Variation within *Lactuca* spp. for Resistance to *Impatiens necrotic spot virus*

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Abstract

Lettuce (*Lactuca sativa* L.) production in coastal California, one of the major lettuce-producing areas of the United States, is regularly affected by outbreaks of *Impatiens necrotic spot virus* (INSV), a member of the genus *Orthospovirus*. Transmission of INSV among lettuce crops in this growing region has been attributed predominantly to the western flower thrips (*Frankliniella occidentalis*). INSV is acquired by first- or second-instar thrips nymphs feeding on infected host plants (not necessarily lettuce). The virus replicates within the insect vector, and is transmitted to new plants by adult thrips as they feed on epidermal and mesophyll cells of susceptible host plants. All currently grown cultivars of lettuce are susceptible to the disease. Screening lettuce for resistance to INSV under field conditions is problematic because natural infections appear sporadically and the virus is not evenly distributed across infected fields. We have developed a greenhouse-based assay that uses viruliferous thrips in combination with mechanical inoculation that allows dependable, year-round screening for

resistance. In all, 89 cultivars, breeding lines, and plant introductions of cultivated lettuce, together with 53 accessions from 11 other *Lactuca* spp., 4 accessions from two dandelion (*Taraxacum*) species, and 4 tomato (*Solanum lycopersicum* L.) lines were evaluated for resistance to INSV. All tested material was susceptible to INSV to varying degrees, with the exception of two tomato lines that carry the *Sw-5* gene that confers resistance to *Tomato spotted wilt virus*, a virus closely related to INSV. In cultivated lettuce, a partial resistance to INSV was observed in cultivars Amazona, Ancora, Antigua, Commodore, Eruption, Iceberg, La Brillante, Merlot, Telluride, and Tinto. Limited comparison of the greenhouse-based screening results with the data from opportunistic evaluations of resistance on 775 lettuce accessions from six field trials indicates consistency of results from both greenhouse and field environments. The most resistant lettuce accessions are being incorporated into our breeding program for introgression of resistance into lettuce breeding lines.

California is the main lettuce-producing area of the United States, providing approximately 75 to 80% of the total U.S. production (Simko et al. 2014). Since 2006, the coastal California lettuce crop has been affected by frequent and recurring outbreaks of *Impatiens necrotic spot virus* (INSV) (Kuo et al. 2014), a virus that belongs to genus *Orthospovirus*, family *Tospoviridae* in the order *Bunyavirales* (https://talk.ictvonline.org/ictv-reports/ictv_online_report/). Infection of lettuce (*Lactuca sativa* L.) plants with orthospoviruses in this important growing region was first reported over 80 years ago (Tompkins and Gardner 1934) but recent outbreaks brought renewed attention to diseases caused by orthospoviruses in lettuce. Two orthospoviruses cause spotted wilt disease on lettuce in California. *Tomato spotted wilt virus* (TSWV) is the primary orthospovirus responsible for disease outbreaks on both lettuce and tomato in California's Central Valley, whereas the closely related INSV is the predominant orthospovirus causing economic damage to lettuce in coastal California (Kuo et al. 2014). Two other members of the *Orthospovirus* genus have also been shown to infect lettuce—*Groundnut ringspot virus* (GRSV) (Gracia et al. 1999) and *Tomato chlorotic spot virus* (TCSV) (Colariccio et al. 2004; de Jensen and Adkins 2014; Gilbertson et al. 2015; Webster et al. 2015)—although neither GRSV nor TCSV has been identified in California or in the western United States to date.

Plants may become infected with the INSV at any age, though infection of plants in earlier stages of development usually results in more pronounced symptoms that include stunted growth and severely distorted leaves. Initial symptoms of spotted wilt disease on

lettuce appear as small brown lesions or spots, often associated with veins. These expand into larger necrotic sections on infected leaves (Fig. 1). Plants infected as young seedlings may die but plants infected later in development show yellowing and wilting, and petioles may develop necrotic spots that often appear to sink into the midrib. Infected plants may develop disproportionate growth, with reduced plant growth on the side with the most symptoms, and leaf petioles bending to grow around the diseased tissue (Gilbertson and Koike 2017). Disease incidence (DI) within fields increases with the age of the crop as INSV is transmitted from initial points of infection throughout the field. A recent survey of fields in coastal California determined the mean DI in romaine lettuce was 5.7%, with a range between 0.5 to 27% (Kuo et al. 2014). Because stunted, necrotic, or otherwise symptomatic plants are not marketable, high DI results in substantial economic losses to growers.

INSV is also frequently observed infecting lettuce plants grown in greenhouses. Our observations indicate that, in years highly conducive to the disease, as many as 50 to 70% of greenhouse-grown lettuce plants in coastal California can be infected with this virus if the population of thrips is not well managed (I. Simko, unpublished data). Many greenhouse-grown plants in this region are used for early filial generation seed production. Lettuce plants that become infected in later stages of development can survive until maturity but they remain weak and may not produce seed. This is particularly problematic when only a limited number of plants are grown for seed production, such as with the single-seed descent method used in plant breeding (Lee et al. 2012) and genetic studies (Hayes et al. 2014; Simko et al. 2013).

Orthospoviruses are transmitted by several species of thrips but spread of INSV among lettuce crops in the coastal growing regions of California, as well as TSWV in the Central Valley, has been attributed mostly to the western flower thrips (*Frankliniella occidentalis*) (Kuo et al. 2014). Thrips transmit orthospoviruses in a persistent, propagative manner. These viruses can also be transmitted experimentally by sap, although sap transmission is not reliable. Orthospoviruses lose thrips transmissibility after one or two transmissions if the virus does not pass through the thrips vector (Whitfield et al. 2005; W. M. Wintermantel, unpublished results). Orthospoviruses can only be acquired by larval thrips (first or second instar) feeding on infected host plants, and adult thrips can acquire these viruses but are unable to transmit them to other plants (Moritz et al. 2004). Virus acquisition can occur with short feeding periods but longer feeding results in more efficient acquisition of virus

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(Linford 1932). Once acquired, INSV replicates in the thrips vector over a period of several days as the insect matures. It is retained through molting and can only be transmitted to new plants by the adult thrips as they feed on epidermal and mesophyll cells of the host plant (Whitfield et al. 2005). Transmission occurs quickly and can be effective with feeding periods as short as 5 min (Gilbertson and Koike 2017; Whitfield et al. 2005).

In addition to lettuce, INSV can cause disease in a large number of plant species, including cultivated crops such as basil, bell pepper, blackberry, celery, faba bean, peanut, pepino, potato, radicchio, spinach, sweet pepper, tobacco, tomatillo, and tomato (Kuo et al. 2014; Turina et al. 2012). The wide range of host species that can be infected by INSV and TSWV complicates disease control; therefore, the best strategy to control orthotospoviruses in affected crops is the use of commercial cultivars carrying resistance genes. This approach was successfully employed to control TSWV in tomato and pepper, though strains of TSWV recently emerged that overcome the *Sw-5* resistance gene in California (Batuman et al. 2017) and Europe (Turina et al. 2012). No INSV-resistant cultivar of lettuce is currently available, and only very limited information is known about resistance to the disease in lettuce. A partial resistance to TSWV was described in cultivars Ancora and Tinto, two cultivars that carry the same or closely linked resistance genes (O'Malley and Hartmann 1989). When inoculated with TSWV, plants of these two cultivars

showed delayed appearance of symptoms compared with the susceptible check but all plants eventually developed disease symptoms (O'Malley and Hartmann 1989). Because these resistance tests were performed before TSWV and INSV were recognized as serologically distinct and independent virus species (de Avila et al. 1990; Law and Moyer 1990; Soler et al. 2003), it is not possible to determine whether Ancora and Tinto were tested against TSWV or INSV.

Screening lettuce for resistance to INSV under field conditions is problematic because artificial inoculation is not practical, reliable, or advisable. Because natural INSV infections appear sporadically, it is not possible to accurately predict when and where the disease would occur and with what frequency. A low or uneven DI does not allow for accurate evaluation of resistance, because many plants may escape infection. Therefore, a greenhouse-based test that can provide higher and more consistent disease pressure than is observed in the field is desirable. Mechanical inoculation alone may not provide high and reproducible DI (Shrestha et al. 2015). Therefore, use of viruliferous thrips would seem to be a more effective approach. The utilization of viruliferous thrips, however, brings its own problems regarding maintenance of the populations and preventing transmission of viruliferous thrips to other plant-growing facilities and fields. The main objectives of the research described herein were to (i) develop a reliable, greenhouse-based method for testing lettuce resistance to INSV; (ii) screen lettuce cultivars, breeding lines, and plant

Fig. 1. Symptoms of *Impatiens necrotic spot virus* (INSV) infection on lettuce plants grown under field or greenhouse conditions. **A, B, and C,** Symptoms of INSV on field-grown plants. **D, E, and F,** Disease symptoms on plants inoculated with INSV and grown in a greenhouse. **G,** Enlargement of the leaf area heavily infested with western flower thrips (*Frankliniella occidentalis*), causing severe damage on the leaf, although there are no discernable INSV symptoms yet on this greenhouse-grown plant. **H,** Enlargement of the leaf area with severe disease symptoms developing on a plant inoculated with INSV and grown in a greenhouse.

introductions for their reaction to INSV; and (iii) determine the range of resistance in other *Lactuca* spp.

Material and Methods

Plant material. Eighty-nine cultivars, breeding lines, and plant introductions (collectively called accessions) of cultivated lettuce were evaluated for their reaction to inoculation with INSV. In addition, 53 accessions from 11 other *Lactuca* spp. and 4 accessions from two dandelion (*Taraxacum*) species that belong to the same Asteraceae/Compositae family as genus *Lactuca* were also analyzed. Furthermore, tests were performed on four tomato (*Solanum lycopersicum* L.) accessions that differ for the presence of *Sw-5*, a gene locus that confers resistance in tomato to several strains of TSWV (Brommonschenkel et al. 2000).

Greenhouse-based evaluations. Each experiment consisted of approximately seven accessions, one of which was always a known INSV-susceptible cultivar (usually Conquistador or Triple Threat). Approximately 20 seeds of each accession were planted into 72-cell plug trays in a standard greenhouse potting soil (Premium Growers Mix; Sun Land Garden Products, Watsonville, CA) and maintained in a cool growth chamber at 10°C with no light for 2 days to improve uniformity of germination. Next, seedlings were transferred to a greenhouse until two true leaves were fully emerged, then individually transplanted to 10-by-10-cm pots containing potting soil. An average of 13 plants per accession were transplanted and used per experimental replication, with a total of 47 experiments conducted over a 3-year period.

Three methods were used for inoculation of young plants: mechanical (rub) inoculation, feeding by viruliferous thrips, and a combination of both approaches. Inoculations were performed once seedlings reached the three- to four-true-leaf stage. Inoculum for use in rub inoculations was prepared by adding 1 to 2 g of INSV-infected leaf tissue from a plant that had been inoculated with INSV by thrips transmission, suspended in approximately 3 ml of mechanical inoculation buffer (100 mM sodium phosphate buffer, pH 7.0 containing 0.2% sodium sulfite), and 0.05 g of celite as an abrasive. Inoculum was usually INSV-infected *Emilia sonchifolia* (L.) DC. leaves but, occasionally, INSV-infected lettuce was used as well. Using these inoculum sources interchangeably did not affect resistance test results. It is critical that inoculum be derived from a plant infected by thrips transmission of virus, because INSV infectivity decreases greatly with successive mechanical (rub) inoculations without passage through the insect host. Plants were rinsed with water following inoculation to remove remaining celite. Leaves to be used as inoculum sources for transmission experiments were tested prior to inoculation using immunostrips (Agdia, Elkhart, IN) to confirm infection with INSV and to verify that inoculum source plants were not infected with TSWV. Plants were maintained in a greenhouse with a day and night temperature range of 14 to 22°C to allow recovery from inoculation. At 3 to 5 days following inoculation, mechanically inoculated plants were moved to the experimental greenhouse containing INSV-infected lettuce and *E. sonchifolia* plants with a stable population of INSV-infected western flower thrips. In addition to the combined approach involving both mechanical and thrips transmission, experiments were also conducted using plants inoculated only mechanically or by transmission from viruliferous thrips.

Test plants were monitored for thrips damage and symptom development over the course of the experiments. After 4 weeks, plants were given a final visual evaluation of symptoms and 0.5 g of leaf tissue was collected from plants for determination of virus infection and titer by enzyme-linked immunosorbent assay (ELISA) using the Agdia INSV ELISA detection kit, which uses a polyclonal antiserum against INSV, according to manufacturer's recommendations. Tissue used for the tests originated from leaves that were younger than those mechanically inoculated with INSV to confirm virus replication and systemic movement in the lettuce plant. Each ELISA plate included three positive and three negative controls. Only the central 60 wells of the 96-well plate were used for tests to ensure high uniformity of conditions. Positive controls were *E. sonchifolia* or lettuce plants previously confirmed to be infected by INSV. Negative controls were healthy lettuce plants. Samples were considered to be positive if

the ELISA absorbance readings were 2.5× higher than the mean of negative controls. DI was expressed as the percentage of positive plants out of the total number of plants (DI%).

Field evaluations. Opportunistic evaluation of lettuce resistance to the disease was performed in six field trials in 2015 and 2016 (three trials in each year) that were naturally infected with INSV. Presence of INSV and absence of TSWV was confirmed on randomly selected symptomatic plants with virus-specific immunostrips (Agdia). These trials were established to evaluate yield or postharvest quality of lettuce. The trials were set up in the randomized complete block design, with three replications and 20 to 30 plants per plot. The DI was recorded from individual plots, and data across all three replications were averaged for each accession prior to statistical analyses. DI was expressed as the percentage of symptomatic plants out of the total number of plants (DI%).

Statistical analyses. Values of DI were logit-transformed (Warton and Hui 2011) prior to statistical analyses. To avoid negative and positive infinity, 0 and 100 DI% were replaced with 0.1 and 99.9 DI%, respectively, before logit transformation. The results of statistical analyses performed on logit-transformed data were back transformed for more suitable presentation. Marginal means of DI were estimated using the least squares means (LS-means) approach. Because LS-means values are adjusted for covariates, they may lie outside of the original 0 to 100% range for DI%. LS-means of accessions were subsequently compared with the overall LS-mean of all accessions that was computed as a weighted mean of the LS-means. Comparison of LS-means and the overall LS-mean was performed with analysis of means (ANOM). ANOM can separate accessions into three groups, thus allowing differentiation of host plant reaction to virus infection: those with LS-means below the confidence interval of the overall LS-mean (relatively resistant accessions), those with LS-means within the confidence interval of the overall LS-mean (accessions with the intermediate resistance), and those with LS-means above the confidence interval of the overall LS-mean (relatively susceptible accessions). LS-means and ANOM were calculated separately for data obtained from 47 greenhouse tests and from 6 field evaluations. Confidence intervals of the overall means in ANOM were calculated at $\alpha = 0.05$. All statistical analyses were performed with JMP (v. 11.1.1; SAS Institute, Cary, NC).

Results

Different inoculation approaches for infection of plants under the greenhouse conditions were compared using the susceptible Conquistador. Results of these tests showed that the combination of mechanical and viruliferous thrips inoculation was more efficient (DI% = 90) than either inoculation by thrips alone (DI% = 60) or when only mechanical inoculation was used (DI% = 50). Uninoculated check plants were all virus free based on ELISA confirmation. Statistical analyses performed on ELISA values confirmed DI% results and identified significant differences among the inoculation methods. The highest mean ELISA values were detected in the plants that were inoculated using the combined approach of mechanical inoculation followed by thrips-based inoculation (mean reading of 0.76, 95% confidence interval 0.66 to 0.85), and this was significantly higher than when plants were inoculated using viruliferous thrips only (mean reading of 0.44, confidence interval 0.35 to 0.54) or by mechanical inoculation only (mean reading of 0.37, confidence interval 0.28 to 0.47). Virus titers as determined by mean ELISA readings using only mechanical inoculation or only thrips inoculation were not significantly different from one another. The lowest mean ELISA readings were identified in the uninoculated check plants (mean reading of 0.13, confidence interval 0.04 to 0.23). Because the combined inoculation method provided the highest DI%, only this method was used in the subsequent tests.

Reactions of 4,381 plants from 150 accessions and 15 species to INSV inoculation was evaluated in 47 greenhouse-based experiments. The overall DI% among all accessions was 49.6, while the overall LS-mean was 50.1. LS-means for individual accessions ranged from -0.6 for lettuce breeding line SMF-005 to 114.1 for two *L. serriola* accessions (ARM-09-161-10-1-1 and ARM-09-161-10-1-3). Twenty-three accessions were classified by ANOM as relatively more resistant

than the overall LS-mean (Fig. 2; Table 1). Of these accessions, 15 were *L. sativa*, 2 were tomato cultivars carrying the *Sw-5* allele, 2 accessions were *L. serriola*, and 1 accession originated from each of *L. indica*, *L. inermis*, *L. virosa*, and *Taraxacum alaskanum* species. In contrast, 21 accessions were classified by ANOM as relatively more susceptible than the overall LS-mean. These accessions belonged to species of *L. sativa* ($n = 15$), *L. aculeata* ($n = 2$), *L. serriola* ($n = 2$), *L. indica* ($n = 1$), and *L. saligna* ($n = 1$). The most resistant lettuce cultivars among those evaluated were Amazona, Ancora, Antigua, Commodore, Eruption, Iceberg, La Brillante, Merlot, Telluride, and Tinto, while the most susceptible cultivars were Alamo, Amadeus, Antares, Balady Bahera, Balady Banha, Conquistador, Da Ye Wo Sun, Deer's Tongue, Green Wave, and Triple Threat (Supplementary Table S1).

Opportunistic evaluation of resistance to INSV was performed on 775 lettuce accessions tested in six field trials that were naturally

infected with the virus. However, because these field trials were not originally established to evaluate INSV incidence, 740 of the accessions (95%) were evaluated from only a single trial in which the experimental field became infected with INSV. The overall mean DI% across all trials was 7.2, with a range of 2.6 to 16.3 (overall LS-mean = 9.7, range = 6 to 12.9). Because there was only a limited overlap among accessions tested in different trials, and because disease pressure in field trials was substantially lower compared with that in the greenhouse experiments, data from field evaluations were highly heterogeneous and did not allow identification of accessions that were significantly more resistant than the overall LS-mean. However, field trials did facilitate the identification of 46 accessions that are likely more susceptible to INSV than the overall LS-mean (Fig. 3). All of these accessions are inbred lines (highest LS-mean of 62.4) with the exception of cultivar Balady Barrage (LS-mean of

Fig. 2. Comparison of least square means (LS-means) of disease incidence data from greenhouse experiments using analysis of means. LS-means were calculated for 150 accessions tested in 47 greenhouse experiments. The black horizontal line shows the overall LS-mean of all accessions (50.1). The two jagged lines indicate the upper and lower limits of the 95% confidence interval for the overall mean. Accessions with LS-means below the confidence interval were classified as relatively resistant (23 accessions), accessions with LS-means within the confidence interval were classified as intermediate (106 accessions), and accessions with LS-means above the confidence interval were classified as highly susceptible (21 accessions). For easier viewing, accessions were arranged in the order of increasing size of the confidence interval.

Table 1. Reaction of 150 accessions from 15 species to inoculation with *Impatiens necrotic spot virus* in greenhouse conditions

Species	Number tested ^b	Number of accessions with DI% ^a		
		Resistant	Intermediate	Susceptible
<i>Lactuca aculeata</i> Boiss.	3	0	1	2
<i>L. floridana</i> (L.) Gaertn.	1	0	1	0
<i>L. indica</i> L.	4	1	2	1
<i>L. inermis</i> Forssk.	2	1	1	0
<i>L. ludoviciana</i> (Nutt.) Riddell	1	0	1	0
<i>L. perennis</i> L.	2	0	2	0
<i>L. quercina</i> L.	1	0	1	0
<i>L. saligna</i> L.	7	0	6	1
<i>L. sativa</i> L.	89	15	59	15
<i>L. serriola</i> L.	25	2	21	2
<i>L. tatarica</i> (L.) C.A. Mey.	1	0	1	0
<i>L. virosa</i> L.	6	1	5	0
<i>Solanum lycopersicum</i> L.	4	2	2	0
<i>Taraxacum alaskanum</i> Rydb.	1	1	0	0
<i>T. kok-saghyz</i> L. E. Rodin	3	0	3	0
Total	150	23	106	21

^a Least square means (LS-means) of disease incidence (DI%) were compared using analysis of means. Accessions with LS-means below the confidence interval of the overall LS-mean were classified as relatively resistant, accessions with LS-means within the confidence interval of the overall LS-mean were classified as intermediate, and accessions with LS-means above the confidence interval of the overall LS-mean were classified as highly susceptible.

^b Number of tested accessions.

35.5). Although, the field trials did not allow identification of accessions that were significantly more resistant than the overall LS-mean, very low LS-means may indicate putative sources of resistance. These include the cultivars Eruption (−3.1, evaluated in one trial), Merlot (−0.1, evaluated in four trials), and Blonde Lente a Monter (0.3, evaluated in two trials) (Supplementary Table S2). No TSWV was detected in any field experiments in the Salinas Valley over the course of these studies and INSV was confirmed infecting symptomatic plants from field trials, indicating that the orthospovirus present in field evaluations was indeed INSV.

Thirty-three lettuce accessions were evaluated for their resistance to INSV in both greenhouse experiments and at least one of the field trials. Accessions classified in the greenhouse experiments as being significantly more resistant than the overall LS-mean also had relatively low LS-means in the field trials (Eruption = −3.1, Iceberg = 6.0, Merlot = −0.1, and Telluride = 0.7), whereas those classified as being significantly more susceptible than the overall LS-mean also had relatively high LS-means in the field trials (Balady Banha = 13.2, Conquistador = 8.7, and Triple Threat = 10.2). There were two notable exceptions to this pattern, when cultivars classified as being more susceptible than the overall LS-mean in greenhouse tests may have escaped INSV infection in the field: Amadeus (LS-mean of 0.9) and Deer's Tongue (LS-mean of 0.5).

Discussion

None of lettuce cultivars or plant introductions that were tested for resistance to INSV in multiple greenhouse-based assays were immune to infection. We confirmed, however, a relatively high level of resistance, based on low virus titers and reduced DI, in Ancora (DI% mean = 26.1, LS-mean = 21.1) and Tinto (DI% mean = 28.5, LS-mean = 25.2) that were previously determined to carry genes for partial resistance to TSWV (O'Malley and Hartmann 1989). That study reported similar levels of DI for both Ancora (DI% averaged across five trials was 25.0) and Tinto (DI% = 19.8), whereas the DI% in susceptible check cultivar Green Mignonette was 70 (our susceptible check was Conquistador, with DI% = 69.6 and LS-mean = 67.7). Ancora and Tinto, along with another 13 *L. sativa* accessions that were identified as being more resistant than the overall mean, were incorporated into our breeding program for further development of this material. Other possible sources of resistance genes against INSV could be wild *Lactuca* spp. that are sexually compatible with

cultivated lettuce, either directly or through the use of bridging species that allow production of fertile hybrids. Among the noncultivated *Lactuca* spp. that were included in our tests, high resistance to INSV was observed in two accessions of *L. serriola* (ARM-09-191-5 and ARM-09-191-9, LS-means of 8.5) and one accession of *L. virosa* (IVT280, LS-mean of 16.8). However, none of these accessions completely suppressed symptom development, and the virus was present in plants when tested 3 and 4 weeks later, as confirmed by ELISA. The only *Lactuca* accession that showed consistently high levels of resistance to INSV in multiple experiments was SAL 063 (*L. inermis*), in which only 1 of 35 plants tested positive in greenhouse tests. Results were confirmed even when the plants were retested for the presence of the virus a month later (although plants were not grown to full maturity). Unfortunately, *L. inermis* is not sexually compatible with *L. sativa* and cannot be used to introgress resistance genes into cultivated lettuce. Our results show that accessions from all tested *Lactuca* and *Taraxacum* spp. are susceptible to INSV to some extent and, thus, are potential reservoirs from which the virus can be acquired by thrips and transmitted to lettuce and other susceptible crops.

Field trials that were used to evaluate resistance to INSV showed mean DI% of 7.2, with a range of 2.6 to 16.0. Similar DI was previously reported in fields of romaine lettuce grown in coastal California (average DI% = 5.7, with a range of 0.5 to 27) (Kuo et al. 2014). These levels of infection may be high enough to cause economic damage but they are too low and uneven to allow reliable screening of a large number of accessions for resistance to INSV. The sample size for finding infection in a field can be estimated as $n = \log \beta / \log p$, where β is the probability of committing a type II error ($1 - \beta$ is the chosen power) and p represents the proportion of plants that are not infected (Dell et al. 2002). Using this formula, it can be determined that approximately 90 plants need to be evaluated to have a 99% chance of detecting INSV infection that effects only 5% of plants ($\log 0.01 / \log 0.95 = 89.8$) (Fig. 4). However, when the infection rate increases to 50% (as in our greenhouse assays), the number of plants that need to be evaluated decreases to approximately 7 ($\log 0.01 / \log 0.50 = 6.6$, about 13× less). These data show that the number of plants evaluated in our field trials was too small ($n = 60$ to 90) to accurately identify resistant accessions at the observed levels of infection. However, even with this limited number of evaluated plants, statistical analyses show that the accessions with high DI% are significantly

Fig. 3. Comparison of least square means (LS-means) of disease incidence data from field trials using analysis of means. LS-means were calculated for 775 accessions evaluated in six field trials. The black horizontal line shows the overall LS-mean of all accessions (9.7). The two jagged lines indicate the upper and lower limits of the 95% confidence interval for the overall mean. Accessions with LS-means below the confidence interval were classified as relatively resistant (no accession), accessions with LS-means within the confidence interval were classified as intermediate (729 accessions), and accessions with LS-means above the confidence interval were classified as highly susceptible (46 accessions). For easier viewing, accessions were arranged in the order of increasing size of the confidence interval.

more susceptible to INSV than the overall mean of the accessions that were tested. Despite the limitations of field evaluations, the classification of accessions based on DI and virus titer in the greenhouse assays demonstrated consistency with the results of field evaluations (e.g., Eruption, Iceberg, Merlot, and Telluride show good resistance under both conditions, whereas Balady Banha, Conquistador, and Triple Threat were consistently highly susceptible). Statistical analyses differentiating resistant and susceptible lettuce based on DI% and virus titer were nearly identical when performed on greenhouse-grown plants. Substantially more data are needed, however, to perform reliable statistical analyses of field trials.

The greenhouse assay that utilized the combination of mechanical inoculation and viruliferous thrips revealed that the two tomato accessions carrying the *Sw-5* gene locus were completely resistant (mean DI% = 0, LS-mean = 2.1) to INSV whereas two accessions that do not carry this locus were susceptible to the virus (means 54.9 and 55, LS-means 57 and 57.2). These results demonstrate that the *Sw-5* locus in tomato provides resistance against the INSV isolate collected in Salinas, CA and likely to other isolates of this virus. Several other major genes were described in tomato and pepper that provide complete resistance to strains of TSWV (Turina et al. 2012). However, neither our present study nor previous extensive screening efforts (Cho et al. 1996; O'Malley and Hartmann 1989) have been able to identify lettuce accessions with the high levels of resistance to INSV or TSWV that are found in tomato. Therefore, lettuce breeding efforts may need to focus on pyramiding several genes that confer partial resistance to viruses that cause this disease. Because INSV is

transmitted by thrips, disease spread within fields may also be indirectly limited by planting of lettuce cultivars that attract fewer thrips or that limit their reproduction. Alternatively, cultivars with complete resistance to INSV and TSWV could be developed by using transgenic approaches, such as RNA interference or a posttranscriptional gene-silencing mechanism (Pang et al. 1996; Peng et al. 2014). However, to the best of our knowledge, this technology has not been used for the development of commercial cultivars of lettuce.

A recent patent application describes development of lettuce plants with a genetic determinant that confers resistance to both TSWV and INSV (Schut et al. 2014). The origin of a resistant plant of the invention 08D.121730 is not fully explained but information states that a single recessive gene located on chromosome 2 provides complete resistance against both viruses. Seed of this material were deposited into the National Collection of Industrial, Marine and Food Bacteria (NCIMB) depository under the accession number NCIMB 42023; however, we were unable to obtain seed for testing because the seed will not be available for distribution until the patent is granted.

Development of a greenhouse-based method for evaluation of lettuce germplasm for resistance to orthospoviruses became necessary due to the inconsistent presence and unpredictable distribution of INSV in lettuce fields. A greenhouse-based approach was necessary to develop a reliable method to systematically evaluate *Lactuca* germplasm for resistance to the virus. Initial studies demonstrated low inoculation efficiency when using only viruliferous thrips as inoculum, as well as when mechanical inoculation was used for virus delivery even when this inoculum came from a source directly

Fig. 4. Required number of plants that need to be evaluated to detect infection. The sample size for finding infection was estimated as $n = \log \beta / \log p$, where β is the probability of committing a type II error ($1 - \beta$ is the chosen power) and p represents the proportion of plants that are not infected (Dell et al. 2002). The black arrows show two examples for the number of plants that need to be evaluated to have 99% chance of detecting infection that effects only 5% of plants (approximately 90 plants need to be evaluated) or 50% of plants (approximately 7 plants need to be evaluated), respectively. Note that the x-axis is in logarithmic scale.

infected by viruliferous thrips. Testing of different inoculation approaches revealed that the combination of mechanical inoculation followed by exposure to and inoculation by viruliferous thrips leads to significantly higher DI than either inoculation method alone. We suspect that this is due to the complex nature of orthospovirus transmission. As noted previously, INSV must be acquired by first- or second-instar nymphs, replicates within the insect, and can only be transmitted by adult thrips. The use of mechanical inoculation of INSV to lettuce plants provides a more consistent source of inoculum for virus acquisition by the next generation of thrips, leading to perpetuation of the INSV transmission cycle.

An intimate relationship exists between orthospoviruses and their plant and insect vector hosts, such that the viruses require replication in both insect and plant for reliable infection of plant hosts and transmission by thrips. Indeed, studies have shown changes in the feeding behavior of thrips based on the presence or absence of TSWV within the western flower thrips (Stafford et al. 2011). Furthermore, virus transmission is rapidly lost when orthospoviruses are not passed through their insect hosts. This was a problem for previous studies of INSV and TSWV in lettuce (Cho et al. 1996; Kuo et al. 2014), and appears to be a unique characteristic of viruses in the genus *Orthospovirus*. *Orthospovirus* spp. contain not only a protein coat protecting the nucleic acid, as is found with most plant viruses, but also a lipid membrane surrounding the virus particle that contains specific glycoproteins (Kikkert et al. 2001; Naidu et al. 2004; Ribeiro et al. 2009); a trait more commonly associated with viruses of animals. Most likely some of the components of the lipid membrane can only be acquired from the thrips host. Therefore, without passage through the insect, it is likely that these lipid membrane components may be lost, and infectivity of some host plants reduced. Because of this unique need for passage through both host insect and host plant, all mechanical inoculations in our study were performed with sap from plants that were infected by INSV passaged directly by viruliferous thrips. The presence of an active thrips population containing all life stages of the insects facilitated maintenance of a viable and steady inoculum source. By conducting experiments continuously, rotating groups of plants in and out of a greenhouse containing an active viruliferous thrips population with INSV replicating in two preferred hosts (lettuce and *E. sonchifolia*), we stabilized the disease cycle to facilitate efficient infection of susceptible lettuce plants.

Similarly, age of plants at the time of inoculation is also critical. Plants that were inoculated after four to six true leaves had developed showed a low infection rate by INSV. This is true regardless of the inoculation method, as well as susceptibility or resistance of the cultivar, and can confound the results of an experiment. In preliminary experiments, we observed higher DI in susceptible plants that were inoculated when two to four true leaves were present but not fully expanded than when leaves were larger at the time of inoculation. In addition, it is necessary to allow thrips sufficient time to infect plants and for infections to develop in lettuce. If plants are evaluated too early, the ability to differentiate resistant and susceptible plants is reduced due to a low DI%, because early analysis particularly compromises the ability to compare symptom development. However, if plants are evaluated too late, differentiation may also become difficult because all accessions of lettuce (*L. sativa* as well as other *Lactuca* spp.) will become infected if given sufficient time and proper transmission methods.

We visually analyzed 2,396 greenhouse-grown, INSV-inoculated plants for the presence of INSV symptoms before performing ELISA tests. Based on a positive ELISA test as the true indicator of a diseased plant, there were 1,020 (42.6%) true-positive results (visual +/ELISA +), 767 (32.0%) true-negative results (–/–), 427 (17.8%) false-positive results (+/–), and 182 (7.6%) false-negative results (–/+). The coefficient of association between visual observations and ELISA tests ($\phi = 0.502$) was highly significant ($P < 0.0001$), indicating a positive match between the two methods and an overall accuracy of 0.75 (95% confidence interval from 0.73 to 0.76). When visual observations of symptoms and positive ELISA results were compared in a sample of 75 field-grown lettuce plants, the results matched perfectly. In our experience, the INSV symptoms on field-grown plants are readily distinguishable from

symptoms of other diseases and plant defects, with the exception of symptoms caused by TSWV that appear to be identical in lettuce. However, although INSV-infected, greenhouse-grown plants often develop symptoms typical of field infections, symptoms observed on greenhouse-grown plants are frequently more difficult to reliably use for assessment of infection than symptoms observed on field-grown plants. Although the reason for this is not yet clear, it is likely that this is due to the limitations of conducting the evaluations in a greenhouse. Our greenhouse experiments were conducted with lettuce plants grown in 10-by-10-cm pots, which restricts the growth and size of plants over time and also results in steady exposure to thrips feeding, leading to increased plant stress and foliar damage. Misclassification of dying of leaves resulting from severe thrips infestation and associated feeding damage may contribute to the false-positive results obtained in visual evaluation of symptoms in greenhouse experiments. Furthermore, greenhouse evaluations are conducted for a limited period. It is possible that infected lettuce plants transplanted to larger pots and maintained for longer periods in the greenhouse might more consistently develop symptoms typical of field-grown plants. However, this would also diminish contact between lettuce plants, thereby reducing movement of viruliferous thrips within experiments, which might adversely affect the performance of experiments.

There are some additional issues that remain to be resolved with regard to efficiency of lettuce evaluations for resistance to INSV and other orthospoviruses. First, there may be an irregular distribution of INSV within lettuce plants, such that titers may vary by leaf. This may be particularly important to further characterize in partially resistant accessions. Many viruses exhibit differences in both their systemic movement throughout infected plants and their rate of systemic movement. Some viruses remain confined to root, leaf, or even floral tissue, whereas others may move readily throughout all tissues of a plant. We identified variation in symptom development as well as virus titer for INSV during infection of lettuce and wild *Lactuca* accessions. Further characterization of partially resistant materials will be necessary to determine whether systemic movement may be impeded in these accessions compared with fully susceptible cultivars and accessions. Similar information was presented by Kuo et al. (2014), in which INSV was not detected in all leaves but was always detected in flowers of infected plants. Studies on peppermint also found tissue-specific differences for accumulation of INSV (Deangelis et al. 1994). They determined that virus detection in newly developed leaves was inconsistent, whereas older leaves lower on the stem often appeared bronzed and were a more reliable source of virus. Peppermint stem tissue was also consistently found to have low titers of INSV (Deangelis et al. 1994).

We have developed a greenhouse-based assay that uses viruliferous thrips in combination with mechanical (rub) inoculation that allows dependable, year-round screening for resistance to INSV in lettuce. All *Lactuca* accessions were susceptible to the virus; however, a partial resistance was observed in 15 accessions of cultivated lettuce and 3 accessions of wild *Lactuca* spp. that are sexually compatible with cultivated lettuce. These genotypes are being incorporated into our breeding program for introgression of resistance alleles into breeding lines.

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