter day length or lower temperatures will work or if the physiological effects of no darkness period contributes to the ability of the virus to infect and produce symptoms. Recent studies over the course of this project demonstrated that the development of lettuce dieback symptoms is very sensitive to environmental variables, and it is important to standardize lighting, temperature, and day length, for testing purposes, which can only reliably be managed in a growth chamber.

Experiments were conducted to optimize infection of susceptible lettuce cultivars and differentiate these from resistant cultivars. To date our target approach has been focused on systemic infection and induction of lettuce dieback symptoms in susceptible, but not resistant plants. Although we currently have methods that can lead to 50 to 80 percent infection of a susceptible variety, some variables that have not yet been fully characterized sometimes cause infection levels to be much lower. Low infection rates for susceptible controls is not acceptable for a resistance test. Studies focused on treatment combinations temperatures varying from 21 to 30°C, 16 to 24 hour day length, field and greenhouse soils, and varying soil salinity levels in order to optimize methods for systemic infection of susceptible cultivars and to differentiate them from resistant materials.

Studies during the 2011-2012 year indicated that local lesions on the inoculated leaves of resistant plants were generally larger than those on susceptible varieties when exposed to certain conditions in growth chambers. New studies explored the potential for use of the local lesion response to inoculation as a screen for resistance using varying day length and temperature. If reliable, such a screen would lead to the ability to differentiate resistant vs. susceptible lettuce as early as one week post-inoculation. Methods for this were performed in parallel with studies on systemic infection in growth chambers.

Objective 2. Develop and "field-test" methodology for induction of lettuce dieback symptoms in growth chambers for release to industry.

Once parameters for differentiating resistant and susceptible lettuces has been optimized, we will develop and present such a method to the industry both in the form of a peer-reviewed publication and as a laboratory protocol that we will make available to the industry upon request. Objective 2 requires clarification of factors influencing infection addressed in Objective 1.

Following completion of Objective 1, we will “field-test” the method using a range of cultivars of known tombusvirus resistance to confirm effectiveness, followed by optimization as needed. This approach is necessary to assure that our methods are broadly effective for evaluation of a wide range of lettuce and *Lactuca* germplasm.

RESULTS AND DISCUSSION

Through this project we demonstrated successful infection of lettuce in a growth-chamber based assay and demonstrated successful infection of susceptible lettuce varieties in three separate experiments. Furthermore, initial studies indicated the resistant variety, Sturgis, did not develop systemic infection using these conditions, whereas the susceptible variety, Darkland, was infected. Both resistant and susceptible varieties develop local lesions on the inoculated leaves, which differ somewhat in appearance depending on resistance status of the plant, as well as growth conditions.

Brief synopsis of successful experimental setup:

- All inoculated plants were romaine type cultivar Darkland, inoculated by rubbing leaves separately with TBSV- or LNSV-infected sap from *N. benthamiana* suspended in 100 mM sodium phosphate buffer.
- Growing conditions were 29-30°C, constant light, and light intensity of 75-105 μ Einsteins per m^{-2} per s^{-1} . Higher light levels up to 300 μ E per m^{-2} per s^{-1} were successful in later experiments as this reduced excessive elongation of lettuce plants.
- Both field soil and greenhouse potting mix were effective for symptom induction
- First symptoms appeared about 10-14 days after inoculation.
- Control plants were inoculated with buffer only.
- Viral RNA was isolated from inoculated plants and infections were confirmed by RT-PCR using primers specific to the coat protein region of each virus.

Direct foliar inoculation of the susceptible romaine variety, Darkland, in growth chambers with 24-hour light and 30°C in potting mix resulted in over 80% infection with TBSV and approximately 50% infection with LNSV, with both viruses producing clear systemic symptoms of lettuce dieback (Fig. 1A). RT-PCR analysis confirmed infection with the respective viruses, as described in the 2011 report. The experiment was subsequently repeated, with the additional steps of scoring infected plants for disease severity. In the second experiment plants were grown in three types of soil to determine if soil-type in which plants were grown influenced ability of plants to become infected. Results demonstrated that susceptible lettuce plants grown in soil containing either LNSV or TBSV under 24-hour light and 30°C resulted in high numbers of infected plants, and disease severity comparable to that observed in field evaluations (Fig. 1B & C). Soil type did not appear to be a significant factor in determining development of systemic symptoms. Cumulative numbers for Experiment 2 demonstrated 100% infection with TBSV and 70% infection with LNSV. These experiments clearly demonstrated the value of the new method to induce field-like symptoms of lettuce dieback in susceptible lettuce, and that there were no significant differences based on soil in which plants are grown.

Figure 1.



A. Romaine lettuce plants grown with 24 hour day length and 30°C for 2 weeks following inoculation with LNSV or TBSV, exhibiting classic stunting and necrosis characteristic of lettuce dieback disease.



B. TBSV infection



C. LNSV infection.

Following confirmation that infection and disease development can be obtained with high temperature, long day conditions, we began studies to determine if the same method could be used to differentiate between resistant (*Tvr1*) and susceptible (*tvr1*) lettuce, as well as to dissect the specific conditions (light and/or temperature) that lead to disease development in order to fully understand the physiological factors driving disease development. Darkland was used as the susceptible variety for all experiments. Sturgis and Bandit were used as the main resistant varieties.

Plants of both resistant and susceptible varieties developed local lesions on the inoculated leaf in replicated experiments at cumulative rates of near 90% each among all experiments; however, only plants of the susceptible variety developed systemic symptoms (Fig. 2). Interestingly we also observed differences in the type and size of lesions between resistant and susceptible lettuce varieties (Fig. 3), with further variation depending on day length and temperature (data not

shown). Although this may make using lesions to differentiate resistant and susceptible plants challenging, we are exploring this as a possibility, evaluating lesions with different treatment conditions. As described later in this report, however, TBSV and LNSV produce different types of lesions on inoculated lettuce plants. Therefore any evaluations based on lesion size or other physical traits would need to examine the reaction to each virus separately.

Figure 2. Symptoms on susceptible and resistant lettuce when inoculated and maintained at 29°C with 24 hour day length. **A.** Some plants of susceptible lettuce (Darkland) showing systemic symptoms of lettuce dieback. Four uninoculated plants are shown in front of 20 inoculated plants (rear). **B.** All plants of resistant lettuce (Sturgis) remained healthy. Yellowed leaves were those initially inoculated. All new growth remained healthy. Four uninoculated plants are shown in front of 20 inoculated plants (rear).

A.



B.



Although lesions on inoculated leaves of resistant plants are often larger and more distinct than those on susceptible plants, the resistant plants did not develop systemic symptoms of stunting and necrosis in experiments to date. Further testing will be needed, however, as only one successful experiment was completed that yielded both high rates of infection on susceptible plants and no infection of resistant plants. Other experiments involving resistant plants did not provide conclusive results due to low or no infection of susceptible controls as described below. Without significant numbers of infected susceptible control plants in subsequent experiments it was not possible to conclude lack of infection in resistant varieties results from effectiveness of resistance, or if it simply is the result of conditions that are not conducive to development of systemic symptoms.

Figure 3. Differences in lesion morphology on leaves of susceptible (left) and resistant (right) lettuce inoculated with LNSV. Lesions on susceptible leaves were usually smaller and more diffuse, whereas those on resistant varieties generally exhibited more distinct borders. Further testing will be necessary as there is some variation in lesion response based on variety and environmental factors.



After the three very successful initial experiments we suddenly ran into difficulty obtaining systemic infection of susceptible lettuce in mid-summer 2011 while trying to evaluate effectiveness of the method for screening resistant material. Although no obvious changes occurred in our evaluation system (lighting periods and temperatures remained the same as in previous experiments), we were unable to establish systemic infection of Darkland or other susceptible lettuce varieties. Without successful infection of susceptible lettuce, evaluation of resistance was not possible. Consequently, we began a series of studies to determine what was responsible for the sudden loss of infectivity under long day, high temperature conditions. Light intensity was varied from slightly below $100 \mu\text{Einsteins per m}^{-2} \text{ per s}^{-1}$ to above $300 \mu\text{Einsteins per m}^{-2} \text{ per s}^{-1}$, keeping day length constant. Multiple varieties of susceptible lettuce were used, and virus inoculum was concentrated prior to inoculation, all in an attempt to identify the missing variable. There is clearly more to tombusvirus infectivity during mechanical inoculation of lettuce than simply exposing plants to 30C and 24 hour light; otherwise we would not have had the decline in performance. Although we have achieved systemic infection of susceptible lettuce in additional experiments, rates are still far too low (approximately 5 percent), compared with studies during 2011 when we obtained infection in replicated experiments for each virus in the range of 40 to 82 percent systemic infection.

In the course of our resistance evaluations we became concerned about inability to continue to obtain the high rates of infectivity achieved in our early studies. One possible explanation for this was that tombusviruses are known to generate defective interfering RNAs (DI RNAs) in some host plants that can reduce symptom severity. These are incomplete viral nucleic acids. The virus genome is made of RNA, a type of nucleic acid, and the DI RNAs are shorter than full-length pieces of virus RNA that affect replication of the virus and other virus functions. DI RNAs can emerge within a few days after infection on many host plants, and greatly reduce

disease severity. We suspected this might have reduced effectiveness of our lettuce resistance evaluation based on loss of severity following sequential propagation of the viruses in *Nicotiana benthamiana*. To eliminate this as an issue in our lettuce inoculations, we now use cloned versions of TBSV and LNSV to inoculate source plants. The LNSV clone was developed in our lab associated with the sequencing of LNSV, described below (Wintermantel and Hladky, 2013); whereas the TBSV clone (clone of an isolate known to infect lettuce) was a gift from Dr. Herman Scholthof of Texas A&M. Although using virus generated from a cloned source of RNA alone was not sufficient to restore high rates of infection seen in early tests, we continue to use cloned sources as inoculum for tests in order to eliminate potential concerns with DI RNAs.

Cloning and Sequencing the Genome of LNSV and importance for lettuce resistance evaluations (LNSV is now re-named as MPV)

A separate but related project in the Wintermantel Lab sequenced the genome of a LNSV isolate from the Salinas Valley and found it to have an organization typical of the genus, *Tombusvirus*, as expected (Wintermantel and Anchieta, 2012). Much of the genome was most closely related to TBSV; however, the coat protein was nearly identical to that of *Moroccan pepper virus* (MPV), a poorly characterized tombusvirus from Mediterranean regions. Subsequent studies allowed us to sequence the genomes of three MPV isolates as well (generously provided by Dr. H.J. Vetten, Braunschweig, Germany), which confirmed LNSV and MPV are actually the same virus, sharing 97% genomic sequence identity (Wintermantel and Hladky, 2013). Collectively these results demonstrated that LNSV should be classified as MPV (which was known by the virology community since the late 1970s) within the family *Tombusviridae*, genus *Tombusvirus*, and confirms the presence of MPV in North America. Symptoms of LNSV matched those described previously for MPV on a select series of host plants, and showed clear differences in symptoms on some hosts between LNSV and TBSV (Wintermantel and Hladky, 2013); a result that had not been clearly demonstrated in previous studies on MPV. Interestingly, LNSV (MPV) and TBSV produced very definitive types of lesions when inoculated to lettuce. When seedling lettuce plants were rub-inoculated with each virus and maintained under 16 hour days and 23°C, LNSV(MPV) produced chlorotic local lesions on inoculated leaves of susceptible lettuce plants in less than a week, whereas TBSV produced necrotic lesions (**Fig. 4**).

To date we have confirmed that the plant stress-inducing conditions of longer day length and higher temperatures both influence infection, but these are clearly not the only factors necessary. Studies in the past year have also examined the role of soil salinity and soil moisture in restoring the high rates of transmission achieved in early experiments. At present we are still observing relatively low rates of infection compared to early studies, indicating further clarification of environmental effects on infection are necessary. Studies will continue to examine the potential for salinity and/or soil moisture in lettuce dieback symptom induction.

Although we have learned a great deal in the course of these studies on environmental factors influencing disease development, we have not yet achieved consistent, reliable rates of infection. Efforts toward development of a resistance assay using controlled environments are continuing, focusing on various combinations of high temperature vs. moderate temperature, long and moderate day length (photoperiod) conditions, and elevated soil salinity and soil moisture, in attempts to clarify what conditions led to the successful results early in these studies. Until we

can establish reliable parameters to provide significant rates of infection, controlled environment or greenhouse evaluations will not provide the desired benefit to the seed industry. It is clear this project will require considerable in depth study that will likely take some time. We have not requested additional funds for these efforts, but will continue to work toward the goal of achieving a reliable and effective assay for resistance to tombusvirus infection and lettuce dieback disease.

Figure 4. Susceptible seedling lettuce plants showing differential symptoms inoculated with RNA of LNSV (MPV) and TBSV, and maintained in a growth chamber with 16 hour days and 23°C. LNSV (MPV) produces chlorotic lesions under these conditions (A), whereas TBSV produces necrotic lesions (B).

A. LNSV (MPV)

B. TBSV



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