I. Abstract.

Project Title: Development of a method for conducting tests for resistance to tombusviruses and lettuce dieback in the greenhouse.

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Summary: Lettuce dieback causes necrosis, stunting and death of lettuce plants throughout all western lettuce production regions in California and Arizona. Two tombusviruses, *Tomato bushy stunt virus* (TBSV) and the closely related *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. 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, causes this disease to persist in soils for many years. Due to the significant economic threat lettuce dieback poses to the industry, it is critical that the industry have 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, and there are advantages to a greenhouse resistance testing program. A method for greenhouse induction of lettuce dieback symptoms was developed several years ago by the Wintermantel lab, but the original method was exceptionally labor-intensive and time consuming, and was not cost effective for routine screening. 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 conditions. This project was initiated during the summer of 2010, and has made significant progress toward the goal of a reliable greenhouse testing method. Previous studies demonstrated that the viruses responsible for lettuce dieback produce different symptoms when lettuce is infected through the soil than when the lettuce is inoculated by rubbing leaves with plant sap. Although root infection results in lettuce dieback symptoms, manually rubbing leaves with virus infected sap at ambient temperature and standard lighting conditions has been shown to produce necrotic local lesions on the inoculated leaf, but no systemic infection (infection of entire plant). Recent studies have shown that rub-inoculation of plants maintained at high temperature and 24 hour day length resulted in full systemic infection of many inoculated plants, resembling lettuce dieback symptoms from the field. However, not all experiments have such high success rates. Current studies are attempting to clarify whether day length or temperature is the primary determinant for the ability of the viruses to infect systemically, and whether these conditions will facilitate reliable selection of resistant materials.

II. Main Body of Report

Project Title: Development of a method for conducting tests for resistance to tombusviruses and lettuce dieback in the greenhouse.

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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. 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. 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 two or three 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. Infected lettuce plants have also been obtained annually from San Luis Obispo County, Santa Barbara County, and southern desert regions.

Previously, the USDA-ARS virology lab demonstrated that tombusviruses were responsible for the 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. The USDA-ARS lettuce genetics group also identified a dominant resistance gene from 'Salinas' lettuce. 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.* *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. Additionally, the USDA-ARS Virology lab has developed diagnostic methods for confirmation of infection by both TBSV and LNSV, including RT-PCR, immunocapture-RT-PCR, Enzyme-linked immunosorbent assay (ELISA), western blot analyses, and immuno-specific electron microscopy.

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.

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 2011-2012:
Test new methods for rapid greenhouse pre-screening of lettuce cultivars/lines for tombusvirus resistance.

1. Continue testing plants to determine specific parameters for day-length and temperature induction of lettuce dieback symptoms.

2. Confirm effectiveness of new method for screening lettuce and other *Lactuca* species for lettuce dieback resistance.

PROCEDURES

Objective 1. ***Continue testing plants to determine specific parameters for day-length and temperature induction of lettuce dieback symptoms.***

Experiments were conducted to clarify which environmental factors allow full systemic infection of susceptible lettuce plants with the tombusviruses, LNSV and TBSV. Initial studies demonstrated that 24 hour day length and high temperatures of 30°C facilitated systemic infection in growth chambers, when plants were rub-inoculated on leaves with virus infected

plant sap. In contrast, 16 hour day length and 22°C did not lead to systemic infection, although local lesions were produced on rub-inoculated leaves under both sets of conditions. Full light for 24 hours is not a 'normal' situation. Therefore we wanted to ascertain whether slightly shorter day length would work or if the physiological effects of no darkness period is what leads to infection. Similarly, we wanted to clarify what temperature was necessary for induction, and if this requirement is based on the need for long days alone, high temperatures alone, or if both are necessary for the systemic infection to occur. Initial experiments were conducted with the lettuce dieback-susceptible variety, Darkland, and were designed to confirm performance and clarify hours and temperatures using the four treatments listed below. Initially plans were to include an 8 hour photoperiod; however, this was eliminated as the 16 hour period provided a clear differential.

- Temperature 30°C – 24 h photoperiod
- Temperature 30°C – 16 h photoperiod
- Temperature 20°C – 24 h photoperiod
- Temperature 20°C – 16 h photoperiod

Objective 2. ***Confirm effectiveness of new method for screening lettuce and other Lactuca breeding materials for lettuce dieback/tombusvirus resistance.***

Resistant and susceptible cultivars were inoculated with optimized parameters as determined in Objective 1 (30°C, 24 h photoperiod) to confirm that the method allows infection of susceptible cultivars, but not resistant cultivars. These studies began in summer 2011 and are continuing. Initial focus has been on romaine cultivars, but some leaf types have been included in initial testing. Treatments included the following:

- Susceptible– Inoculated with TBSV
- Susceptible– Inoculated with LNSV
- Susceptible– Inoculated with buffer only
- Resistant– Inoculated with TBSV
- Resistant– Inoculated with LNSV
- Resistant– Inoculated with buffer only

Plants were analyzed for type and size of local lesions on inoculated leaves, as well as development of systemic infections in both susceptible and resistant cultivars. Confirmation of infection was determined by RT-PCR using primers specific to LNSV or TBSV.

Further studies were planned to analyze effects of resistance on a third tombusvirus, *Cucumber necrosis virus* (CNV) in the conditions conductive to TBSV/LNSV (temperature 30°C and 24 h photoperiod). CNV is another tombusvirus related to TBSV and LNSV. It infects lettuce, but based on previous studies does not cause disease on lettuce. Due to complications with infectivity in late summer and fall of 2011, these tests were delayed in order to focus efforts on why infection rates were dropping.

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 30°C, constant light, and light intensity of 75-105 µEinsteins per m^{-2} per s^{-1}. Higher light levels up to 300 uE per m^{-2} per s^{-1} were successful in later experiments as this reduced excessive elongation of lettuce plants.
- 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 inoculuation 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 can be used to differentiate between resistant (*Tvr*1) 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 observed differences in the type and size of lesions between resistant and susceptible lettuce varieties, with further variation depending on day length and temperature (data not shown).

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 Darkland showing systemic symptoms of lettuce dieback. Four uninoculated plants are shown in front of 20 inoculated plants (rear). **B.** All plants of resistant 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 than those on susceptible plants, the resistant plants do 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 have not provided conclusive results due to low or no infection of susceptible controls as described below. Without significant numbers of infected susceptible control plants it is 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.

After 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 µEinsteins per m^{-2} per s^{-1} to above 300 µEinsteins per m^{-2} 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 appears to be more to infectivity 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 again this spring, rates are still low (approximately 5 percent), compared with studies last spring and summer when we obtained infection in replicated experiments for each virus in the range of 40 to 82 percent systemic infection.

It was determined last summer that some of our virus source plants in the greenhouse were "cross-contaminated," such that our single infections with LNSV and TBSV may have contained both viruses as a mixed infection, as determined during recent RT-PCR testing. Consequently, the stocks were cleaned up and from that point on we used known pure cultures of each virus. Although most virus synergism involves distantly or unrelated viruses, it is possible that co-infection by TBSV and LNSV (closely related tombusviruses) may have led to the high rates of systemic infection observed in the first three successful experiments. The coincidence of identifying the cross contamination and cleaning up virus source material, with the sudden loss of systemic infection necessitates further testing of this as a possible explanation for the decline in infectivity. Studies are in progress using single and mixed infections with 30C and 24 hour light to determine if this impacts systemic infection of lettuce.

We anticipate resolution of the environmental factor(s) interfering with successful tests, the clarification of screening parameters, and completion of all studies by March 2013.

Supplemental: Related studies in our laboratory determined the complete genome sequence of *Lettuce necrotic stunt virus* (LNSV), one of the two tombusviruses commonly found to cause lettuce dieback (Wintermantel and Anchieta, 2012; Archives of Virology DOI 10.1007/s00705-012-1307-x), and comparison with related tombusviruses suggest it is very closely related to a partially characterized tombusvirus identified in the Middle East and Mediterranean regions known as *Moroccan pepper virus*. Studies are in progress to determine conclusively if the two viruses are actually the same species because information was lacking on the other virus, but our studies demonstrate near to complete identity in the portion of the genome of the other virus sequenced to date. If these are the same species, virus movement across the Atlantic likely happened at least several decades ago. Additionally, a full length infectious clone of an LNSV isolate from the Salinas Valley has been developed by our lab for future studies on lettuce dieback and its interaction with lettuce.