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). However, studies conducted through this project have shown that rub-inoculation of plants maintained at high temperature and 24 hour day length resulted in full systemic infection of nearly all inoculated plants, resembling lettuce dieback symptoms from the field. Further 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 selection of resistant materials. Experiments in progress are comparing high and low temperature with 24 hour day length, as well as differential day length at high temperatures previously shown to induce systemic infection, and whether this method will allow effective differentiation of resistant and susceptible genetic materials.

II. Main Body of Report

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INTRODUCTION

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 65% of all lettuce production in Monterey County (2009 Monterey County, CA Crop Reports). 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 soil-borne 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 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, 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: Test new methods for rapid greenhouse pre-screening of lettuce cultivars/lines for tombusvirus resistance.

1. Application of standard and concentrated virus inoculum to lettuce to induce lettuce dieback symptom development

2. Inoculation of lettuce with cloned tombusviruses (LNSV and TBSV) to induce lettuce dieback symptom development

PROCEDURES

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

Objective 1. Application of standard and concentrated virus inoculum to lettuce to induce lettuce dieback symptom development:

Our plan was to explore greenhouse-based testing methods for determination of resistance in lettuce to tombusvirus infection, the cause of lettuce dieback disease. Lettuce can develop a localized necrotic reaction when leaves are mechanically (rub-inoculation) inoculated with

plant sap containing LNSV or TBSV; however, this symptom can be inconsistent and does not resemble the normal symptoms of lettuce dieback that are observed under field conditions which develop when virus infects through the roots. Similarly, previous efforts to replicate field-like conditions were labor and time intensive and not conducive to large-scale greenhouse testing. Our goal in this objective was to determine if it will be possible to develop a field-like lettuce dieback symptom in plants inoculated in either greenhouses or growth chambers. Such a breakthrough would allow routine greenhouse testing for dieback resistance. Initial plans were to deliver purified virus directly to the roots or stems of a lettuce seedling to induce symptoms typical of lettuce dieback. This was subsequently expanded to examine many different approaches with the hope that one of the methods would be effective.

Concentrated inoculum was generated in the virus propagation host, *Nicotiana benthamiana,* by grinding large quantities of leaves infected with either LNSV or TBSV, in small amounts of inoculation buffer (100 mM sodium phosphate solution). Using this inoculum source, several greenhouse-based methods were attempted to determine if a greenhouse screening method to evaluate resistance to lettuce dieback and tombusvirus infection in lettuce germplasm would be feasible. The following approaches were evaluated:

1) Injecting inoculum directly into the vascular system of lettuce seedlings with a syringe.
2) Cutting seedling roots to create wound sites, and dipping the roots in inoculum for 15s, followed by planting and maintaining plants in saturated soil (Saturated soil has been shown to facilitate disease induction through previous studies).
3) Rubbing roots with inoculum, followed by planting and maintaining plants in saturated soil.
4) Direct inoculation of leaves under greenhouse conditions, with approx. 14-hour day length and 22°C.
5) Direct inoculation of leaves in growth chambers with 24 hour light and 30°C.
6) Concentration of inoculum by centrifugation, followed by inoculation using approaches 1 through 4 listed above.

Confirmation of infection involved visual inspection of plants for lettuce dieback symptoms, as well as molecular confirmation of infection through RT-PCR using the primers developed in the Wintermantel lab that have already been confirmed to work efficiently on our laboratory isolates of TBSV and LNSV.

Objective 2: Inoculation of lettuce with cloned tombusviruses to induce lettuce dieback symptom development. ***(Skipped to focus on refinement of successes achieved in Objective 1 as this method would be more costly and likely less successful than our newly developed method using sap inoculation with plants maintained at high temperature and long days.)***

Originally we planned to explore inoculation of cloned versions of TBSV (or LNSV) into lettuce roots or stems to induce tombusvirus infections that would resemble lettuce dieback field symptoms. This should be possible with direct inoculation of RNA transcripts, or could

be done using an *Agrobacterium tumefaciens*-mediated delivery of the full virus genome. Either molecular biology based method would be much more costly than traditional inoculation methods, but these methods were to be explored in case other methods were unsuccessful. Once we achieved success with the long-day, high temperature treatments, we focused our efforts on development of that method as it was much more cost-effective, and likely much more reliable based on results to date.

RESULTS AND DISCUSSION

Evaluation of new methods continued until one of the methods was successful in producing high levels of infection. None of the first four approaches resulted in infection. Injection of inoculum directly into the stem of lettuce plants (Method 1 from list above) allowed us to visually observe inoculum as it was injected inside major veins of lettuce leaves. Nevertheless, none of the plants developed infection using this method. It should be noted that plants were larger (6 leaf stage) than those normally used for mechanical inoculation (2 leaf stage) in order to prevent leaf midribs from collapsing during injection. Cutting seedling roots and dipping them in inoculum (2) and rubbing roots with inoculum (3) also failed to result in infection, and also caused significant damage to the root systems of plants that may have affected plant growth rates. Direct inoculation of leaves under greenhouse conditions (4) was considered a 'control' method. As expected based on previous studies, this method merely resulted in the development of local lesions on the inoculated leaf and no systemic infection. The symptoms for this form of inoculation differ dramatically from what one observes under field conditions with lettuce dieback.

Finally…Success!

Direct inoculation of leaves of the cultivar Darkland in growth chambers with 24-hour light and 30°C in potting mix (Method 5); however, resulted in 19 of 23 TBSV-inoculated plants and 10 of 23 LNSV- inoculated plants infected systemically with classic symptoms of lettuce dieback (Fig. 1). RT-PCR analysis confirmed infection with the respective viruses (Table 1). The experiment was subsequently repeated, with the additional steps of scoring infected plants for disease severity. Results demonstrated that susceptible lettuce plants grown in field soils 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. 1). These experiments clearly demonstrated the value of the new method to induce field-like symptoms of lettuce dieback in susceptible lettuce; however, these experiments did not examine the ability of this method to evaluate resistant germplasm carrying the *Tvr1* gene. Performance of resistant germplasm is currently being evaluated. Preliminary results with resistant lettuce germplasm are discussed later in this report.

Table 1. Number of inoculated lettuce plants with confirmed tombusvirus infections (Experiment 1).

Inoculum	No. of inoculated plants	No. of symptomatic plants*	Percent of symptomatic plants
TBSV	23	19	82.6
LNSV	23	10	43.5
Healthy	4	0	0

* Symptomatic plants confirmed infected with TBSV and LNSV, respectively, based on testing a subset of plants (5 per virus) by RT-PCR with virus-specific primers along with positive and negative controls.

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} (measured in Experiment 1 only, but same settings used for subsequent experiments).
- 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.

Figure 1.

A. Romaine lettuce plants (cv. Darkland) 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 inoculation with several symptomatic plants.

C. LNSV inoculation with several symptomatic plants.

Following the success of the first experiment involving high temperature and long day conditions, a second experiment was set up examining performance of both LNSV and TBSV from different soils in order to determine if soil type influenced the ability of the infection to develop. This was important since previous studies, including method 4, demonstrated that mechanical inoculation of plants using standard greenhouse conditions results in a type of symptom that is entirely different from that observed in the field. By conducting these tests with both standard greenhouse potting mix as well as using different field soils with and without a history of lettuce dieback, we intended to determine if the nature of the soil influenced our successful induction of lettuce dieback symptoms using long days and high temperatures. In addition to standard potting mix as used in Experiment 1, two field soils were selected. These were Field A, located on the USDA-ARS campus at 1636 East Alisal Street in Salinas; and Carr Lake, a field located off Laurel Drive in Salinas, with a history of lettuce dieback problems. The Carr Lake field has been used for evaluation and selection of dieback resistant lettuces for many years. Experiment two was conducted using the same growth chamber conditions as in the first experiment (24-hour light and 30°C), and plants were maintained for approximately six weeks. At the conclusion of the experiment plants were evaluated using a disease severity scale of 1 to 4, with one appearing healthy and four being a dead plant. As plants were scored, a leaf sample was taken from each plant for molecular confirmation of infection. Results of scoring are presented in Table 2, and confirmed that the long day, high temperature mechanical inoculation treatment is functional in diverse soil types. Disease severity ratings varied somewhat among soil types, and may relate to rate of plant growth in each soil type, since some previous studies have suggested that plants with a faster growth rate can reach larger sizes before symptoms are induced (Wintermantel and Grube, unpublished). Interestingly, the highest disease severity ratings were observed with the field soils, indicating these soils might be more conducive to symptom development than potting mix, although such information would require further confirmation in additional experiments. Field A soil, which has no history of lettuce dieback, produced lettuce with the highest disease severity of all (Table 2), demonstrating infection can occur using the high temperature, long day method in soil types with no history of disease.

Table 2. Disease severity evaluation of lettuce plants grown in different soil types, and infected with LNSV and TBSV in a growth chamber inoculation experiment with 24 hour day length and high temperature (Experiment 2)[1].

Soil origin[2]	Inoculum	Number of plants with each severity rating							
		1	1.5	2	2.5	3	3.5	4	Mean
Field A	TBSV					2		6	3.75
	LNSV							7	4.00
	Healthy	1							1.00
Carr Lake	TBSV			2		3	1	4	3.25
	LNSV			1	5			3	2.94
	Healthy	1							1.00
Potting mix	TBSV			2					2.00
	LNSV			3			1		2.38
	Healthy	1							1.00

[1] Plants were evaluated for symptoms of dieback on the scale from 1 to 4; 1 is completely healthy plant and 4 is dead plant. Table shows how many plants from a particular soil/inoculum combination were in each category. Weighted mean for each treatment (soil/inoculum combination) was calculated as

$$Mean = \frac{\sum(S \times n_s)}{n}$$

where S is the score category (1 to 4), n_s is number of plants in the particular category, and n is total number of plants in a treatment (soil/inoculum combination).

[2] Soil types: Field A, located on the USDA-ARS campus at 1636 East Alisal Street in Salinas; Carr Lake, a field located off Laurel Drive in Salinas, with a history of lettuce dieback problems; and standard greenhouse potting mix as used in Experiment 1.

Can the method differentiate performance of resistant and susceptible lettuces?

A third experiment was conducted to determine if the high temperature, long day treatment of mechanically-inoculated plants would facilitate differentiation of resistant and susceptible reactions to inoculation with tombusviruses. These studies continue, but at the time of this report, one experiment had been completed. Plants of the susceptible variety, Darkland, and the resistant variety, Sturgis, were grown in potting mix at 24 hour day length, with constant 29°C demonstrated in initial experiments to induce systemic lettuce dieback symptom development on susceptible lettuces. Results on inoculated leaves suggested a differential physiological response between resistant and susceptible lettuce inoculated and maintained at high temperatures with long days.

Susceptible lettuce produced an initial necrotic local lesion response on the inoculated leaf (Fig. 2A), while Sturgis plants, containing the *Tvr*1 resistance gene, exhibited chlorotic halos around such lesions (Fig. 2B). Importantly, as time progressed, the inoculated leaves of Sturgis turned yellow, but newer leaves remained symptomless (Fig. 2C) and virus was not detected in these leaves at the conclusion of the experiment. In contrast, some (4/20) of the susceptible Darkland

(*tvr*1) developed full systemic symptoms of lettuce dieback (Fig. 2D). Although a lower percentage of the susceptible Darkland controls became systemically infected in this experiment compared with previous experiments, we are encouraged by the lack of systemic symptoms in the resistant Sturgis plants. Further studies will be necessary to confirm and expand upon these results.

Figure 2. Symptoms on susceptible and resistant lettuce when inoculated and maintained at 29°C with 24 hour day length. **A.** Susceptible lettuce variety, Darkland, with small necrotic local lesions on inoculated leaf resulting from mechanical inoculation at 29°C and 24 hour day length. **B.** Resistant lettuce variety, Sturgis, showing small necrotic local lesions on inoculated leaf surrounded by chlorotic halos, suggesting a different reaction to inoculation. **C.** All plants of resistant Sturgis remained healthy throughout the experiment (one plant died due to damping off). Yellowed leaves were those initially inoculated. All new growth remained healthy. Four uninoculated plants are shown in front of 20 inoculated plants (rear). **D.** Some plants of susceptible Darkland showing systemic symptoms of lettuce dieback at the conclusion of the experiment. Four uninoculated plants are shown in front of 20 inoculated plants (rear).

A.

B.

C.

D.

Additional studies planned for the upcoming 2011-2012 project year are designed to refine conditions and to clarify whether both long day and high temperature are required for induction of systemic lettuce dieback symptoms under controlled conditions or if less ‘stressful’ conditions could be used to induce the resistance. Although such conditions could be replicated in a greenhouse, it would be preferable for plant quality if lower temperatures could be used. Initial studies have focused primarily on documenting performance of the method. The upcoming year should allow us to refine the method and determine optimal parameters for testing in growth chambers, and hopefully in greenhouses as well.