DISSERTATION

BIOLOGY AND OVER-WINTER SURVIVAL OF *IRIS YELLOW SPOT VIRUS* IN COLORADO

Submitted by

Stephanie Aspen Szostek

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2014

Doctoral Committee:

Advisor: Howard F. Schwartz

Michael Bartolo
Whitney Cranshaw
Ned Tisserat

ABSTRACT

BIOLOGY AND OVER-WINTER SURVIVAL OF *IRIS YELLOW SPOT VIRUS* IN COLORADO

Iris yellow spot virus (IYSV) (Family: Bunyaviridae, Genus: *Tospovirus*) and its insect vector, *Thrips tabaci* Lindeman, are of economic concern in onion (*Allium cepa* L.) growing regions worldwide. IYSV symptoms appear on onion foliage as tan or straw colored, elongate diamond shaped lesions. Accumulated lesions may coalesce on the foliage or girdle the scape, causing lodging and loss of seed. There is no evidence that Tospoviruses, including IYSV, are seed transmitted. Onion seed included in double antibody sandwich enzyme linked immunosorbent assays (DAS-ELISA) to detect IYSV occasionally yielded a positive result. IYSV was detected in the pedicels, petals, anthers, and fruits of onion flowers by reverse transcriptase polymerase chain reaction (RT-PCR). Onion seed collected from several cultivars of IYSV symptomatic plants was grown out under greenhouse and growth chamber conditions. IYSV was not detected in the six week old seedlings. Further investigation of onion seeds revealed IYSV could be detected in the seed coat, but not the emerging radicle. It is highly unlikely that IYSV can pass from the seed coat to the new plant during germination, and seeds remain an unlikely source of IYSV inoculum.

Several weed species have been described as additional hosts and likely green bridges for IYSV survival, however, there is little work regarding the overwintering habits of *T. tabaci* and its potential to act as a source of inoculum during the following season. The results presented in this work close the loop, and show that both *T. tabaci* and IYSV are present near onion fields

throughout the winter, *T. tabaci* will reproduce on several weed species, and larvae can acquire IYSV from non-allium sources. Thrips activity was monitored via sticky trap during the winter months from 2011 to 2013. Thrips activity appeared to cease once the average temperature fell below 0°C and resumed once the average temperature rose above 0°C. Onion cull piles were constructed, and while these piles provided an environment conducive to thrips survival, few live thrips were recovered from the piles after the onset of bulb decay. IYSV was detected by RT-PCR in live adult and larval thrips recovered from onion, *Malva neglecta* Wallr. (common mallow), *Taraxacum officinale* Weber in Wiggers (dandelion), *Descurainia sophia* (L.) Webb. Ex Prantl (flixweed), *Lactuca serriola* L. (prickly lettuce), and *Tragopogon dubius* Scop. (salsify) during the winters from 2010 to 2013. Of these plants, IYSV was detected in prickly lettuce and flixweed. These five weed species were grown from seed in the greenhouse and exposed to viruliferous thrips to further elucidate their potential role as green bridges. Of the five, IYSV was detected in salsify and the thrips larvae reared on this plant. Results indicate winter annuals play a role in onion thrips and IYSV over-winter survival, providing inoculum the next growing season, and that weed management during the winter may be warranted.

IYSV distribution throughout onion leaves is uneven and patchy. A reverse transcription quantitative real time PCR (RT-qPCR) was developed to compare relative amounts of IYSV within leaves and between cultivars. The amount of IYSV was greatest at the lesion site itself and decreased as distance from the lesion increased. No statistically significant differences were found in the amount of IYSV between susceptible cultivar Granero and tolerant cultivar Advantage. This assay may be useful for additional comparative studies with other crops and viruses.

ACKNOWLEDGEMENTS

I would like to thank my committee for their support and for being all around good guys. Dr. Myron Bruce provided technical assistance constructing the plasmid for the RT-qPCR. Jim zumBrunnen provided statistical assistance. Thanks to Jillian Lang for the LAMP conversations, and to Janet Hardin for identifying thrips. Kris Otto and Mark McMillan, I have appreciated your field expertise. Special thanks to the undergraduate assistants, Matt Miller, Robin Ward, Aubrey Smeal, and Libby Atwater, who did a lot of really tedious work, and probably wish they had never heard of thrips. Finally, thank you Peter Forrence, for listening and conversing, and for your patience.

TABLE OF CONTENTS

INTRODUCTION

Onions

Taxonomy

Allium cepa L., the common onion, is currently taxonomically situated as follows: Class: Monocotyledones, Order: Asparagales, Family: Alliaceae, Genus: *Allium* (15). Unless otherwise specified, *Allium cepa* L. will hereafter be referred to as common onion, or onion, denoting the crop grown for its fleshy bulb.

Center of origin and domestication

Approximately 780 *Allium* species (15) occur worldwide, primarily in the Northern hemisphere between the Arctic Circle and Tropic of Cancer (39). Only one *Allium* species has been described in the southern hemisphere (39). Species diversity is greatest from the Mediterranean through Central Asia, followed by North America (39). Most *Allium* species grow in arid climates, however, adaptation to diverse environments has occurred (39).

The common onion grown today for its bulb-like storage structure does not exist in the wild (15). Domestication likely first occurred in the Middle East or southwest Asia in what is now Iran and Turkmenistan when the people of ancient civilizations transplanted wild onions into gardens (15, 39). Historical records and artifacts show that onions have been cultivated for at least 4,700 years (15). Ancient Egyptians placed onions in the eviscerated body cavity during mummification (1), and onions are depicted in carvings (39). The people of ancient India, as well as the Greeks and Romans are known to have used onions (39). The Romans likely

introduced onions throughout Europe and the Europeans later introduced onions to the Americas (39).

Description and growth (15, 39)

A representative onion is shown in Figure 1.1. The onion stem, also referred to as the basal plate, is a flat, disk-like structure at the base of the onion bulb. Leaves grow in an alternate and opposite arrangement, with the youngest leaf emerging from the center of the stem. The leaves are hollow and somewhat cylindrical, tapering to a point at the tip. The older leaves form a sheath around the young, emerging leaves, and this sheath is known as a pseudostem. The onion bulb is formed as the base of the leaves swell. A subglobose umbel consisting of up to several hundred individual white flowers is borne at the end of a scape (Figure 1.2). Onion scapes are hollow stalks, can grow to over one meter in height, and typically have a bulge in the lower half.

Figure 1.1 Onions forming bulbs. (Photo: Howard F. Schwartz, Colorado State University, Bugwood.org)

Figure 1.2. Onion scapes with umbels. (Photo: Howard F. Schwartz, Colorado State University, Bugwood.org)

The growth stages of onions are shown in Figure 1.3. Upon seed germination the cotyledon emerges as a loop. The cotyledon eventually senesces as leaves develop. As new leaves grow, older leaves continue to progressively senesce. When the plant has formed 8 to 13 leaves the bulb begins to swell. The foliage may fall over (also known as cropping) after new leaves have stopped forming and the pseudostem is left hollow. Finally the outer bulb skins dry, forming a thin, papery covering over the bulb, and the leaves senesce. Flowering may occur during the next growing season after a period of vernalization (15). Following pollination by honey bees (64) small, black seeds are produced in capsules.

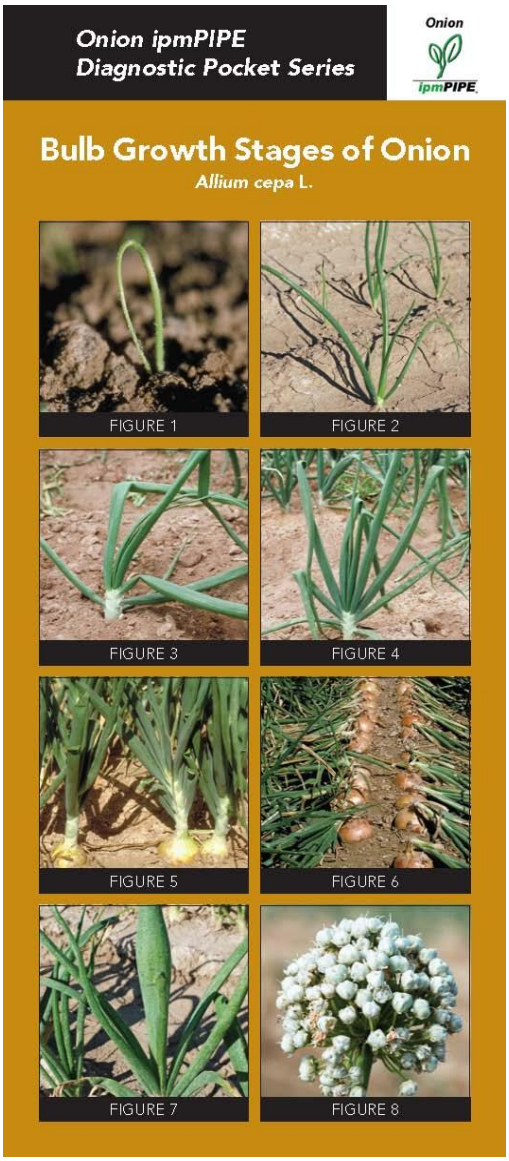


Figure 1.3. The growth stages of onion, beginning with cotyledon emergence (FIGURE 1), proceeding through vegetative growth (FIGURES 2 to 4), bulb formation (FIGURE 5), cropping (FIGURE 6), and scape formation and flowering (FIGURES 7 and 8). (Image: Howard Schwartz, Colorado State University)

Current use

Onions are primarily grown for consumption and are used by individuals cooking at home, in restaurants, and in the manufacture of prepared foods. Onions have also long been used in traditional medicine and have since proven to contain compounds with antimicrobial, antiasthmatic, and anti-tumorigenic properties (13). In addition, onion consumption has been

linked to decreased platelet aggregation. Negative effects of onion consumption may include bad breath, acid reflux, or allergic response (13).

Economic value

Worldwide onion production was approximately 83 million metric tons harvested from approximately 4.2 million hectares during 2012-2013 (38). During 2013 in the United States 61,326 hectares were planted to onion and 58,007 hectares were harvested at a value of $969,183,000. In Colorado during 2013, 2,428 hectares were planted to onion and 1,618 were harvested with a value of $28,539,000 (84).

***Thrips tabaci* Lindeman**

Taxonomy and Center of Origin

Thrips tabaci Lindeman (Figure 1.4), commonly known as onion thrips, is a worldwide pest of onion (25). This insect is placed in the Order: Thysanoptera, Suborder: Terebrantia, Family: Thripidae, Subfamily: Thripinae, and Genus: *Thrips* (28). *T. tabaci* may have arisen in the eastern Mediterranean (94).

Figure 1.4. Adult *Thrips tabaci* Lindeman. (Photo: Alton N. Sparks, Jr., University of Georgia, Bugwood.org)

Life Cycle

Thrips undergo hemimetabolous metamorphosis as they develop from egg to adult (Figure 1.5). Onion thrips development from egg to adult typically takes two to three weeks (25), however, total development time is temperature dependent (28) and will vary. Eggs are typically laid in the leaf tissue, but *T. tabaci* will lay eggs in any part of the onion plant including flower petals (28). After hatching, thrips feed on during two larval instars, followed by nonfeeding prepupal and pupal instars spent in the soil at the base of the plant, before emerging as winged adults (25). *T. tabaci* are capable of various types of parthenogenic reproduction which contributes to their success as a pest of onion. Unfertilized eggs may develop into females

(thelytoky), into males and females (deuterotoky), or unfertilized eggs can develop into males while fertilized eggs develop into females (arrhentoky) (28).

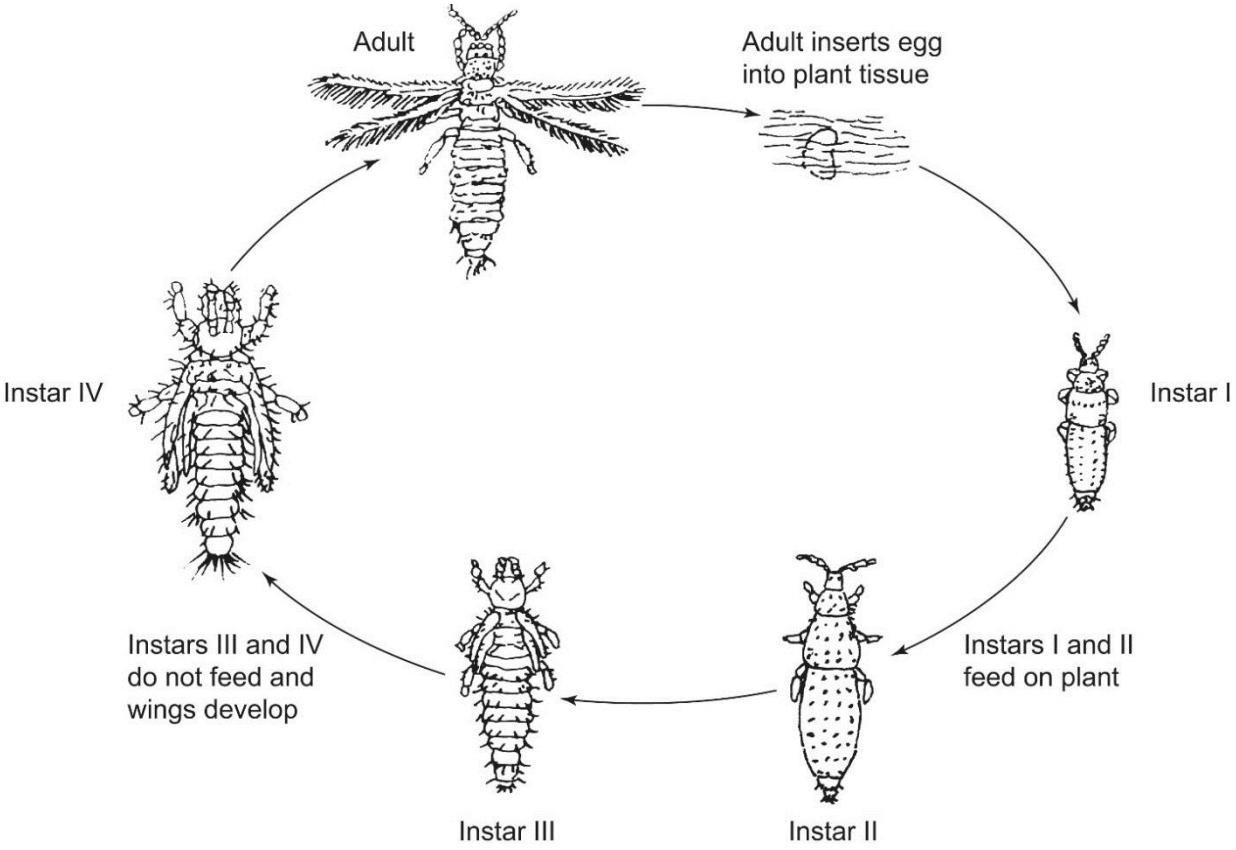


Figure 1.5. The life cycle of onion thrips. (Image: W.S. Cranshaw in Compendium of Onion and Garlic Diseases and Pests 2nd ed., APS Press)

T. tabaci are minute insects and adult female onion thrips (measuring 1.0 to 1.3 mm) are typically longer than males (0.7 mm) (28). These insects are visible with the naked eye, but microscopy is necessary to distinguish any morphological features used in classification.

Habits of onion thrips

Eggs are typically laid along the length of older leaves and after hatching the larvae move down the leaves and congregate in the crevices where new leaves have emerged. Adults spend more of their time on exposed leaf surfaces where insecticides are more likely to reach them

(66). *T. tabaci* concentrations vary randomly throughout an onion field, but areas with higher numbers, such as edges, may be present (28).

T. tabaci thrive in warm, dry environments especially when onions, their preferred host, are present in large numbers (94). Thrips numbers tend to increase in hot, dry weather, but it is unclear if the increase is due to onion plant quality, favorable temperatures, or to the lack of rain which can wash thrips from onions and destroy prepupal and pupal thrips in the soil (28).

T. tabaci are phytophagous and will feed from leaf, petal, stamen and style cells, pollen, developing seeds, fruit, and nectar. Onion thrips will occasionally prey on other small arthropods (94). Thrips feed by first puncturing a cell wall with their mandible, then by sucking out the cellular contents through their maxillary stylets. This feeding strategy has been termed "punch and suck" (94). Plant tissue areas where thrips have fed have a silvery appearance (25).

Thrips cause injury to onion plants by reducing the photosynthetic capacity of the plant which can result in decreased yield, and by introducing fungal, viral, and bacterial pathogens (25, 28, 34). Wounds created by onion thrips can facilitate infection by fungal pathogens (28), and *T. tabaci* is the vector of *Iris yellow spot virus* (21).

Iris yellow spot virus

Taxonomy

Although still awaiting formal species approval by the International Committee on Taxonomy of Viruses (98), available evidence supports *Iris yellow spot virus* as a member of the genus *Tospovirus* in the family Bunyaviridae. Viruses within the Bunyaviridae consist of three negative and/or ambisense single stranded RNA (ssRNA) molecules, each of which is surrounded by a protein coat. The tripartite genome is enveloped by an 80 to 120 nm diameter

spherical lipid bilayer derived from host cell Golgi membranes. Glycoproteins project from the membrane envelope. The three ssRNA segments are known as S RNA, M RNA, and L RNA, due to their size differences. Each of the three ssRNA segments has complimentary termini allowing each strand to form a closed loop.

Members of the genus *Tospovirus* are characterized by: conserved terminal sequences on each RNA segment; the S and M RNAs are ambisense; glycoproteins are encoded in the viral-complimentary sense of the M RMA, while the NSm is encoded in the viral sense of the same RNA; the N protein is encoded in the vc-sense and the NSs is encoded in the v-sense of the S RNA; and they are transmitted by thrips.

Tospovirus species are determined by their vector specificity, plant host range, and N protein characteristics. In order to be considered a species, the N protein amino acid sequence must be at least 90% different from the other *Tospovirus* species. Currently 9 species are formally recognized and 14 are awaiting approval (98, 99).

Iris yellow spot virus was first isolated in The Netherlands from *Iris hollandica* Tub. plants displaying yellow to necrotic spots (21). The 30 kDa IYSV nucleoprotein (N) was found to be distinct from other known tospovirus nucleoproteins. The IYSV N protein did not interact with the anti-N sera of the other known tospoviruses, nor did the N proteins of the other tospoviruses react with the IYSV N antiserum in DAS-ELISA tests (21).

Symptoms on onion

Pozzer et al. (75) described IYSV symptoms on onion as, "necrotic eyelike spots on leaves and flower stems, followed by abortion of the flowers in the umbel, and finally resulted in death of flowers." Kritzman et al. (61) described the symptoms on onion as "straw-colored,

chlorotic, and necrotic lesions on leaves." Lesions on scapes can coalesce, causing the scape to collapse (40), which has a negative impact on seed production.

Figure 1.6. IYSV symptoms on an onion plant (left) and in an onion field (right). (Photos: Howard F. Schwartz, Colorado State University, Bugwood.org)

Infection by IYSV has economic consequences. IYSV incidence has been associated with a reduction in colossal and jumbo onion yield (42). At times, IYSV has caused complete loss of onion bulb and seed crops in Brazil (75). IYSV has been attributed to a 50 to 60% loss in onion bulb crop in Israel (60). While conducting cultivar trials, du Toit and Pelter (31) found a negative correlation between IYSV incidence and total marketable and jumbo onion yield. Shock et al. (86) also found a negative correlation between IYSV incidence and bulb yield. Decreased onion seed yield has been correlated to increased insecticide use to control thrips in an attempt to prevent IYSV infection (64). Economic losses in the form of increased labor costs

may occur when lesioned leaves must be removed from bunching onions before they are acceptable in the marketplace (59).

IYSV distribution throughout the world

IYSV may have been present in Brazil as early as 1981, causing a disease called "sapeca" by local growers. The cause of "sapeca" was not confirmed as IYSV until 1998 (75). Symptoms typical of IYSV were first observed on onion scapes in Idaho and Oregon in 1989 (47) and electron microscopy revealed enveloped particles consistent with the morphology of a Tospovirus (47). Unfortunately, mechanical transmission to onion was unsuccessful and Koch's postulates remained uncompleted.

Symptoms later understood to be caused by IYSV were observed in field grown onion accessions in 1999 in Washington. In 2005, IYSV was confirmed in field grown accessions of the wild onion species *Allium pskemense*, *A. vavilovii*, and *A. altaicum* (71). Since its first observation and characterization, IYSV has been detected in onion crops throughout the world (Table 1.1).

Table 1.1. Chronology of IYSV infection of onion crops around the world.

Year[a]	Location	Year	Location	Year	Location
1998	Israel (43)	2007	Germany (59)	2010	Austria (59)
1999	Brazil (75)	2007	The Netherlands (48)	2010	Mauritius (63)
2001	Slovenia (59)	2007	South Africa (32)	2010	Mexico (97)
2001	USA, Colorado (82)	2007	USA, New York (51)	2010	Uruguay (20)
2003	Australia (23)	2007	Western Oregon (41)	2010	USA, Hawaii (85)
2004	USA, Georgia (68)	2008	Canada (49)	2010	USA, Pennsylvania (50)
2004	New Mexico (26)	2008	France (59)	2011	Eastern Africa (93)
2004	Washington (33)	2008	Serbia (18)	2011	Kenya, Uganda (12)
2005	Chile (79)	2009	Greece (19)	2011	Tajikistan (2)
2005	Egypt (35)	2009	Italy (59)	2013	Bosnia and Herzegovina (96)
2005	USA, Oregon (27)	2009	New Zealand (100)	2013	Indonesia (72)
2006	India (76)	2009	USA, Nevada and California (7)	2013	Pakistan (55)
2006	Peru (67)				
2006	Reunion Island (78)				
2006	USA, Texas (65)				

[a]Year IYSV was first observed or formally reported.

Additional plant hosts of IYSV

Nearly 50 plants other than *A. cepa* have been shown to host IYSV (Table 1.2). The early reports on IYSV claim that the host range is narrow (21, 60, 75); however, new plant hosts are frequently identified, thus expanding the known host range. Currently, plants from 18 families have been documented as IYSV hosts (89). Plant hosts of IYSV have also been summarized by Smith et al. (89) and Schwartz (81) and their summaries contain additional taxonomic information and references.

Acquisition and transmission of IYSV by Thrips tabaci

Thrips tabaci was confirmed as a vector of IYSV by Cortes et al. (21). IYSV is transmitted in a persistent, propagative and transstadial manner. There is no evidence of transovarial transmission. There is no evidence of IYSV transmission by *Frankliniella occidentalis* (21), or *Frankliniella schultzei* (75). Studies by Kritzman et al. (61) confirmed that *T. tabaci* was able to acquire IYSV from infected onion plants and transmit to healthy plants, but that *F. occidentalis* was not able to vector IYSV. They were able to detect IYSV in 33 to 50% of the onion leaf pieces they placed single field collected thrips on, which they interpreted as a 33 to 50% transmission rate. Without knowing if the initial thrips were infected with IYSV, this rate can only be an approximation.

Inoue et al. (57) found no statistically meaningful differences in larval mortality, development time, or fecundity of thelytokous *T. tabaci* exposed or unexposed to IYSV; they suggested that the neutral effect of IYSV on onion thrips may explain the rapid spread of the disease. They also found that both larvae and adults can transmit IYSV with efficiencies ranging from 17.3% to 44.1%, and that second instar larvae and adults transmitted at about the same

efficiency. *N. benthamiana* and impatiens were used as indicator plants in the experiment, so it is possible that the results may differ when *T. tabaci* are reared on their preferred host plant.

The exact route a tospovirus (including IYSV) travels through a thrips is unknown, but a probable route has been suggested (101). Detection of labeled virions by electron microscopy suggests ingested virus particles pass through the thrips' foregut into the midgut. From the midgut the virions must pass through the membranes of the microvilli, columnar epithelial cells, and muscle cells surrounding the midgut, and finally into the salivary glands. Viral replication may occur in any of these cells. During the larval stages the salivary glands, midgut, and visceral muscles are in direct contact with each other which may facilitate viral movement from the foregut into the salivary glands. As thrips mature these structures lose contact. These anatomical changes are currently the most favored explanation of tospoviral movement through thrips and serve as an explanation why adult *F. occidentalis* cannot transmit TSWV not acquired as a larvae.

Assuming *T. tabaci* undergo similar anatomical changes, *T. tabaci* would presumably have to acquire IYSV as larvae in order to transmit the virus. Hoedjes et al. (48) were able to detect the IYSV N and NSs proteins in larval, pupal, and adult *T. tabaci* after a period of feeding on infected followed by uninfected plant material, thus providing evidence that IYSV replicates in the insects.

Table 1.2. Additional *Allium* and non-*Allium* host plants of IYSV.

Host plant		Location and year of first published report
Allium altaicum	Wild onion	Washington, USA, 2006 (71)
Allium ampeloprasum	Egyptian leek	Egypt, 2011 (45)
Allium cepa var. ascalonicum	Shallot	Réunion Island, 2006 (78)
Allium pskemense	Wild onion	Washington, USA, 2006 (71)
Allium porrum	Leek	Réunion Island, 2006 (78)
Allium sativum	Garlic	Réunion Island, 2006 (78)
Allium roylei	Wild onion	New Mexico, USA, 2011 (24)
Allium schoenoprasum	Chive	New Mexico, USA, 2011 (24)
Allium tuberosum	Garlic chives	New Mexico, USA, 2011 (24)
Allium vavilovii	Wild onion	Washington, USA, 2006 (71)
Alstroemeria sp.	Alstroemeria	Netherlands, 2004 (48)
Amaranthus retroflexus	Redroot pigweed	Idaho and Washington, USA, 2007 (80)
Arctium minus Bernh.	Common burdock	New York, USA, 2011 (53)
Atriplex micrantha Ledeb.	Twoscale saltbush	Utah, USA, 2009 (37)
Bessera elegans	Coral drops	Japan, 2005 (58)
Capsicum annuum L.	Pepper	Tunisia, 2005 (10)
Cichorium intybus L.	Chicory	New York, USA, 2011 (53)
Chenopodium album	Common lambsquarters	Idaho and Washington, USA, 2007 (80)
Chenopodium amaranticolor		Brazil, 1999 (75)
Chenopodium quinoa Wild.	Quinoa	Brazil, 1999 (75)
Clivia minata	Natal lily	Japan, 2005 (58)
Cycas sp.		Iran, 2005 (44)
Datura stramonium	Datura	Netherlands, 2005 (21)
Dendranthema grandiflora (D.C.)Desmoul.	Chrysanthemum	Poland, 2005 (8)
Eustoma grandiflorm	Lisianthus	United Kingdom, 2008 (69)
Eustoma russellianum	Lisianthus	Israel, 2000 (60)
Geranium carolinianum	Carolina geranium	Georgia, USA 2006 (40)
Gomphrena globosa L.	Globe amaranth	Brazil, 1999 (75)
Iris hollandica Tub.	Iris	Netherlands, 1998 (21)
Kochia scoparia	Kochia	Idaho and Washington, USA, 2007 (80)
Lactuca serriola	Prickly lettuce	Idaho and Washington, USA, 2007 (80)
Linaria canadensis	Canada toadflax	Georgia, USA 2006 (40)
Nicotiana benthamiana	Wild tobacco	Brazil, 1999 (75)
Nicotiana rustica L.	Wild tobacco	Brazil, 1999 (75)
Pelargonium hortorum	Zonal geranium	Iran, 2005 (44)
Petunia hybrida	Petunia	Netherlands, 1998 (21)
Portulaca oleracea	Common purslane	Brazil, 1999 (75)
Rosa sp.		Iran, 2005 (44)
Rumex crispus L.	Curly dock	New York, USA, 2011 (53)
Scindapsus sp.		Iran, 2005 (44)
Setaria viridis (L.) Beauv.	Green foxtail	Utah, USA, 2009 (36)
Solanum lycopersicum L.	Tomato	Tunisia, 2005 (10)
Solanum tuberosum L.	Potato	Tunisia, 2005 (10)
Sonchus asper	Spiny sowthistle	Georgia, USA, 2007 (70)
Taraxacum officinale G. H. Weber ex. Wiggers	Dandelion	New York, USA, 2011 (53)
Tribulus terrestris	Puncturevine	Idaho and Washington, USA, 2007 (80)
Vicia sativa	Common vetch	Georgia, USA 2006 (40)
Vigna unguiculata	Cowpea	Iran, 2005 (44)

Additional thrips hosts of IYSV

Srinivasan et al. (92) reported IYSV replication in and transmission by *Frankliniella fusca* on the indicator plant lisianthus (*Eustoma russellianum*). This result must be interpreted cautiously. *Frankliniella fusca* larvae were reared on lisianthus plants that had been inoculated by *T. tabaci*. Although the authors claimed that *T. tabaci* did not survive on lisianthus, experience has shown that when examined microscopically, plants that appear devoid of thrips may actually harbor a small number. It is difficult to distinguish thrips species while larvae, and the authors did not specify their method of species determination. It is possible that a small population of *T. tabaci* survived on lisianthus among the *F. fusca* that were transferred to the plant. If this were the case it would appear that *F. fusca* acquired and transmitted IYSV. Verifying the larvae were *F. fusca* by PCR would strengthen the claim.

In Georgia, USA, *F. fusca* is observed more frequently on onion than *T. tabaci* (91). The presence of both species on IYSV infected onions does raise the possibility of *F. fusca* becoming an additional vector.

Other modes of transmission

IYSV is not known to be seed transmitted (17, 61). Kritzman et al. (61) collected 25 onion bulbs from IYSV infected plants and planted them. The resulting plants did not develop symptoms nor was IYSV detected in the leaf tissue, suggesting that IYSV does not remain in onion bulbs between growing seasons.

IYSV structure and genomic organization

The IYSV genome is contained within an 80 to 120 nm spherical membrane (60) derived from the host (101). The tripartite genome of IYSV consists of three RNA segments designated S, M, and L, of approximately 2.9, 4.8, and 8.9 kb, respectively (21).

Cortes et al. (21) sequenced the 3,105 nucleotide IYSV S RNA. Several features of this sequence including a conserved 5' to 3' terminal sequence, complementary sequences allowing the RNA strand to form a panhandle structure, and ambisense open reading frames (ORF) allowed them to confirm that IYSV was a new tospovirus.

Nucleotide composition among IYSV isolates is somewhat variable. Cortez et al. (22) sequenced the M RNA segment of an IYSV isolate from The Netherlands and found it was comprised of 4838 nt, while Bag et al. (4) found the M RNA from an isolate from Washington, USA to be comprised of 4821 nt. Both groups found that the M RNA contained an ORF in both the viral (v-) and viral complimentary (vc-) sense. Amino acid sequence comparisons with other tospoviruses led both groups to conclude that the ORF in the v-sense likely encodes a small non-structural protein termed NSm, a predicted movement protein. The ORF in the vc-sense encodes a glycoprotein precursor, which is later processed into the G1 and G2 (or Gn and Gc, depending on author) glycoproteins (4, 22). Like the S RNA, the M RNA has an AU rich region between the protein coding regions that allow the formation of a hairpin structure, and two complimentary regions at the 5' and 3' termini (4, 22). These two regions are thought to give the molecule stability (21).

The L RNA consists of 8,880 nucleotides (3). The 5' and 3' termini are complimentary and conserved, and there is an ORF in the vc-sense. Based on amino acid sequence comparison with other tospoviruses, the L RNA ORF likely encodes an RNA-dependent RNA polymerase

(RdRp) (3). No ORFs were found in the v-sense, and it was concluded that the L RNA functions as a negative sense RNA (3).

IYSV proteins

Cortes et al. (21) predicted that in the v-sense the S RNA segment encoded a 50.1 kDa protein named NSs with unknown function. Due to structural similarities with the NSs of *Rift Valley fever virus* and the diversity of the NSs sequence within the tospoviruses, Cortes et al. (21) suggest that the NSs protein may not be directly involved in replication but is important in determining host range and pathogenesis. The NSs protein of other tospoviruses has been reported to suppress RNA silencing during plant infection (101). Work by Bag et al. (5) supports the putative RNA silencing suppression function of the NSs. They demonstrated a synergistic effect of TSWV and IYSV when both were co-inoculated into Datura plants. It is not uncommon for a single plant to be infected with more than one Tospovirus. Infection with more than one virus may have a synergistic effect, worsening symptoms, or may provide an avenue for genetic recombination and emergence of new viruses. Symptoms were more severe, and the Datura plants died earlier when infected with both TSWV and IYSV. They attributed the effect to the systemic expression of the IYSV NSs gene in addition to the TSWV NSs gene, which likely acted as an additional suppressor of RNA silencing.

Further support of the NSs protein acting as an RNA silencing suppressor comes from Hafez et al. (46) who found some changes in gene expression in leek in response to infection with IYSV. Proteins involved with plant response to pathogens including mitogen-activated protein kinase, pathogenesis-related protein, and serine/threonine-protein kinase, were up-regulated. They also found that the alpha-tubulin suppressor-like protein was down regulated.

This is interesting and supports the idea that the NSs suppresses the manufacture of the alpha-tubulin suppressor protein, allowing the NSm protein to form tubules (101).

Through cloning and expressing the S RNA vc-sense ORF in *E. coli*, Cortes et al. (21) were able to confirm that the nucleoprotein (N protein) is encoded by this RNA segment.

The exact function of the NSm is unknown, but Silva at al. (88) propose several possibilities including interaction with plasmodesmata proteins, the viral N proteins, or the viral RNA. Studies of the NSm proteins of other plant viruses revealed that the protein is expressed early, alters plasmodesmata, and forms tubules, all of which suggest this protein facilitates viral cell to cell movement (101).

Tospoviral glycoproteins contain hydrophobic regions that anchor them in the viral membrane. Glycoproteins may be involved with virion assembly or with host cell membrane interactions (101). The specific functions of the IYSV G1 (Gn) and G2 (Gc) glycoproteins are still unknown, but sequence homology with TSWV and several animal infecting Bunyaviruses suggests that G1 (Gn) may bind to the insect midgut (22). The two glycoproteins associated with TSWV were shown binding the virion to the thrips midgut and fusing the viral and host cell membranes (101). It would not be unreasonable for the IYSV glycoproteins to function in a similar manner.

IYSV replication

The specific details of IYSV replication are not known, but IYSV replication is likely similar to the replication process of other tospoviruses. The general replication process of a tospovirus begins with virion attachment to a host cell. The RNA genome enters the cell and becomes uncoated. The RdRp transcribes mRNA which is translated by the host cell's

ribosomes. RNA strands complimentary to the original viral genomic RNA are synthesized and used as templates for genomic replication. The new viral genomic RNA strands are coated with nucleoprotein, and the tripartite genome is enveloped with a host derived membrane (98).

Virus receptors have not yet been identified in thrips (101); however, tospovirus replication within thrips is thought to begin when the glycoproteins in the viral envelope bind and fuse with the host cell. Upon membrane fusion the N protein enveloped RNA genome is released into the host cell's cytoplasm where replication occurs (54, 101). The TSWV RdRp removes the cap ('cap-snatching') and an additional 10 to 20 nt from host mRNA and attaches it to viral mRNA. The host cell then recognizes the viral mRNA as its own and initiates translation (101).

Birithia et al. (11) found evidence of IYSV replication in *T. tabaci* by using direct antigen-coated ELISA to follow NSs protein accumulation over time. They found greater absorbance over time which they associated with accumulation of the NSs protein. They did not observe increases in absorbance in *F. occidentalis* or *F. schultzei*, or in any of the thrips that fed on healthy plants. Non-structural proteins such as NSs are not present in virions, but are present during replication and movement.

Emergence/Evolution

It is unclear how IYSV became a distinct *Tospovirus* species, but analysis of the nucleotide and amino acid sequences give clues as to how new viruses evolve. The RdRp is error prone, and genomic reassortment can occur in plants infected with multiple viruses, thus contributing to genetic variability and driving evolution (101). The approximately 60 amino acid

N-terminal sequence of the NSm protein is the most variable region among tospoviruses (22, 88) and this variability may be one of the determining factors of host range (22).

Phylogenetic analysis of the N and NS_M proteins place IYSV in a group with a Eurasian origin. GBNV (*Groundnut bud necrosis virus*) and WSMV (*Watermelon silver mottle virus*) are also in this group (88). Sequence comparison of the RdRp among tospoviruses continues to group IYSV with tospoviruses of Eurasian origin (3). Phylogenetic analysis of the N gene of IYSV isolates in Australia, group the Australian isolates with Japanese isolates (90). The authors suggest the Australian and Japanese isolates share a common ancestor, and that IYSV did not originate in Australia.

Later analysis of 98 IYSV N gene sequences from isolates collected worldwide revealed temporal and spatial changes (56). Most IYSV isolates fit into one of two genotypes: Netherlands ($IYSV_{NL}$) or Brazil ($IYSV_{BR}$). Most of the IYSV isolates collected in North America and Europe fit best with the $IYSV_{NL}$ genotype, while isolates from Asia and Australia grouped with the $IYSV_{BR}$ genotype. The percentage of isolates belonging to either genotype has shifted slightly from just over half belonging to the $IYSV_{NL}$ genotype prior to 2005, to just under half since then. Recombination detection analysis suggests that $IYSV_{BR}$ may have evolved from $IYSV_{NL}$.

Detecting IYSV

IYSV can be detected by serological and molecular methods. DAS-ELISA, reverse-transcriptase PCR, and real time reverse transcriptase PCR methods have been described (73). The availability of IYSV sequences in GenBank allows any researcher to easily develop primers suitable for various PCR methods. A DAS-ELISA kit, and an ImmunoStrip kit are commercially

available through Agdia, Inc. (Elkhart, IN). Reduction of false positives can be reduced by following the modification to DAS-ELISA described by Gent et al. (42).

False negatives may also occur if the IYSV isolate is serologically distinct from those recognized by a given antibody (95), in which case detection of a conserved region by RT-PCR may be necessary to confirm a suspected infection.

IYSV distribution in onion

IYSV is unevenly distributed throughout onion plants, but has been detected in each leaf in pre- and post-bulbing onions (14). IYSV has not been detected in onion bulb, basal plate, or root samples by DAS-ELISA (14, 61). Kritzman et al. (61) performed ELISA on eight onion leaves infected with IYSV and found the highest absorbance readings in the portion of the leaves closest to the bulb, which may indicate a higher concentration of virus particles in those samples relative to samples obtained toward the leaf tip.

Mechanical inoculation

Reliable mechanical inoculation of IYSV into onion plants has not yet been achieved. Kritzman et al. (61) were able to detect IYSV by DAS-ELISA from onion plants that had been mechanically inoculated but the plants did not develop symptoms. It is possible they were detecting the inoculum and not newly replicated virus. Pozzer et al. (75) reported that although they did not observe symptoms on mechanically inoculated onion plants, they did observe symptoms on *Nicotiana benthamiana* after inoculation with an extract prepared from the mechanically inoculated asymptomatic onion plants. This suggests either the inoculum remained infectious, or IYSV replicated within the onion plants without causing symptoms. Beikzadeh et

al. (9) were also able to observe symptoms on indicator plants after mechanical inoculation with IYSV extracts, but did not observe symptoms on onion after attempting mechanical inoculation.

Sources of IYSV inoculum

How IYSV arrives in new locations and persists in the environment is still under investigation. There is evidence that IYSV and *T. tabaci* are transported in onion transplants (40). IYSV may persist in volunteer onions. Gent et al. (42) found IYSV in volunteer onions growing amidst other crops, but pointed out that it was unclear how IYSV arrived at the volunteers. It is still unknown if IYSV overwinters in onion bulbs or if volunteer onions are infected by thrips. Many of the additional plant hosts listed in Table 1.2 are common weeds found around onion fields and likely play a role in the persistence of thrips and IYSV in the environment. Onion cull piles may be another source of inoculum as 35.7% of thrips collected from cull piles in Georgia were viruliferous (6). More work needs to be done to determine if (or which) crops adjacent to onion fields influence and contribute to IYSV incidence (42).

Thrips and IYSV in the field

Within a field, IYSV incidence is highest along edges and decreases toward the center of the field (42). du Toit and Pelter (31) also observed a higher incidence of IYSV at a field's edge than in the center and proposed that thrips migrated from adjacent fields. Secondary movement of thrips within the field is thought to be limited compared with thrips migration (31, 42).

Hsu et al. (52) found that thrips numbers were greater in transplanted onion fields than seeded onion fields early in the season, but later in the season thrips numbers were greater in seeded fields. Thrips numbers were greater in fields harvested later in the season than earlier.

Whether the field was transplanted or seeded did not affect when IYSV was first detected or how the disease would develop, but IYSV incidence was greater in seeded fields. IYSV incidence increased as the growing season progressed and was greater in fields harvested later in the season.

Bag et al. (6) developed antibodies against the NSs and were able to use them in a DAC-ELISA assay to distinguish viruliferous thrips from thrips carrying non-replicating IYSV. In thrips collected from fields in Hermiston, Oregon the greatest proportion of viruliferous thrips was found in mid-July. Thrips samples from Georgia were also checked, and 58.9% of those collected from IYSV infected onion plants were viruliferous by this assay.

IYSV and thrips management

Several strategies should be utilized to manage *T. tabaci* and IYSV. Insecticides are typically used to suppress thrips populations in the hope that IYSV transmission will also be suppressed, however, thrips populations are prone to developing insecticide resistance (25). Schwartz et al. (83) found that a conventional insecticide treatment did not control thrips, but there was a reduction in thrips numbers when reduced-risk insecticides were used. Conventional insecticide regimes may not be effective because thrips can continue to migrate from other crops; viruliferous thrips that escape the insecticide treatment can continue to infect plants; and thrips tend to congregate in tight spaces (such as the onion neck) inaccessible to insecticides (74). The timing and order of the insecticides used can affect thrips numbers and onion yield (77). In order to prevent thrips from becoming resistant to insecticides, it is recommended that no more than two applications of a particular chemistry be used in a season; growers may need to use several different insecticides with different modes of action throughout the season (77). Controlling

thrips late in the season to reduce the number that migrate into the field may prevent new introductions of IYSV to fields harvested later (52).

Cultural practices may also prevent or diminish the effects of onion thrips and IYSV (40). If transplants are used, they should be free of *T. tabaci* and IYSV. If possible, seed producers should choose plants with shorter scapes, as shorter scapes are less likely to lodge than tall scapes when numerous lesions develop. Volunteer onions should be removed and onions should not be planted in close proximity to other *Allium* species. As of 2006, the role of weed control to manage IYSV was unknown, but it is generally advised that weeds be controlled. Physically separating seed and bulb crops with large distances is also speculated to reduce the spread of IYSV, however, the appropriate separation distance is unknown. Plant density may affect IYSV incidence, but this relationship may be cultivar dependent. Thrips numbers often decrease on onion plants after a heavy rain, and overhead irrigation may provide a similar function, thus reducing thrips and IYSV incidence.

Although research on the relationship to onion plant stress and IYSV is lacking, it is advised that plant stress be minimized (40). Shock et al. (87) found that IYSV symptoms were more severe and yield was lower on onion plants that received less than an optimal amount of water.

Buckland et al. (16) found an association of high rates of nitrogen fertilizer with high numbers of adult onion thrips. They also found that when onions were planted following wheat, adult onion thrips were greater than if onions followed corn and attributed this to a lower nitrogen level in the soil following corn than wheat. They were unable to find any factors that influenced the incidence of IYSV, but noted that incidence was low during the years the study was conducted. They do suggest that planting onions after corn may be a better option than

planting onions after wheat. Rotation of onions with nonhost crops may reduce the spread of IYSV, however, this is speculation (40).

Schwartz et al. (83) reported that applying straw mulch to a depth of 10 cm to onion beds reduced thrips numbers compared to onions grown on bare soil, however, the straw mulch did not appear to affect IYSV incidence. Larentzaki et al. (62) found that straw mulch reduced the number of thrips larvae, but not adults, and suggested the mulch may impede thrips development within a field, but did not prevent adult migration from other fields. The straw mulch did allow delayed insecticide application.

If harvest dates are to be staggered, it may be best to place some distance between fields to prevent thrips from migrating between harvested and unharvested fields (52). An appropriate distance between fields that would prevent thrips migration is yet to be determined.

Strategies used to manage other tospoviruses have included: planting non-host barriers between crops; planting up wind of the inoculum source; adjusting agronomic practices; and planting resistant cultivars (74). Onion cultivars resistant to IYSV are not available.

Resistance

While there is as yet no evidence of resistance to IYSV, some cultivars appear less susceptible than others. IYSV incidence differed in cultivars Granero and Sterling planted adjacent to each other (42). Cultivar trials conducted in Washington showed IYSV incidence differed among 46 onion cultivars, but each cultivar was susceptible (31). In cultivar trials conducted by Shock et al. (86), all cultivars were susceptible to IYSV, but some differences in response, such as incidence and yield, were noted.

Onion cultivars that are resistant to onion thrips are not necessarily resistant to IYSV (30). Resistant cultivars tend to support fewer thrips larvae than susceptible plants. Diaz-Montano et al. (29) identified several onion cultivars resistant to onion thrips, but the same cultivars were not resistant to IYSV. A yellow-green leaf color was associated with resistance to onion thrips. In the absence of IYSV, onion plants exhibiting resistance to thrips were still impacted as plant height and fresh plant weight were reduced in all but one cultivar. Despite lower thrips numbers on the resistant cultivars, it appeared that the thrips still caused enough leaf damage to negatively impact the plants.

Objectives

The objectives of this work were to investigate onion seed as a potential source of IYSV inoculum, to identify additional host plants that may play a role in IYSV survival over the winter, and to develop a quantitative real time PCR assay to make comparisons about the amount of IYSV present in a sample. The presence of IYSV in onion seed is examined in Chapter 2. In Chapter 3 onion cull piles were monitored and several plant species examined as potential overwintering sites of IYSV and *Thrips tabaci*. Chapter 4 describes the development of a real time quantitative reverse transcriptase PCR. The relative amount of IYSV between two onion cultivars was compared, and IYSV distribution in onion leaves described. Appendices 1 to 3 describe work supportive to developing the methods used in Chapters 2 and 3. Appendix 4 includes observations regarding volunteer onions, onion thrips, and IYSV. Appendix 5 is a report on the use of thrips lures in a field setting, and Appendix 6 investigates the use of reverse transcription-loop mediated isothermal amplification as a potentially new IYSV diagnostic tool.

LITERATURE CITED

1. Abdel-Maksoud, G., and El-Amin, A. R. 2011. A review on the materials used during the mummification processes in ancient Egypt. Mediterr. Archaeol. Archaeom. 11 (2):129-150.
2. Alabi, O. J., Saidov, N., Muniappan, R., and Naidu, R. A. 2012. First report of *Iris yellow spot virus* in onion in Tajikistan. New Disease Reports 26:28-28.
3. Bag, S., Druffel, K. L., and Pappu, H. R. 2010. Structure and genome organization of the large RNA of *Iris yellow spot virus* (genus *Tospovirus*, family Bunyaviridae). Arch. Virol. 155 (2):275-279.
4. Bag, S., Druffel, K. L., Salewsky, T., and Pappu, H. R. 2009. Nucleotide sequence and genome organization of the medium RNA of *Iris yellow spot virus* from the United States. Arch. Virol. 154 (4):715-718.
5. Bag, S., Mitter, N., Eid, S., and Pappu, H. R. 2012. Complementation between two tospoviruses facilitates the systemic movement of a plant virus silencing suppressor in an otherwise restrictive host. PLoS One 7 (10).
6. Bag, S., Rondon, S. I., Druffel, K. L., Riley, D. G., and Pappu, H. R. 2014. Seasonal dynamics of thrips (*Thrips tabaci*) (Thysanoptera: Thripidae) transmitters of *Iris yellow spot virus*: A serious viral pathogen of onion bulb and seed crops. J. Econ. Entomol. 107 (1):75-82.
7. Bag, S., Singh, J., Davis, R. M., Chounet, W., and Pappu, H. R. 2009. *Iris yellow spot virus* in onion in Nevada and Northern California. Plant Dis. 93 (6):674-674.
8. Balukiewicz, A., and Kryczyn´ski, S. 2005. Tospoviruses in chrysanthemum mother stock plants in Poland. Phytopathologia Polonica (37):59-67.
9. Beikzadeh, N., Jafarpour, B., Rouhani, H., Peters, D., and Hassani-Mehraban, A. 2012. Molecular diagnosis of *Iris yellow spot virus* (IYSV) on onion in Iran. J. Agric. Sci. Technol. 14 (5):1149-1158.
10. Ben Moussa, A., Marrakchi, M., and Makni, M. 2005. Characterisation of Tospovirus in vegetable crops in Tunisia. Pp. 299-322 in: 9th International Workshop on Virus Evolution and Molecular Epidemiology Infection, Genetics and Evolution, Stanford.
11. Birithia, R., Subramanian, S., Pappu, H. R., Muthomi, J., and Narla, R. D. 2013. Analysis of *Iris yellow spot virus* replication in vector and non-vector thrips species. Plant Pathol. 62 (6):1407-1414.
12. Birithia, R., Subramanian, S., Pappu, H. R., Sseruwagi, P., Muthomi, J. W., and Narla, R. D. 2011. First report of *Iris yellow spot virus* infecting onion in Kenya and Uganda. Plant Dis. 95 (9):1195-1196.
13. Block, E. 1992. The organosulfur chenistry of the genus *Allium* - implications for the organic-chemistry of sulfur. Angew. Chem.-Int. Edit. Engl. 31 (9):1135-1178.
14. Boateng, C. O., and Schwartz, H. F. 2013. Temporal and localized distribution of *Iris yellow spot virus* within tissues of infected onion plants. Southw. Entomol. 38 (2):183-199.
15. Brewster, J. L. 2008. Onions and Other Vegetable Alliums. CABI, Wallingford, UK.

16. Buckland, K., Reeve, J. R., Alston, D., Nischwitz, C., and Drost, D. 2013. Effects of nitrogen fertility and crop rotation on onion growth and yield, thrips densities, *Iris yellow spot virus* and soil properties. Agric. Ecosyst. Environ. 177:63-74.
17. Bulajic, A., Djekic, I., Jovic, J., Krnjajic, S., Vucurovic, A., and Krstic, B. 2009. Incidence and distribution of *Iris yellow spot virus* on onion in Serbia. Plant Dis. 93 (10):976-982.
18. Bulajic, A., Jovic, J., Krnjajic, S., Petrov, M., Djekic, I., and Krstic, B. 2008. First report of *Iris yellow spot virus* on onion (*Allium cepa*) in Serbia. Plant Dis. 92 (8):1247-1247.
19. Chatzivassiliou, E. K., Giavachtsia, V., Mehraban, A. H., Hoedjes, K., and Peters, D. 2009. Identification and incidence of *Iris yellow spot virus*, a new pathogen in onion and leek in Greece. Plant Dis. 93 (7):761-761.
20. Colnago, P., Achigar, R., Gonzalez, P. H., Peluffo, S., Idiarte, H. G., Pianzzola, M. J., and Galvan, G. A. 2010. First report of *Iris yellow spot virus* on onion in Uruguay. Plant Dis. 94 (6):786-786.
21. Cortes, I., Livieratos, I. C., Derks, A., Peters, D., and Kormelink, R. 1998. Molecular and serological characterization of *Iris yellow spot virus*, a new and distinct tospovirus species. Phytopathology 88 (12):1276-1282.
22. Cortez, I., Aires, A., Pereira, A. M., Goldbach, R., Peters, D., and Kormelink, R. 2002. Genetic organisation of *Iris yellow spot virus* M RNA: indications for functional homology between the G((C)) glycoproteins of tospoviruses and animal-infecting bunyaviruses. Arch. Virol. 147 (12):2313-2325.
23. Coutts, B. A., McMichael, L. A., Tesoriero, L., Rodoni, B. C., Wilson, C. R., Wilson, A. J., Persley, D. M., and Jones, R. A. C. 2003. *Iris yellow spot virus* found infecting onions in three Australian states. Austral. Plant Pathol. 32 (4):555-557.
24. Cramer, C. S., Bag, S., Schwartz, H. F., and Pappu, H. R. 2011. Susceptibility of onion relatives (*Allium* spp.) to *Iris yellow spot virus*. Plant Dis. 95 (10):1319-1319.
25. Cranshaw, W. S. 2008. Thrips. Pp. 89-91 in: Compendium of Onion and Garlic Diseases and Pests, 2nd ed., H. F. Schwartz and S. K. Mohan, eds. American Phytopathological Society, St. Paul, MN.
26. Creamer, R., Sanogo, S., Moya, A., and Romero, J. 2004. *Iris yellow spot virus* on onion in New Mexico. Plant Dis. 88 (9):1049-1049.
27. Crowe, F. J., and Pappu, H. R. 2005. Outbreak of *Iris yellow spot virus* in onion seed crops in central Oregon. Plant Dis. 89 (1):105-105.
28. Diaz-Montano, J., Fuchs, M., Nault, B. A., Fail, J., and Shelton, A. M. 2011. Onion thrips (Thysanoptera: Thripidae): A global pest of increasing concern in onion. J. Econ. Entomol. 104 (1):1-13.
29. Diaz-Montano, J., Fuchs, M., Nault, B. A., and Shelton, A. M. 2010. Evaluation of onion cultivars for resistance to onion thrips (Thysanoptera: Thripidae) and *Iris yellow spot virus*. J. Econ. Entomol. 103 (3):925-937.
30. Diaz-Montano, J., Fuchs, M., Nault, B. A., and Shelton, A. M. 2012. Resistance to onion thrips (Thysanoptera: Thripidae) in onion cultivars does not prevent infection by *Iris yellow spot virus* following vector-mediated transmission. Fla. Entomol. 95 (1):156-161.
31. du Toit, L. J., and Pelter, G. Q. 2005. Susceptibility of storage onion cultivars to iris yellow spot in the Columbia Basin of Washington, 2004. Biological & Cultural Tests 20: (V006).

32. du Toit, L. J., Burger, J. T., McLeod, A., Engelbrecht, M., and Vjljoen, A. 2007. *Iris yellow spot virus* in onion seed crops in South Africa. Plant Dis. 91 (9):1203-1203.
33. du Toit, L. J., Pappu, H. R., Druffel, K. L., and Pelter, G. Q. 2004. *Iris yellow spot virus* in onion bulb and seed crops in Washington. Plant Dis. 88 (2):222-222.
34. Dutta, B., Barman, A. K., Srinivasan, R., Avci, U., Ullman, D. E., Langston, D. B., and Gitaitis, R. D. 2014. Transmission of *Pantoea ananatis* and *P. agglomerans*, causal agents of center rot of onion (*Allium cepa*), by onion thrips (*Thrips tabaci*) through feces. Phytopathology 104 (8):812-819.
35. Elnagar, S., El-Sheikh, M. A. K., and Wahab, A. S. A. 2008. *Iris yellow spot virus* (IYSV): a newly isolated thrips-borne tospovirus in Egypt. in: 7ème Conférence Internationale sur les Ravageurs en Agriculture, Montpellier, 26-27 octobre, 2008 Association Française de Protection des Plantes (AFPP), Alfortville; France.
36. Evans, C. K., Bag, S., Frank, E., Reeve, J., Ransom, C., Drost, D., and Pappu, H. R. 2009. Green foxtail (*Setaria viridis*), a naturally infected grass host of *Iris yellow spot virus* in Utah. Plant Dis. 93 (6):670-671.
37. Evans, C. K., Bag, S., Frank, E., Reeve, J. R., Ransom, C., Drost, D., and Pappu, H. R. 2009. Natural infection of *Iris yellow spot virus* in twoscale saltbush (*Atriplex micrantha*) growing in Utah. Plant Dis. 93 (4):430-430.
38. Food and Agriculture Organization of the United Nations, S. D. 2014. Food and agriculture organization of the United Nations.
39. Fritsch, R. M., and Friesen, N. 2002. Evolution, domestication and taxonomy. Pages 5-30 in: Allium Crop Science: Recent Advances, H. D. Rabinowitch and L. Currah, eds. CABI Pub, Wallingford, UK ;.
40. Gent, D. H., du Toit, L. J., Fichtner, S. F., Mohan, S. K., Pappu, H. R., and Schwartz, H. F. 2006. *Iris yellow spot virus*: An emerging threat to onion bulb and seed production. Plant Dis. 90 (12):1468-1480.
41. Gent, D. H., Martin, R. R., and Ocamb, C. M. 2007. First report of *Iris yellow spot virus* on onion and leek in western Oregon. Plant Dis. 91 (4):468-468.
42. Gent, D. H., Schwartz, H. F., and Khosla, R. 2004. Distribution and incidence of *Iris yellow spot virus* in Colorado and its relation to onion plant population and yield. Plant Dis. 88 (5):446-452.
43. Gera, A., Cohen, J., Salomon, R., and Raccah, B. 1998. Iris yellow spot tospovirus detected in onion (*Allium cepa*) in Israel. Plant Dis. 82 (1):127-127.
44. Ghotbi, T., Shahraeen, N., and Winter, S. 2005. Occurrence of tospoviruses in ornamental and weed species in Markazi and Tehran provinces in Iran. Plant Dis. 89 (4):425-429.
45. Hafez, E. E., Abdelkhalek, A. A., El-Morsi, A. A., and Ei-Shahaby, O. A. 2012. First report of *Iris yellow spot virus* infection of garlic and Egyptian leek in Egypt. Plant Dis. 96 (4):594-594.
46. Hafez, E. E., Abdelkhalek, A. A., El-Wahab, A., and Galal, F. H. 2013. Altered gene expression: Induction/supression in leek elicited by *Iris yellow spot virus* infection (IYSV) Egyptian isolate. Biotechnol. Equip. 27 (5):4061-4068.
47. Hall, J. M., Mohan, K., Knott, E. A., and Moyer, J. W. 1993. Tospoviruses associated with scape blight of onion (*Allium-cepa*) seed crops in Idaho. Plant Dis. 77 (9):952-952.
48. Hoedjes, K., Verhoeven, J. T. J., Goldbach, R., and Peters, D. 2011. *Iris yellow spot virus* in the Netherlands: Occurrence in onion and confirmation of transmission by *Thrips*

tabaci. Pages 199-206 in: Xii Inter. Symp. on Virus Diseases of Ornamental Plants, A. Derks, E. T. M. Meekes and C. Stijger, eds. Int. Soc. Hort. Sci., Leuven 1.
49. Hoepting, C. A., Allen, J. K., Vanderkooi, K. D., Hovius, M. Y., Fuchs, M. F., Pappu, H. R., and McDonald, M. R. 2008. First report of *Iris yellow spot virus* on onion in Canada. Plant Dis. 92 (2):318-318.
50. Hoepting, C. A., and Fuchs, M. F. 2012. First report of *Iris yellow spot virus* infecting onion in Pennsylvania. Plant Dis. 96 (8):1229-1229.
51. Hoepting, C. A., Schwartz, H. F., and Pappu, H. R. 2007. First report of *Iris yellow spot virus* on onion in New York. Plant Dis. 91 (3):327-327.
52. Hsu, C. L., Hoepting, C. A., Fuchs, M., Shelton, A. M., and Nault, B. A. 2010. Temporal dynamics of *Iris yellow spot virus* and its vector, *Thrips tabaci* (Thysanoptera: Thripidae), in seeded and transplanted onion fields. Environ. Entomol. 39 (2):266-277.
53. Hsu, C. L., Hoepting, C. A., Fuchs, M., Smith, E. A., and Nault, B. A. 2011. Sources of *Iris yellow spot virus* in New York. Plant Dis. 95 (6):735-743.
54. Hull, R. 2002. Matthews' Plant Virology. Academic Press, San Diego, Calif.
55. Iftikhar, R., Bag, S., Ashfaq, M., and Pappu, H. R. 2013. First report of *Iris yellow spot virus* infecting onion in Pakistan. Plant Dis. 97 (11):1517-1517.
56. Iftikhar, R., Ramesh, S. V., Bag, S., Ashfaq, M., and Pappu, H. R. 2014. Global analysis of population structure, spatial and temporal dynamics of genetic diversity, and evolutionary lineages of *Iris yellow spot virus* (Tospovirus: Bunyaviridae). Gene 547 (1):111-118.
57. Inoue, T., Murai, T., and Natsuaki, T. 2010. An effective system for detecting *Iris yellow spot virus* transmission by *Thrips tabaci*. Plant Pathol. 59 (3):422-428.
58. Jones, D. R. 2005. Plant viruses transmitted by thrips. Eur. J. Plant Pathol. 113 (2):119-157.
59. Krauthausen, H. J., Leinhos, G. M. E., Muller, J., Radtke, P. C., and Jehle, J. A. 2012. Identification and incidence of *Iris yellow spot virus* in Allium field crops in Southwest Germany. Eur. J. Plant Pathol. 134 (2):345-356.
60. Kritzman, A., Beckelman, H., Alexandrov, S., Cohen, J., Lampel, M., Zeidan, M., Raccah, B., and Gera, A. 2000. Lisianthus leaf necrosis: A new disease of lisianthus caused by *Iris yellow spot virus*. Plant Dis. 84 (11):1185-1189.
61. Kritzman, A., Lampel, M., Raccah, B., and Gera, A. 2001. Distribution and transmission of *Iris yellow spot virus*. Plant Dis. 85 (8):838-842.
62. Larentzaki, E., Plate, J., Nault, B. A., and Shelton, A. M. 2008. Impact of straw mulch on populations of onion thrips (Thysanoptera: Thripidae) in onion. J. Econ. Entomol. 101 (4):1317-1324.
63. Lobin, K., Saison, A., Hostachy, B., Benimadhu, S. P., and Pappu, H. R. 2010. First report of *Iris yellow spot virus* in onion in Mauritius. Plant Dis. 94 (11):1373-1373.
64. Long, R. F., and Morandin, L. 2011. Low hybrid onion seed yields relate to honey bee visits and insecticide use. Calif. Agric. 65 (3):155-158.
65. Miller, M. E., Saldana, R. R., Black, M. C., and Pappu, H. R. 2006. First report of *Iris yellow spot virus* on onion (*Allium cepa*) in Texas. Plant Dis. 90 (10):1359-1359.
66. Mo, J. H., Munro, S., Boulton, A., and Stevens, M. 2008. Within-plant distribution of onion thrips (Thysanoptera : Thripidae) in onions. J. Econ. Entomol. 101 (4):1331-1336.

67. Mullis, S. W., Gitaitis, R. D., Nischwitz, C., Csinos, A. S., Mallaupoma, Z. C. R., and Rojas, E. H. I. 2006. First report of onion (*Allium cepa*) naturally infected with *Iris yellow spot virus* in Peru. Plant Dis. 90 (3):377-377.
68. Mullis, S. W., Langston, D. B., Gitaitis, R. D., Sherwood, J. L., Csinos, A. C., Riley, D. G., Sparks, A. N., Torrance, R. L., and Cook, M. J. 2004. First report of Vidalia onion (*Allium cepa*) naturally infected with *Tomato spotted wilt virus* and *Iris yellow spot virus* (Family Bunyaviridae, genus *Tospovirus*) in Georgia. Plant Dis. 88 (11):1285-1285.
69. Mumford, R. A., Glover, R., Daly, M., Nixon, T., Harju, V., and Skelton, A. 2008. *Iris yellow spot virus* (IYSV) infecting lisianthus (*Eustoma grandiflorum*) in the UK: first finding and detection by real-time PCR. Plant Pathol. 57 (4):768-768.
70. Nischwitz, C., Gitaitis, R. D., Mullis, S. W., Csinos, A. S., and Langston, D. B. 2007. First report of *Iris yellow spot virus* in spiny sowthistle (*Sonchus asper*) in the United States. Plant Dis. 91 (11):1518-1518.
71. Pappu, H. R., Hellier, B. C., and Dugan, F. M. 2006. Wild *Allium* spp. as natural hosts of *Iris yellow spot virus*. Plant Dis. 90 (3):378-378.
72. Pappu, H. R., and Rauf, A. 2013. First report of *Iris yellow spot virus* infecting green onion in Indonesia. Plant Dis. 97 (12):1665-1665.
73. Pappu, H. R., Rosales, I. M., and Druffel, K. L. 2008. Serological and molecular assays for rapid and sensitive detection of *Iris yellow spot virus* infection of bulb and seed onion crops. Plant Dis. 92 (4):588-594.
74. Persley, D. M., Thomas, J. E., and Sharman, M. 2006. Tospoviruses - an Australian perspective. Austral. Plant Pathol. 35 (2):161-180.
75. Pozzer, L., Bezerra, I. C., Kormelink, R., Prins, M., Peters, D., Resende, R. D., and de Avila, A. C. 1999. Characterization of a tospovirus isolate of *Iris yellow spot virus* associated with a disease in onion fields in Brazil. Plant Dis. 83 (4):345-350.
76. Ravi, K. S., Kitkaru, A. S., and Winter, S. 2006. *Iris yellow spot virus* in onion: a new tospovirus record from India. Plant Pathol. 55 (2):288-288.
77. Reitz, S. R. 2014. Onion thrips (Thysanoptera: Thripidae) and their management in the Treasure Valley of the Pacific Northwest. Fla. Entomol. 97 (2):349-354.
78. Robene-Soustrade, I., Hostachy, B., Roux-Cuvelier, M., Minatchy, J., Hedont, M., Pallas, R., Couteau, A., Cassam, N., and Wuster, G. 2006. First report of *Iris yellow spot virus* in onion bulb- and seed-production fields in Reunion Island. Plant Pathol. 55 (2):288-288.
79. Rosales, M., Pappu, H. R., Lopez, L., Mora, R., and Aljaro, A. 2005. *Iris yellow spot virus* in onion in Chile. Plant Dis. 89 (11):1245-1245.
80. Sampangi, R. K., Mohan, S. K., and Pappu, H. R. 2007. Identification of new alternative weed hosts for *Iris yellow spot virus* in the pacific northwest. Plant Dis. 91 (12):1683-1683.
81. Schwartz, H. F. 2014. IYSV & Hosts. Page Alliumnet.
82. Schwartz, H. F., Brown, W. M., Blunt, T., and Gent, D. H. 2002. *Iris yellow spot virus* on onion in Colorado. Plant Dis. 86 (5).
83. Schwartz, H. F., Gent, D. H., Fichtner, S. M., Hammon, R., Cranshaw, W. S., Mahaffey, L., Camper, M., Otto, K., and McMillan, M. 2009. Straw mulch and reduced-risk pesticide impacts on thrips and *Iris yellow spot virus* on western-grown onions. Southw. Entomol. 34 (1):13-29.
84. Service, N. A. S. 2014. Vegetables 2013 Summary. Pages 32, 34. United States Department of Agriculture.

85. Sether, D. M., Borth, W. B., Shimabuku, R. S., Pappu, H. R., Melzer, M. J., and Hu, J. S. 2010. First report of *Iris yellow spot virus* in onion in Hawaii. Plant Dis. 94 (12):1508-1508.
86. Shock, C. C., Feibert, E., Jensen, L., Mohan, S. K., and Saunders, L. D. 2008. Onion variety response to *Iris yellow spot virus*. HortTechnology 18 (3):539-544.
87. Shock, C. C., Feibert, E. B. G., Saunders, L. D., Jensen, L. B., Pappu, H. R., Mohan, S. K., and Sampangi, R. K. 2009. Cultural practices to reduce the expression of *Iris yellow spot virus* in onion. Report No. 0018-5345. Pp. 1135-1135 in: Amer.Soc. Hort. Sci.
88. Silva, M. S., Martins, C. R. F., Bezerra, I. C., Nagata, T., de Avila, A. C., and Resende, R. O. 2001. Sequence diversity of NSM movement protein of tospoviruses. Arch. Virol. 146 (7):1267-1281.
89. Smith, E. A., Ditommaso, A., Fuchs, M., Shelton, A. M., and Nault, B. A. 2011. Weed hosts for onion thrips (Thysanoptera: Thripidae) and their potential role in the epidemiology of *Iris yellow spot virus* in an onion ecosystem. Environ. Entomol. 40 (2):194-203.
90. Smith, T. N., Jones, R. A. C., and Wylie, S. J. 2006. Genetic diversity of the nucleocapsid gene of *Iris yellow spot virus*. Austral. Plant Pathol. 35 (3):359-362.
91. Sparks, A. N., Diffie, S., and Riley, D. G. 2011. Thrips species composition on onions in the Vidalia production region of Georgia. J. Entomol. Sci. 46 (1):40-45.
92. Srinivasan, R., Sundaraj, S., Pappu, H. R., Diffie, S., Riley, D. G., and Gitaitis, R. D. 2012. Transmission of *Iris yellow spot virus* by *Frankliniella fusca* and *Thrips tabaci* (Thysanoptera: Thripidae). J. Econ. Entomol. 105 (1):40-47.
93. Subramanian, S., Pappu, H. R., Birithia, R., Shem, O., Muthomi, J., Sseruwagi, P., and Narla, R. 2011. Diversity and distribution of *Iris yellow spot virus* (genus *Tospovirus*) infecting onion in Eastern Africa. Phytopathology 101 (6):S172.
94. Thrips as crop pests. 1997. CAB International, Wallingford, Oxon, UK ;.
95. Tomassoli, L., Tiberini, A., Masenga, V., Vicchi, V., and Turina, M. 2009. Characterization of *Iris yellow spot virus* isolates from onion crops in northern Italy. J. Plant Pathol. 91 (3):733-739.
96. Trkulja, V., Salapura, J. M., Kovacic, D., Stankovic, I., Bulajic, A., Vucurovic, A., and Krstic, B. 2013. First report of *Iris yellow spot virus* infecting onion in Bosnia and Herzegovina. Plant Dis. 97 (3):430-430.
97. Velásquez-Valle, R., and Reveles-Hernández, M. 2011. *Iris yellow spot virus* detection in the onion cultivation of Zacatecas, Mexico. Revista Mexicana de Ciencias Agrícolas 2 (6):971-978.
98. Virus taxonomy classification and nomenclature of viruses : ninth report of the Inter. Comm. on Taxonomy of Viruses. 2012. Academic Press, London.
99. Virus Taxonomy: 2013 Release. 2014. Page Taxonomy of viruses, I. C. o. T. o. Viruses, ed. http://ictvonline.org/virusTaxonomy.asp
100. Ward, L. I., Perez-Egusquiza, Z., Fletcher, J. D., Corona, F. M. O., Tang, J. Z., Liefting, L. W., Martin, E. J., Quinn, B. D., Pappu, H. R., and Clover, G. R. G. 2009. First report of *Iris yellow spot virus* on *Allium cepa* in New Zealand. Plant Pathol. 58 (2):406-406.
101. Whitfield, A. E., Ullman, D. E., and German, T. L. 2005. Tospovirus-thrips interactions. Annual Review of Phytopathology 43:459-489.

CHAPTER TWO: *IRIS YELLOW SPOT VIRUS* PRESENCE IN ONION SEED

Introduction

When *Iris yellow spot virus* (IYSV) was first described, it was shown to be transmitted by *Thrips tabaci* (4). Since that time it has been observed that IYSV can be introduced via onion transplants (or viruliferous *T. tabaci* on the transplants), (7) and persist in volunteer onions (8), cull piles (9), and weeds (19). Onion seed was not expected to be a source of inoculum.

Tospoviruses are generally not known to be seed transmitted; however, seed transmission of a *Tospovirus* is listed in some publications (10, 14, 20). In 2001, Kritzman (13) tested 535 onion seedlings grown from seed obtained from infected onion plants and found that none of the 8 week old plants displayed IYSV symptoms or tested positive by ELISA. It was concluded that IYSV is not seed transmitted. At the time the following study was conducted, this was the only published account of testing onion seed for IYSV transmission that this author was aware of. Since then, Bulajic et al. (3) reported that they found no evidence of IYSV transmission by seed in 5,000 onion seedlings grown from infected plants. Neither author stated how many, or which cultivars were used in their seed grow outs.

The published accounts of a seed transmitted *Tospovirus*, the small sample size tested by Kritzman (13), personal observation that seeds could yield a positive result in a DAS-ELISA test for IYSV, and detection of IYSV in onion flowers suggested that further investigation of the possibility of IYSV being seed transmitted was prudent. The objectives of this study were to i) investigate the results of Kritzman (13) by testing a greater number of seeds from several cultivars of onion for the presence of IYSV in seed and evidence of transmission to the new plant, and ii) to determine the location of IYSV within the seed.

Materials and Methods

Collection of onion seed

Onion seed was collected from three cultivars in 2010 and from seven cultivars in 2011 at the CSU Agricultural Research Development and Education Center (ARDEC) near Fort Collins, Colorado. In 2010, umbels and scapes were collected from IYSV symptomatic and non-symptomatic onion plants that had bolted (Table 2.1). In 2011, umbels and scapes were collected only from IYSV symptomatic plants that had bolted (Table 2.1). Red, yellow, and white cultivars were represented each year. The goal was to select enough umbels and scapes to have sufficient seed to grow out from at least ten IYSV infected plants per cultivar per year. The number of umbels and scapes collected per cultivar varied as the number of bolted onions was not equal among cultivars in both years. With the exception of some Red Defender volunteers, the cultivars selected in 2010 were unavailable in 2011. The diameter of each umbel was measured, as was the diameter of each scape 1, 5, and 10 cm from the base of the umbel. Seeds were separated from each umbel, counted, and weighed, and the remaining chaff from each umbel was weighed. These measurements were taken to make comparisons between IYSV infected and uninfected onion scapes and seed; however, these comparisons were not feasible as IYSV was detected in the majority of non-symptomatic plants. Scapes were tested for IYSV by DAS-ELISA using commercially available antisera (Agdia, Inc., Elkhart, IN). Seed, chaff, and scapes were stored in the lab until use.

In addition, an umbel from a symptomatic onion plant (cultivar unknown) was collected from CSU-ARDEC in August 2011 and taken to the lab for IYSV detection by RT-PCR. Thrips were removed from the flowers and collected in a 1.5 ml microcentrifuge tube. Ten florets were dissected into pedicels, petals, anthers, and fruits. Each part was pooled and stored in groups of

five in 1.5 ml microcentrifuge tubes. Two 0.1 g samples from the scape were excised and placed in 1.5 ml microcentrifuge tubes. All samples were stored at -80°C until further use.

Selection of seed for grow-out

Of the plants collected in 2010, scapes from ten umbels containing the most seed of each cultivar were tested for IYSV by DAS-ELISA (Table 2.1). The intention was to compare seed from infected and uninfected plants; however, this was not feasible as IYSV was detected in the majority of non-symptomatic plants. Scapes that tested negative for IYSV may have been infected at a different location. In the end, seed from thirty symptomatic plants (ten umbels from each of three cultivars) was chosen regardless of the DAS-ELISA result.

Each scape collected in 2011 was tested for IYSV by DAS-ELISA. If a scape was positive, and sufficient seed was available from the associated umbel, that single plant seed source was used in the subsequent grow-outs. Seed from twenty-nine plants (from five cultivars) was selected for growing out (Table 2.1). Commercially produced seed from cultivar Tequila (dpSeeds, Yuma, AZ) was used as a presumptive uninfected control in each grow-out. A seed lot consisted of seed collected from a single umbel. Designations given to seed lots from selected umbels are listed in Table 2.1.

Table 2.1. Onion cultivars collected from the field in 2010 and 2011, DAS-ELISA results, number of umbels selected for grow-outs, and seed lot source designations.

Cultivar 2010	Number of umbels/ scapes collected	Number of IYSV positive scapes	Number of umbels selected for grow out	Seed lot source designations of selected umbels
Pentium (symptomatic)	34	10 of 10	10	P1, P2, P3, P7, P10, P20, P24, P25, P27, P30
Pentium (non-symptomatic)	16	6 of 10	0	
Red Defender (symptomatic)	24	10 of 10	10	RD3, RD5, RD9, RD11, RD12, RD13, RD15, RD16, RD21, RD22
Red Defender (non-symptomatic)	15	7 of 10	0	
Solid Gold (symptomatic)	17	8 of 10	10	SG1, SG4, SG5, SG6, SG8, SG9, SG13, SG15, SG16, SG17
Solid Gold (non-symptomatic)	15	6 of 10	0	
2011				
Granero	25	19 of 25	10	G1, G7, G11, G12, G13, G17, G18, G19, G20, G25
Irish Eyes	5	0 of 5	0	
Red Defender, 2011 volunteers	13	3 of 13	2	RD'1, RD'11
Red, unknown cultivar	22	4 of 22	0	
Salsa	14	12 of 14	6	S1, S7, S11, S12, S13, S14
White Cloud	17	5 of 17	1	WC13
White, unknown cultivar	27	16 of 30	10	W8, W9, W12, W15, W17, W19, W20, W21, W24, W28

Greenhouse grow-outs

Sixty seeds from each selected lot of Solid Gold, Pentium, and Red Defender were divided among three petri dishes (20 seeds per dish). Seeds were surface-disinfested in a 10% bleach solution then rinsed in distilled water before placement on moist filter paper in petri dishes. The dishes were held at 18°C in a dark incubator. Water was added as needed to keep the filter paper moist. Once an emerging radicle reached 2 cm long, it was transplanted into a 3.79 liter pot in the greenhouse and marked with a colored toothpick denoting the planting date. Pots contained Pro-Mix BX with biofungicide potting soil (Premier Horticulture, Québec, Canada). Seedlings were best handled with blunt featherweight forceps (BioQuip Products, Rancho Dominguez, CA). Germinated seeds were randomly assigned to pots by drawing a colored token from a pocket. Ten germinated seeds were planted into each of their designated pots for a total of 30 seeds per lot (300 seeds per cultivar). Only ten seeds of the Tequila cultivar were planted in each block.

Pots were arranged along a greenhouse bench in 10 blocks of 10 pots per block (100 pots total). One seed lot from each of the three cultivars was randomly assigned to each block. Within each block, three pots for each seed lot were randomly placed. The tenth pot (also randomly placed) was planted with Tequila seed (control). For example, during the first grow-out block one contained three pots of SG13, three pots of RD3, three pots of P2, and one pot of Tequila seed. Randomization was conducted by a list randomizer (http://www.random.org/lists/).

Plastic and mesh cylinders designed to exclude thrips were placed inside the pots (Figure 2.1). In later experiments, plastic wrap was taped over the intersection of the cylinder and pot in an additional effort to exclude thrips. Plants were immediately measured, pulled from the soil,

and prepared for DAS-ELISA when it was apparent that thrips had breached the barriers. This experiment was performed twice.

Figure 2.1. Example of thrips exclusion cylinders placed inside pots.

Laboratory growth chamber grow-outs

Solid Gold, Pentium, and Red Defender seeds collected in 2010 were grown-out once in an 818 Low Temperature Illuminated Incubator (Thermo Electron Corp., Marietta, OH), and seed collected in 2011 was grown out twice in the same incubator. Seeds were germinated as for the greenhouse grow-outs, however, because fungal growth continued to occur on the germinating seeds, surface disinfestation with bleach was eventually abandoned in favor of rolling the seeds in fungicide (Prevail® K, Gustafson, McKinney, TX) before placing them on moist filter paper. When radicles reached 2 cm, seeds were randomly assigned to and planted into 12.7 cm pots (size limitations of growth chamber) and marked with a colored toothpick.

Plants were grown for 6 weeks after which time they were pulled from the soil, measured for length, and prepared for DAS-ELISA.

A similar random block design was used for the growth chamber as in the greenhouse. However, due to the limited size of the growth chamber, only 5 blocks could be grown at one time. In addition, seed lots were randomly assigned to blocks regardless of cultivar. For example, a single block may have contained two seed lots from cultivar Granero and one from cultivar Salsa. Instead of arranging pots horizontally along a greenhouse bench, pots were arranged in groups of 10 vertically in the growth chamber (10 pots per shelf).

Seedlings did not appear to thrive in the incubator. Subsequently, lighting in the incubator was supplemented with five, T5 High Performance grow lights fitted with warm spectrum bulbs (Hydrofarm, Petaluma, CA). Small dishes were placed under each pot to allow water to drain from the pots and not spill onto the growth chamber. Pots were watered as needed to maintain soil moisture. The incubator was programmed for a 16h light and 28°C (day), and 8h dark and 18°C (night) cycle.

DAS-ELISA

Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) were performed according to the manufacturer's (Agdia, Inc., Elkhart, IN) instructions with the following modifications. Plant tissue was ground in liquid nitrogen as described by Gent et al. (8). A 0.2 g sample of plant tissue was typically used with 1.0 ml extraction buffer. The first four PBST (phosphate buffered saline + tween) rinses were short (<10 seconds) followed by a longer rinse that remained in the plate for 5 minutes. Samples were considered positive if they

measured 2X the background absorbance using an ELx800 Universal Microplate Reader (Bio-Tek Insruments, Inc., Winooski, VT).

Dry scape tissue was not weighed, instead 2.0 cm segments were cut from each end of the scape and from the bulge, then ground in liquid nitrogen before adding 2.0 ml extraction buffer. If the dry tissue absorbed all the extraction buffer, an additional 0.5 ml extraction buffer was added such that 100 µl could be transferred to the plate.

Typically, any leftover germinated seeds were divided among three mortars for grinding and 1.0 ml extraction buffer was added.

Plants harvested from pots were pooled by seed lot and leaves were cut into small segments. Three, 0.2 g samples of pooled leaf tissue were tested for each lot. In the event that insufficient plant material was available, all available plant material was tested.

Selection of seed for shoot vs coat testing

Germinated seeds that had been positive by DAS-ELISA where at least three lots were available per cultivar and the cultivars were known were selected for this study. Thus, three lots each of Granero, Pentium, Red Defender (2010), and Salsa were included.

Growing the shoots, collecting shoots and coats

Seeds were sprouted on moist filter paper as in the grow outs until 50 seeds per lot could be processed and tested. Shoots were pulled from seed coats. Longer shoots tended to pull easily from the seed coat, but shorter shoots tended to break. In that case the seed was broken open and shoot tissue was scraped out with forceps. Shoots and seed coats were pooled separately in groups of five, weighed, and labeled such that pools of seed coats could be matched

to their corresponding pools of shoots. Samples were stored at -80°C until use. For any lot where IYSV was detected, an additional 50 seeds were sprouted and tested (up to 100 seeds were tested for an individual lot).

RNA extraction and cDNA synthesis

Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO) with the 750 µl binding solution option according to the manufacturer's instructions. Total RNA was measured with a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Aliquots of RNA were treated with DNase I according to the manufacturer's instructions (Fermentas, Glen Burnie, MD). cDNA was synthesized from total RNA using M-MLV reverse-transcriptase according to the manufacturer's instructions for use with random primers (Invitrogen, Carlsbad, CA). No more than 1.0 µg total RNA was used in each 20 µl reaction. When possible, cDNA was brought to a final concentration of 10 ng/µl before use in PCR.

RT-PCR Conditions

Reverse-transcriptase PCR (RT-PCR) reactions were performed on a MJ Research PTC-200 thermocycler with an initial step of 94° C for 3 min; followed by 40 repeats of the three step sequence of 94° C for 45 sec, 55° C for 30 sec, 72° C for 90 sec; and a final step of 72° C for 10 min. Each 20 µl reaction consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3 mM MgCl2, 0.15 mM dNTP mix, 1 unit Taq DNA polymerase (New England Biolabs, Ipswich, MA), 20 ng cDNA and autoclaved distilled water. RT-PCR was conducted for each sample to confirm the presence or absence of IYSV. The IYSV nucleoprotein gene was amplified by RT-PCR with primers designed by Coutts et al. (5) , and primers specific to plant NADH dehydrogenase ND2

subunit (21) were used as a control. Reaction products were run in a 1% agarose gel (Bio-Rad Laboratories, Inc., Hercules, CA) stained with SYBRSafe (Invitrogen, Carlsbad, CA) in 1X TAE buffer (Bio-Rad Laboratories, Inc., Hercules, CA) exposed to UV light. A 100 bp molecular weight marker (Fermentas, Glen Burnie, MD) was included on each gel.

Results

Scape testing

Of the 186 scapes that were tested, IYSV was detected in 106 of them (Table 2.1). Due to the uneven distribution of IYSV and over half the non-symptomatic plants testing positive for IYSV, it was possible that IYSV could have been present in the plants that had a negative result. For these reasons non-symptomatic Solid Gold, Pentium, and Red Defender were not included in the grow-outs, but all the tested symptomatic plants of these cultivars were included. Not all scapes from the cultivars collected in 2011 tested positive for IYSV. Only seed from scapes where IYSV was detected was used in the subsequent grow-outs.

RT-PCR of flowers

IYSV was detected in all the flower parts tested, including the petals, pedicels, anthers, and developing seeds. IYSV was also detected in the scape and in thrips removed from the flowers (Figure 2.2).

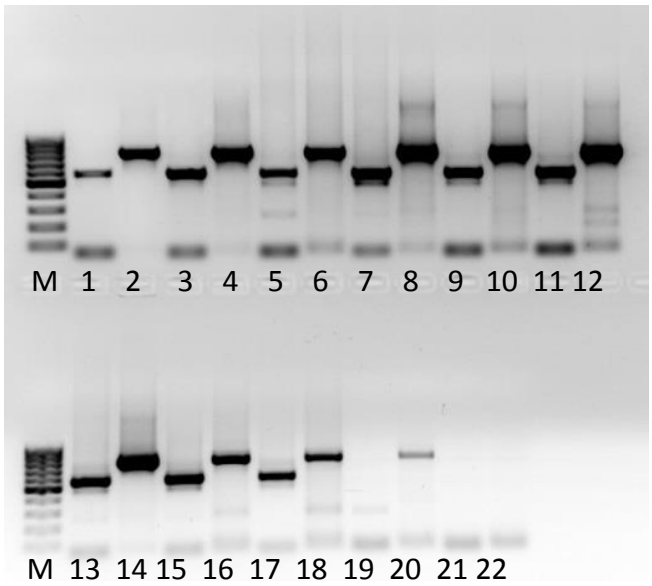


Figure 2.2. RT-PCR of pedicels, petals, anthers, fruit, and thrips removed from an onion umbel. The umbel was collected in 2011 from an unknown cultivar growing in the onion research field at CSU-ARDEC. IYSV was detected in each of the flower parts and in the thrips present on the flowers. Odd numbered lanes: NAD2. Even numbered lanes: IYSV. M: 100bp molecular weight marker. Lanes 1 to 4 and 11 to 12: pedicels; lanes 5 and 6: anthers; lanes 7, 8, 13, and 14: fruit; lanes 9 and 10: petals; lanes 15 to 18: scape; lanes 19 and 20: thrips; lanes 21 and 22: -RT.

Greenhouse grow-outs

During each grow-out in the greenhouse, thrips managed to get through the barriers, thus forcing the early termination of the experiment. Nearly 2,000 germinated seeds were planted, of which 1,736 survived to the point when they were harvested approximately three weeks after being transplanted into pots. Nearly 300 of the surviving plants were infested with thrips, and the only plants that tested positive for IYSV by DAS-ELISA were among this group. Of the thrips infested plants, IYSV was only detected in three plants of cultivar Pentium (seed lot P10) removed from the same pot, and a single plant of cultivar Tequila. Because the three thrips infested Pentium plants were pooled for testing, it is not known what proportion of thrips infested plants was infected with IYSV; however, IYSV was detected in 2 of 69 thrips infested pots (approximately 3%). IYSV was not detected in any plant that was not infested with thrips.

Although the thrips infestation curtailed the experiments by five weeks, it should be noted that onion seedlings can be infected by IYSV by the time they are 21 days old.

Lab grow-outs

Of the 2,545 germinated seeds planted into the growth chamber a total of 1,400 survived. Thrips did not infiltrate this portion of the experiment. No plant tested positive for IYSV by DAS-ELISA.

DAS-ELISA on sprouted seeds

Germinated seeds leftover from the greenhouse and laboratory grow-outs were tested by DAS-ELISA and IYSV was detected in some seed lots (Table 2.3). Three lots each of Granero, Pentium, Red Defender, and Salsa were selected for further investigation.

Table 2.3. Seed lots that tested positive for IYSV by DAS-ELISA. *Italics*: lots selected for further study.

Granero (2011)	Solid Gold (2010)
G7, *G12, G19, G20*	SG8, SG9
Pentium (2010)	White Cloud (2011)
P2, P3, P10, *P30*	WC13
Red Defender (2010)	White, unknown cultivar (2011)
RD3, RD12, RD21	W8, W9, W19
Salsa (2011)	
S1, S13, S14	

Shoots vs. seed coats

With one exception, IYSV was not detected in any of the 940 shoots, however, IYSV was detected in 53 pools of seed coats (Table 2.4). Real-time PCR was performed on the shoot sample that appeared to be infected with IYSV, however, no amplification occurred and this sample was considered negative for IYSV. Germination of RD21 was poor, and the lot ran out of seeds before reaching 50 germinated seeds.

The number of positive pools of seed coats was not equal between reps, or between cultivars. IYSV was detected with the greatest frequency in the seed coats of Granero, followed by Pentium, Salsa, and Red Defender. The weight of the pooled shoots ranged from 0.1 g to 0.004 g, and the weight of the pooled seed coats ranged from 0.032 g to 0.009 g. There was no apparent relationship between the weight of the pooled shoots and their IYSV status, or between the weight of the pooled seed coats and their IYSV status (Table 2.4).

Table 2.4. Weight and RT-PCR result of onion shoots and their corresponding seed coats. Each samples consists of a pool of five shoots or seed coats. A second rep was conducted if IYSV was detected. IYSV detected: + , IYSV not detected: - .

Seed lot *P2*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.059	11	0.020	-/-	1	0.066	11	0.021	-/-
2	0.045	12	0.021	-/-	2	0.046	12	0.024	-/-
3	0.050	13	0.020	-/-	3	0.054	13	0.022	-/-
4	0.054	14	0.022	-/-	4	0.078	14	0.017	-/+
5	0.034	15	0.026	-/-	5	0.061	15	0.015	-/-
6	0.077	16	0.015	-/+	6	0.092	16	0.015	-/-
7	0.071	17	0.015	-/+	7	0.057	17	0.018	-/-
8	0.063	18	0.019	-/-	8	0.060	18	0.023	-/-
9	0.028	19	0.025	-/-	9	0.044	19	0.023	-/-
10	0.018	20	0.020	-/-	10	0.034	20	0.028	-/-
Seed lot *P3*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.100	11	0.020	-/-	1	0.043	11	0.015	-/-
2	0.093	12	0.018	-/+	2	0.031	12	0.019	-/+
3	0.074	13	0.018	-/+	3	0.049	13	0.016	-/-
4	0.063	14	0.022	-/+	4	0.051	14	0.015	-/+
5	0.073	15	0.016	-/+	5	0.019	15	0.022	-/+
6	0.067	16	0.017	-/-	6	0.036	16	0.017	-/-
7	0.046	17	0.024	-/-	7	0.032	17	0.023	-/-
8	0.088	18	0.017	-/+	8	0.028	18	0.024	-/+
9	0.058	19	0.015	-/+	9	0.023	19	0.026	-/-
10	0.052	20	0.016	-/-	10	0.017	20	0.032	-/-
Seed lot *P30*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.051	11	0.015	-/-	1	0.066	11	0.015	-/-
2	0.058	12	0.016	-/-	2	0.075	12	0.014	-/-
3	0.058	13	0.014	-/+	3	0.047	13	0.012	-/-
4	0.044	14	0.014	-/+	4	0.060	14	0.010	-/-
5	0.068	15	0.014	-/-	5	0.058	15	0.012	-/-
6	0.047	16	0.011	-/+	6	0.044	16	0.012	-/-
7	0.040	17	0.017	-/+	7	0.048	17	0.012	-/-
8	0.025	18	0.013	-/-	8	0.034	18	0.013	-/-
9	0.032	19	0.013	-/-	9	0.023	19	0.014	-/-
10	0.047	20	0.012	-/-	10	0.024	20	0.016	-/-

Table 2.4. Continued

Seed lot *RD3*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.051	11	0.016	-/+	1	0.037	11	0.020	-/-
2	0.067	12	0.013	-/+	2	0.047	12	0.016	-/-
3	0.052	13	0.018	-/+	3	0.046	13	0.020	-/-
4	0.067	14	0.013	-/-	4	0.066	14	0.018	-/-
5	0.023	15	0.013	-/+	5	0.063	15	0.016	-/-
6	0.049	16	0.015	-/+	6	0.027	16	0.024	-/-
7	0.051	17	0.015	-/+	7	0.030	17	0.018	-/-
8	0.047	18	0.015	-/-	8	0.030	18	0.019	-/-
9	0.035	19	0.019	-/-	9	0.019	19	0.022	-/+
10	0.033	20	0.019	-/-	10	0.017	20	0.022	-/-
Seed lot *RD12*									
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat					
1	0.054	11	0.018	-/-					
2	0.038	12	0.021	-/-					
3	0.032	13	0.023	-/-					
4	0.007	14	0.018	-/-					
5	0.013	15	0.016	-/-					
6	0.015	16	0.024	-/-					
7	0.004	17	0.024	-/-					
8	0.033	18	0.019	-/-					
9	-	19	-						
10	-	20	-						
Seed lot *RD21*									
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat					
1	0.049	11	0.014	-/-					
2	0.047	12	0.017	-/-					
3	0.027	13	0.019	-/-					
4	0.037	14	0.020	-/-					
5	0.037	15	0.019	-/-					
6	0.052	16	0.013	-/-					
7	0.044	17	0.015	-/-					
8	0.031	18	0.018	-/-					
9	0.029	19	0.014	-/-					
10	0.020	20	0.011	-/-					

Table 2.4. Continued

Seed lot *S1*				
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.062	11	0.017	-/-
2	0.027	12	0.016	-/-
3	0.029	13	0.016	-/-
4	0.016	14	0.017	-/-
5	0.013	15	0.017	-/-
6	0.007	16	0.010	-/-
7	0.013	17	0.024	-/-
8	0.004	18	0.024	-/-
9	0.014	19	0.024	-/-
10	0.013	20	0.029	-/-

Seed lot *S13*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.045	11	0.024	-/-	1	0.083	11	0.011	-/-
2	0.035	12	0.021	-/-	2	0.095	12	0.009	-/+
3	0.038	13	0.019	-/-	3	0.063	13	0.014	-/+
4	0.027	14	0.019	-/-	4	0.062	14	0.016	-/+
5	0.021	15	0.013	-/-	5	0.047	15	0.011	-/+
6	0.015	16	0.013	-/-	6	0.074	16	0.013	-/+
7	0.018	17	0.013	-/-	7	0.065	17	0.014	-/+
8	0.014	18	0.016	-/-	8	0.082	18	0.013	-/+
9	0.041	19	0.013	-/-	9	0.049	19	0.012	-/+
10	0.032	20	0.018	-/+	10	0.047	20	0.015	-/+

Seed lot *S14*				
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.042	11	0.017	-/-
2	0.044	12	0.021	-/-
3	0.066	13	0.022	-/-
4	0.078	14	0.011	-/-
5	0.053	15	0.015	-/-
6	0.016	16	0.016	-/-
7	0.013	17	0.012	-/-
8	0.016	18	0.013	-/-
9	0.010	19	0.022	-/-
10	0.010	20	0.019	-/-

Table 2.4. Continued

Seed lot *G12*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.050	11	0.020	-/-	1	0.075	11	0.015	-/+
2	0.028	12	0.018	-/-	2	0.062	12	0.015	-/+
3	0.035	13	0.022	-/-	3	0.047	13	0.013	-/-
4	0.017	14	0.019	-/-	4	0.079	14	0.012	-/+
5	0.009	15	0.021	-/-	5	0.087	15	0.016	-/+
6	0.055	16	0.014	-/-	6	0.061	16	0.014	-/-
7	0.037	17	0.015	-/+	7	0.067	17	0.019	-/+
8	0.026	18	0.016	-/-	8	0.065	18	0.019	-/-
9	0.018	19	0.016	-/-	9	0.051	19	0.014	-/-
10	0.018	20	0.027	-/-	10	0.043	20	0.016	-/+
Seed lot *G19*									
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat					
1	0.042	11	0.018	-/-					
2	0.038	12	0.018	-/-					
3	0.032	13	0.015	-/-					
4	0.027	14	0.013	-/-					
5	0.023	15	0.019	-/-					
6	0.031	16	0.021	-/-					
7	0.017	17	0.012	-/-					
8	0.013	18	0.017	-/-					
9	0.050	19	0.012	-/-					
10	0.052	20	0.012	-/-					
Seed lot *G20*									
	Rep 1					Rep 2			
Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat	Sample	Shoot weight (g)	Sample	Coat Weight (g)	RT-PCR result Shoot/Coat
1	0.040	11	0.017	-/-	1	0.050	11	0.020	-/+
2	0.027	12	0.017	-/-	2	0.044	12	0.019	-/+
3	0.016	13	0.014	-/-	3	0.048	13	0.025	-/+
4	0.013	14	0.013	-/-	4	0.043	14	0.018	-/+
5	0.026	15	0.022	-/-	5	0.073	15	0.015	-/+
6	0.020	16	0.013	-/-	6	0.058	16	0.012	-/+
7	0.042	17	0.021	-/-	7	0.048	17	0.015	-/+
8	0.031	18	0.018	-/+	8	0.030	18	0.012	-/+
9	0.014	19	0.022	-/-	9	0.034	19	0.010	-/+
10	0.013	20	0.020	-/+	10	0.029	20	0.018	-/+

Discussion

Nearly 3,000 onion plants that were grown from seed collected from IYSV infected plants and not infested with thrips during the grow-outs were tested and did not have IYSV present. Nearly 1,000 onion shoots germinated from seed collected from IYSV infected plants were tested and likewise did not have IYSV present.

The reports of seed transmission of a *Tospovirus*, occasionally specified as *Tomato spotted wilt virus* (TSWV), present in the literature (10, 14, 20) all seem to be based on the findings of Jones (12), which Crowley (6) was unable to replicate. Errors in methodology were pointed out by Baker and Smith (1). Jones (12) did grow out seed, and the resulting plants displayed symptoms of what they suspected to be a virus. They concluded that this virus was seed transmitted, but did note that thrips were not controlled and that this virus could be transmitted by thrips. As the insect vector was not controlled during the seed grow-out experiments, the conclusion that the virus suspected of being TSWV is seed transmitted is not supported. To date there is still no evidence that *Tospoviruses* can be seed transmitted.

In this study, IYSV was only detected in plants that had been infested with thrips. In the absence of thrips, no plant from the grow-outs tested positive. The most reasonable explanation of these results is that thrips transmitted IYSV to those plants in which IYSV was subsequently detected, and that IYSV is not apparently transmitted through onion seed. The results presented here confirm those of Kritzman et al. (13) and Bulajik et al. (3).

It is not possible to prove absolutely that IYSV is not seed transmitted without testing all seeds. The lower the level of infection, the larger the sample size needs to be in order to maximize the probability of detecting a lower incidence of infected seed. Conversely, a large sample size where the pathogen is not detected indicates a low level of infection if the pathogen

in question is actually seed transmitted (17). If all the seeds tested in this work (3,949) were considered to be a single lot and none were positive, the level of infection (if it existed) would be less than 0.001 (Table 4 in (17)). The results of Bulajic et al. (3) who grew 5,000 onion plants from seed collected from IYSV infected plants and found no evidence of IYSV in the 10 week old seedlings further supports a low level of infection, in the unlikely event that infection exists.

When we consider the results of the seed coats separated from their shoots, it appears even less likely that IYSV is transmitted through seed as IYSV was not detected in shoots corresponding to seed coats where IYSV was detected. A similar result was observed by Crowley (6) who dissected tomato and cineraria seeds and found TSWV in seed coats but not embryos. Since the seed coat is formed from the sporophyte, it is reasonable to conclude that seed coats from a virus infected plant would also contain that virus, especially considering that IYSV was detected in various onion flower parts (Figure 2.2). When removing the shoots from the seed coats it was not always possible to separate the endosperm from the seed coat. It is possible that IYSV is present in the endosperm, but the endosperm is unlikely to become infected unless the gametes in the pollen are infected (1).

It is extremely rare for a virus to be transferred from the seed coat or endosperm into the new plant. In the few cases where this does occur, the virus is stable outside the host and is transmitted mechanically as the plant grows (11). It is unlikely that this occurs with IYSV and onion as mechanical transmission of IYSV into onion is extremely difficult (2, 13, 16).

This work underscores the importance of knowing where in the seed a virus is located. That is, if only whole seeds had been tested, the conclusion that IYSV is seed transmitted could have been made. However, separating the plant from the seed coat in combination with grow-outs shows that while IYSV may be present in the seed, it is not present in the new plant.

LITERATURE CITED

1. Baker, K. F., and Smith, S. H. 1966. Dynamics of seed transmission of plant pathogens. Ann. Rev. Phytopath. 4:311-332.
2. Beikzadeh, N., Jafarpour, B., Rouhani, H., Peters, D., and Hassani-Mehraban, A. 2012. Molecular diagnosis of *Iris yellow spot virus* (IYSV) on onion in Iran. J. Agric. Sci. Technol. 14 (5):1149-1158.
3. Bulajic, A., Djekic, I., Jovic, J., Krnjajic, S., Vucurovic, A., and Krstic, B. 2009. Incidence and distribution of *Iris yellow spot virus* on onion in Serbia. Plant Dis. 93 (10):976-982.
4. Cortes, I., Livieratos, I. C., Derks, A., Peters, D., and Kormelink, R. 1998. Molecular and serological characterization of *Iris yellow spot virus*, a new and distinct tospovirus species. Phytopath. 88 (12):1276-1282.
5. Coutts, B. A., McMichael, L. A., Tesoriero, L., Rodoni, B. C., Wilson, C. R., Wilson, A. J., Persley, D. M., and Jones, R. A. C. 2003. *Iris yellow spot virus* found infecting onions in three Australian states. Austral. Plant Pathol. 32 (4):555-557.
6. Crowley, N. 1957. Studies on the seed transmission of plant virus diseases. Austral. J. Biol. Sci. 10 (4):449-464.
7. Gent, D. H., du Toit, L. J., Fichtner, S. F., Mohan, S. K., Pappu, H. R., and Schwartz, H. F. 2006. *Iris yellow spot virus*: An emerging threat to onion bulb and seed production. Plant Dis. 90 (12):1468-1480.
8. Gent, D. H., Schwartz, H. F., and Khosla, R. 2004. Distribution and incidence of *Iris yellow spot virus* in Colorado and its relation to onion plant population and yield. Plant Dis. 88 (5):446-452.
9. Hsu, C. L., Hoepting, C. A., Fuchs, M., Smith, E. A., and Nault, B. A. 2011. Sources of *Iris yellow spot virus* in New York. Plant Dis. 95 (6):735-743.
10. Hull, R. 2002. Matthews' Plant Virology. Academic Press, San Diego, Calif.
11. Johansen, E., Edwards, M. C., and Hampton, R. O. 1994. Seed transmission of viruses - Current perspectives. Ann. Rev. of Phytopath. 32:363-386.
12. Jones, L. K. 1944. Streak and mosaic of cineraria. Phytopath. 34 (11):941-953.
13. Kritzman, A., Lampel, M., Raccah, B., and Gera, A. 2001. Distribution and transmission of *Iris yellow spot virus*. Plant Dis. 85 (8):838-842.
14. Mink, G. I. 1993. Pollen-transmitted and seed-transmitted viruses and viroids. Ann. Rev. of Phytopath. 31:375-402.
15. Nischwitz, C., Mullis, S., Torrance, R., Langston, D., Sparks, A., and Gitaitis, R. 2007. Distribution of *Iris yellow spot virus* in onion leaves. Phytopath. 97 (7):S182-S182.

16. Pozzer, L., Bezerra, I. C., Kormelink, R., Prins, M., Peters, D., Resende, R. D., and de Avila, A. C. 1999. Characterization of a tospovirus isolate of *Iris yellow spot virus* associated with a disease in onion fields in Brazil. Plant Dis. 83 (4):345-350.
17. Roberts S.J., Phelps K., Taylor J.D., Ridout M.S. 1993. Design and interpretation of seed health assays. in: First ISTA PDC Symposium on Seed Health Testing, Ottawa, Canada, www.planthealth.co.uk/downloads/Design_and_interp.pdf.
18. Schwartz, H. F., Otto, K., Szostek, S., Boateng, C., Cranshaw, W. S., Camper, M. A., and Mahaffey, L. 2008. Thrips and IYSV sources in Colorado onion production systems. Pp. 44-47 in: Nat. Allium Res. Conf., Savannah, GA.
19. Smith, E. A., Ditommaso, A., Fuchs, M., Shelton, A. M., and Nault, B. A. 2011. Weed hosts for onion thrips (Thysanoptera: Thripidae) and their potential role in the epidemiology of *Iris yellow spot virus* in an onion ecosystem. Environ. Entomol. 40 (2):194-203.
20. Stacesmith, R., and Hamilton, R. I. 1988. Inoculum thresholds of seedborne pathogens - viruses. Phytopath. 78 (6):875-880.
21. Thompson, J. R., Wetzel, S., Klerks, M. M., Vaskova, D., Schoen, C. D., Spak, J., and Jelkmann, W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria* spp. in combination with a plant mRNA specific internal control. J. Virol. Methods 111 (2):85-93.

CHAPTER 3: *IRIS YELLOW SPOT VIRUS* AND *THRIPS TABACI* OVERWINTER SURVIVAL IN COLORADO

Introduction

Iris yellow spot virus (IYSV) is vectored by onion thrips, *Thrips tabaci*, and both can cause significant damage to onion crops (4). To date there is no evidence of seed transmission ((14, 22) and Chapter 2). IYSV and *T. tabaci* can be introduced into a field via onion transplants (12, 24), however, it is still unclear how IYSV persists between growing seasons over the winter months when onions are not present.

Several non-allium plant species have been reported as additional hosts of IYSV and are summarized by Smith et al. (26). Recent studies investigating non-allium plants as suitable hosts for IYSV or onion thrips have focused on either IYSV (12) or thrips (26), and have been conducted immediately before and after the onion growing season. There are few studies published regarding the overwintering habits of *T. tabaci* that include field sampling in the winter months and those that do exist tend to focus on crop plants. Onion thrips were recovered from alfalfa, clover, grass sod, and onion during the winter from 1945 to 1950 in southern Idaho (25). During the winters of 1952 and 1953, onion thrips were found on red clover, alfalfa, winter wheat, and oat stubble in southwestern Ontario, Canada (2). *T. tabaci* were found during the winter months of 1982 to 1984 in New York on wheat, alfalfa, oat, cabbage, barley, and weeds (20). These studies established that onion thrips can overwinter on plant material in locations where winter temperatures often fall below freezing. It does not appear that any surveys have attempted to connect onion thrips, IYSV, and non-allium host plants during the winter.

After identifying additional host plants of IYSV, Hsu et al. (12) wondered if thrips would complete their life cycle on plants identified as IYSV hosts. They point out that if adult *T. tabaci* were unable to oviposit on the plant, or if larvae were unable to acquire IYSV from the plant, the plant would not be a source of IYSV, and would be considered a "dead-end". This current work addresses that question by rearing thrips on six non-allium plants under greenhouse conditions and monitoring the IYSV status of the plants and thrips larvae developing on those plants.

The objectives of this study were to investigate *T. tabaci* and IYSV survival over the winter and evaluate a selection of weed species to better understand their role as potential IYSV and/or *T. tabaci* host plants.

Materials and Methods

Thrips activity was monitored over two winters. Onion cull piles and weeds were investigated as sources of thrips, IYSV, or both. Living thrips were located on several plant species and the IYSV status of both the plants and thrips was determined. Finally, thrips were reared on a selection of the weed species identified during the study and both the plants and thrips larvae were evaluated for IYSV.

Cull piles

Cull piles were located at CSU-ARDEC north of Fort Collins, CO where a research field planted with onions has been maintained annually since 1997. The cull piles sampled from January to March 2011 were scattered throughout the onion research field, and consisted of remnants from unrelated experiments. These piles varied in size, but were approximately 0.5 m^3 or smaller. Five onions were removed from 5 to 6 piles each month and stored for 1-2 days at

4°C before they were examined. Onions were sliced into quarters and visually examined for presence of thrips larvae and adults, layer by layer. Thrips were removed with a paintbrush and preserved in 95% ethanol for later use. If green neck tissue was present, each leaf was also visually examined for the presence of thrips. Onions collected from cull piles in subsequent years were similarly stored and examined.

In March 2011, onions still standing in the field from the previous growing season were collected in addition to onions recovered from cull piles. Live thrips collected from these onions and from cull piles were transferred to healthy onion plants in the greenhouse to determine if these thrips could transmit IYSV. Thrips were left on these plants in isolation for one month at which time one leaf was removed from each pot of plants and tested for IYSV by DAS-ELISA (modified from (9)). Symptomatic leaves were preferentially selected followed by leaves with the greatest amount of visible thrips damage. Leaves were cut into 0.2 g sections and stored at -80°C until DAS-ELISA was performed.

In October 2011, three cull piles each 1.5 meter diameter by 0.9 meter high were constructed in the onion research field where the previous season's onions remained until spring 2012 (Figure 3.1). Temperature probes (Watchdog data logger, Spectrum Technologies, Inc, Aurora, IL) were fastened to a pole 0, 10, 20, and 30 cm above the soil at the center of each pile. Temperatures were recorded hourly from October 2011 to February 2012. Nine onions were collected from each pile (three each from the top, middle, and bottom) at the beginning of each month until February when the onions were too decayed to separate.

In October 2012, three cull piles were constructed in the same manner as the piles of 2011 to 2012, and were located in an empty field approximately 750 m northwest of the 2012 onion research field. Five onions were collected from the middle of each pile at the beginning of

each month through February when the onions were too decayed to sample. Temperatures were recorded hourly from October 2012 to May 2013.

Figure 3.1. Onion cull piles constructed at CSU-ARDEC for the 2011 to 2012 winter. Temperatures were monitored at 0, 10, 20, and 30 cm above the soil at the center of each pile. Thrips activity was monitored with yellow sticky traps.

Thrips activity

Thrips activity was monitored with 23 x 28 cm yellow sticky traps stapled to stakes about 1.2 m off the ground from November 2011 to June 2012 and from October 2012 to June 2013. In 2011 to 2012, the yellow sticky traps were initially placed near each cull pile, but were later transferred to each corner of the field when the cull piles were transferred for field preparation and planting. In 2012 to 2013 yellow sticky traps were placed at each corner of the field. Traps were collected and replaced at approximately two week intervals. Thrips were counted in the lab and averaged over the number of traps that were counted. Traps were occasionally blown away by strong winds, and often partially impacted and covered with dirt and debris. Air temperatures (1.5 m above ground level) were retrieved from the nearby CoAgMet Hourly Data Access (http://www.coagmet.colostate.edu/hourlysum_form.php), site FTC03 – CSU – ARDEC.

Additional plant hosts

Eleven plant species typically considered as weeds were collected from the vicinity of onion fields from January to March 2012 and 2013 (Table 3.1). Although each species is commonly found in the vicinity of onion fields, not all plants were available at each site during the collection period. Occasionally, snow cover prevented collection at a particular site for a particular date.

Table 3.1. Plant species collected and examined for thrips and IYSV during 2012 to 2013.

Scientific name	Common name	Location[a] and year collected
Bromus inermis Leyss.	Smooth brome	ARDEC (2012)
Chorispora tenella (Pall.) DC.	Blue mustard	Larimer, Weld (2013)
Convolvulus arvensis L.	Bindweed	ARDEC, Hort Farm, Larimer (2012)
Descurainia sophia (L.) Webb. Ex Prantl	Flixweed	ARDEC (2012, 2013), Hort Farm (2012), Weld (2013), Larimer (2013)
Erodium cicutarium (L.) L'Her. Ex Ait.	Redstem filaree	ARDEC (2012)
Helianthus sp.	Sunflower	ARDEC (2012)
Lactuca serriola L.	Prickly lettuce	ARDEC (2012, 2013), Hort Farm (2012), Weld (2013)
Malva neglecta Wallr.	Common mallow	ARDEC (2012, 2013), Hort Farm (2012)
Taraxacum officinale Weber in Wiggers	Dandelion	ARDEC, Hort Farm, Larimer (2012)
Tragopogon dubius Scop.	Salsify	ARDEC (2012), Larimer (2013)
Triticum aestivum	Winter wheat	ARDEC (2012)

[a]ARDEC: CSU Agricultural Research and Development Center; Hort Farm: CSU Horticulture Research Farm; Larimer: Commerical onion field in Larimer County, Colorado; Weld: Commercial onion field in Weld County, Colorado

In 2012, a wide range of green plants was collected at ARDEC from January to March. Additional plants were collected in April, 2012 from the CSU Horticulture Research Farm (Hort Farm) approximately 11 km northeast of Fort Collins, Colorado and from a commercial onion field approximately 32 km north of Fort Collins in Larimer County, Colorado. Thrips and IYSV were present at ARDEC and the Larimer county field during the previous growing season. During the 2011 growing season at ARDEC, thrips density reached just over 6 adults per onion

plant and IYSV incidence was about 60%. IYSV incidence reached 10% in the Larimer County field with <2 adult thrips per onion plant. Few onions were planted previously at the Hort Farm, and it is not known if onions or IYSV were present during the 2011 growing season.

The results of the 2012 samples led to the collection of a more focused range of plants during 2013 from two commercial onion fields (one each in Larimer and Weld Counties, Colorado) in addition to ARDEC. The Larimer County field was approximately 18 km north of Fort Collins, and the Weld County field was approximately 77 km southeast of Fort Collins, Colorado. Thrips and IYSV were present in each field during the previous growing season (Schwartz and Szostek, unpublished). During the 2012 growing season, thrips density reached 65.5 per onion plant at ARDEC, 51.3/plant in the Larimer County field, and >200 thrips per onion plant in the Weld County field. IYSV incidence for each field reached 78%, 87%, and 20%, respectively. Thrips numbers and IYSV incidence for each location were obtained through collaborators with the OnionIPM PIPE project (http://www.alliumnet.com/IPMPipe.html).

Plants were stored in resealable plastic bags at 4°C until inspection for thrips; usually within five days of collection. Each plant was visually inspected for thrips with the aid of a dissecting microscope. Thrips were removed from the plants with a fine tipped (#1, white bristle) artist's paintbrush and preserved in 95% ethanol until prepared for RT-PCR. Larvae were separated from adults. Once thrips had been removed from the plants, the leaves were rinsed and blotted dry, and ten 0.1 g samples were stored at -80°C until RT-PCR analysis.

Larval acquisition of IYSV

To confirm that thrips could complete their life cycle on the collected plants and to confirm that larvae could acquire IYSV from other potential host plants, common mallow, flixweed, dandelion, prickly lettuce, salsify, and winter wheat (cultivar Byrd) were grown from seed in the presence of viruliferous thrips in a greenhouse. Weed seeds were collected from ARDEC and the area around the CSU University Greenhouses, while winter wheat seed was obtained from the CSU wheat breeding project. Seeds were started in flats, then transplanted into 3.8 liter pots. When possible, four plants were transplanted into each of five pots for a total of 20 plants per species. Approximately 10 adult thrips were transferred to each pot from symptomatic onion plants before covering the pot with a barrier (Figure 3.2) to limit thrips movement between pots from exterior sources. At this time, adult thrips from symptomatic onion plants were also collected and tested for IYSV as a check. After four weeks, thrips larvae were removed from each plant to be tested for IYSV by RT-PCR. One leaf from each plant was removed, rinsed, and blotted dry. Sections of each leaf with thrips feeding damage were excised from the leaves. Leaf excisions were pooled, such that a single sample contained 0.1 g of plant material from two leaves. Samples were stored at -80°C until further use.

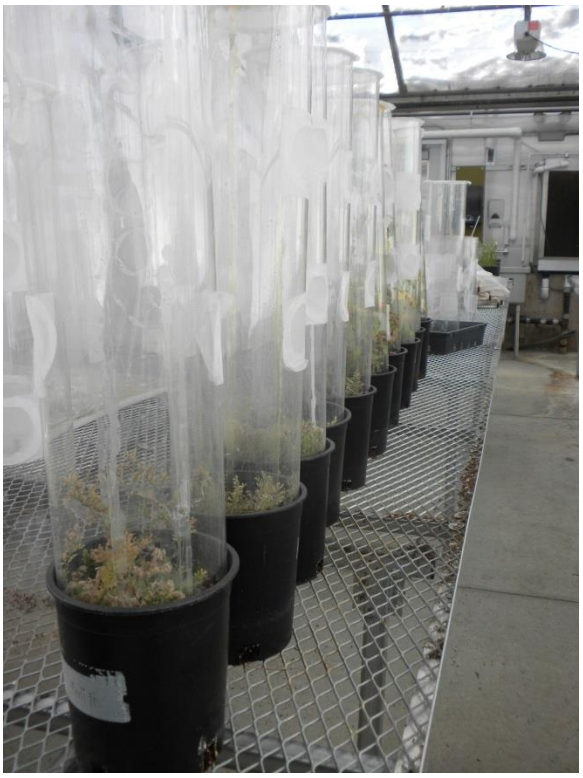


Figure 3.2. Barriers covering pots of weed species.

RNA extraction and cDNA synthesis

Total RNA was extracted using the Spectrum Plant Total RNA kit with the 750 µl binding solution option according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Total RNA was measured with a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Aliquots of RNA were treated with DNase I according to the manufacturer's instructions (Fermentas, Glen Burnie, MD). cDNA was synthesized from total RNA using M-MLV reverse-transcriptase according to the manufacturer's instructions for use with random primers (Invitrogen, Carlsbad, CA). No more than 1.0 µg total RNA was used in each 20 µl reaction. When possible, cDNA was brought to a final concentration of 10 ng/µl before use in PCR.

RT-PCR conditions

Reverse-transcriptase PCR (RT-PCR) reactions were performed on a MJ Research PTC-200 thermocycler (Waltham, MA) with an initial step of 94° C for 3 min; followed by 40 repeats of the three step sequence of 94° C for 45 sec, 55° C for 30 sec, 72° C for 90 sec; and a final step of 72° C for 10 min. Each 20 µl reaction consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3 mM MgCl2, 0.15 mM dNTP mix, 1 unit Taq DNA polymerase (New England Biolabs, Ipswich, MA), 20 ng cDNA and autoclaved distilled water. RT-PCR was conducted for each sample to confirm the presence or absence of IYSV. The IYSV nucleoprotein gene was amplified by RT-PCR with primers designed by Coutts et al. (5) , and primers specific to plant NADH dehydrogenase ND2 subunit (27) were used as a control. Primers were designed with PrimerQuest software (IDT, Coralville, IA) against the cytochrome oxidase subunit I genes for *Thrips tabaci* (GenBank accession number: AM932043) and *Frankliniella occidentalis* (GenBank accession number AM932029) as controls for thrips samples. The primers for *T. tabaci* were 5' ATAAAGAAGGAGCGGGAACGGGAT 3' (forward) and 5'ATAGCTCCCGCTAACACTGGCAAA 3' (reverse). The primers used against *F. occidentalis* were 5' TGCGGGAACGGGATGAACAGTTT 3' (forward) and 5'-CTCCTCTCGGATCTAAGAAGGATGT -3' (reverse). Primers were not developed for other, less frequently encountered thrips species. Reaction products were run in either 1% or 1.5% agarose gels depending on the expected product sizes (Bio-Rad Laboratories, Inc., Hercules, CA), and stained with ethidium bromide in 1X TAE buffer (Bio-Rad Laboratories, Inc., Hercules, CA). A 100bp molecular weight marker (Fermentas, Glen Burnie, MD) was included on each gel.

DAS-ELISA

Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) were performed according to the manufacturer's instructions (Agdia, Inc., Elkhart, IN) with the following modifications. Plant tissue was ground in liquid nitrogen as described by Gent et al. (9). A 0.2 g sample of plant tissue was typically used with 1.0 ml extraction buffer. All PBST rinses were 4 short rinses and one longer rinse that remained on the plate for 5 minutes. Positives were 2X background.

Thrips identification

Adult thrips were identified to species by J. Hardin, Research Associate at CSU, using the keys, *Thysanoptera: an identification guide* (17) and *Thrips of California* (10).

Results

Cull piles

Live thrips were recovered from cull piles during early 2011. Five were recovered in January, eight in February, and 24 in March. Species included *Thrips tabaci* and *Frankliniella occidentalis*, and with the exception of two larvae (one was collected in each of January and February), all were adults. *Thrips tabaci* and *F. occidentalis* were identified by RT-PCR from pooled samples. Subsequently, the proportion of each species present in the 2011 samples is not known. Additional species may have been present in the pooled samples, but remained undetected. IYSV was detected in a pool of four thrips (*T. tabaci* and *F. occidentalis* were present in the pool) collected in January and in a single *T. tabaci* collected in February. IYSV was not detected in *F. occidentalis*. The live thrips collected in March were transferred to seven

healthy onion plants, and IYSV was subsequently detected in five of the seven onion plants one month after thrips exposure.

No live thrips were recovered from the constructed cull piles over the winters of 2011 to 2012 or 2012 to 2013. Onions within the constructed cull piles did not sprout as the onions in the smaller piles of early 2011 did, but instead began to decay. Although live thrips were not found in these piles other small, live arthropods were noted.

Temperatures at each location within the constructed cull piles were similar between cull piles in both 2011 to 2012 and 2012 to 2013 (Figure 3.3). The temperature probe in the center pile (2011 to 2012) and in the south pile (2012 to 2013) occasionally recorded erroneous temperatures and these temperatures were omitted. Temperatures at the bottom of each pile (0 cm) fluctuated the least, while temperature fluctuation increased toward the top of each dome-shaped pile where the insulating layer of onions was not as deep. Each pile was exposed to temperatures below freezing for several weeks and the onions within the piles eventually froze. Temperatures in the cull piles did not fluctuate as dramatically as the surrounding air temperature did (Figure 3.4).

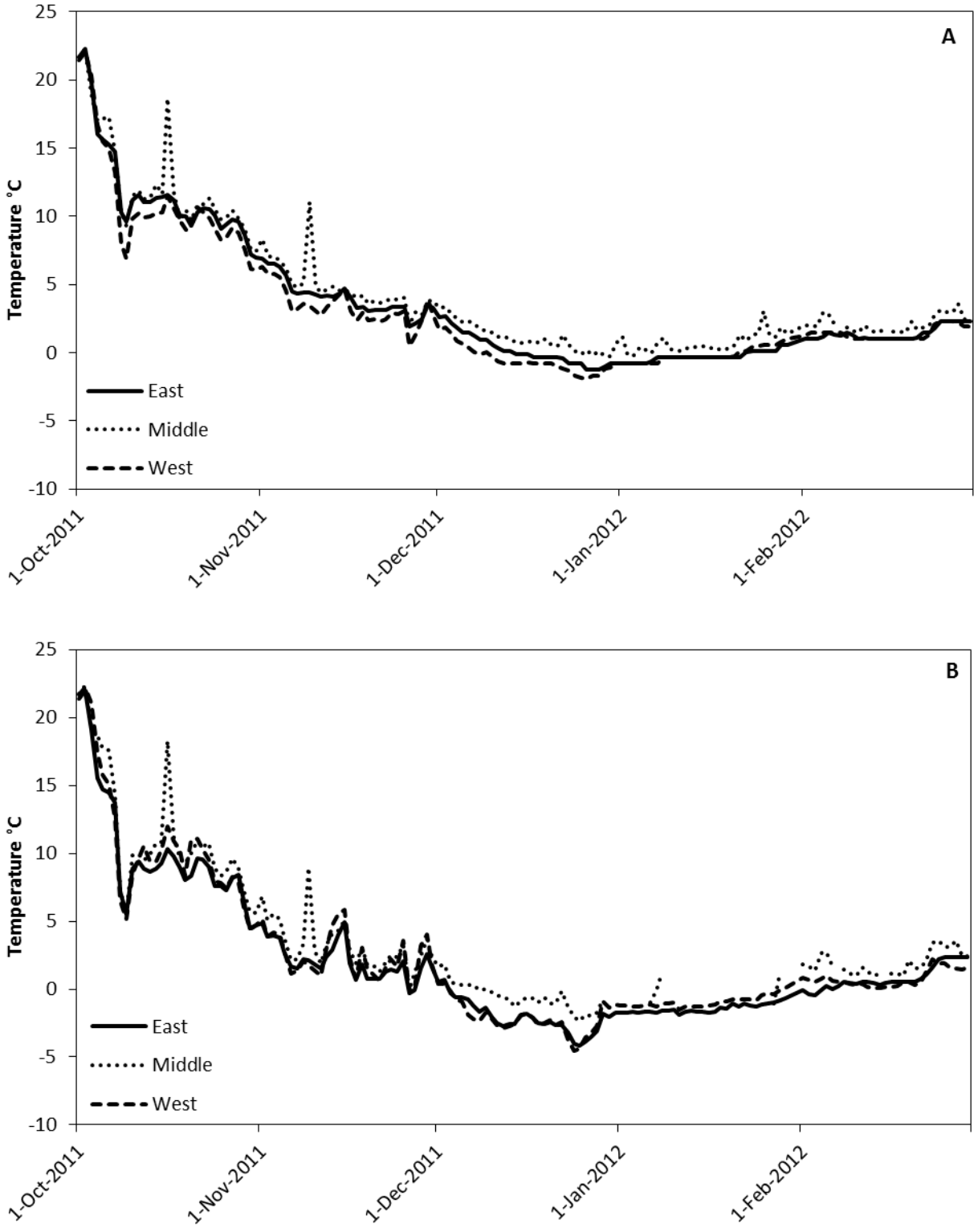


Figure 3.3. Average daily temperature at four locations within each of three cull piles. A-D, 2011 to 2012 temperatures. E-H, 2012 to 2013 temperatures. A, E: 0 cm above soil. B, F: 10 cm above soil. C, G: 20 cm above soil. D, H: 30 cm above soil.

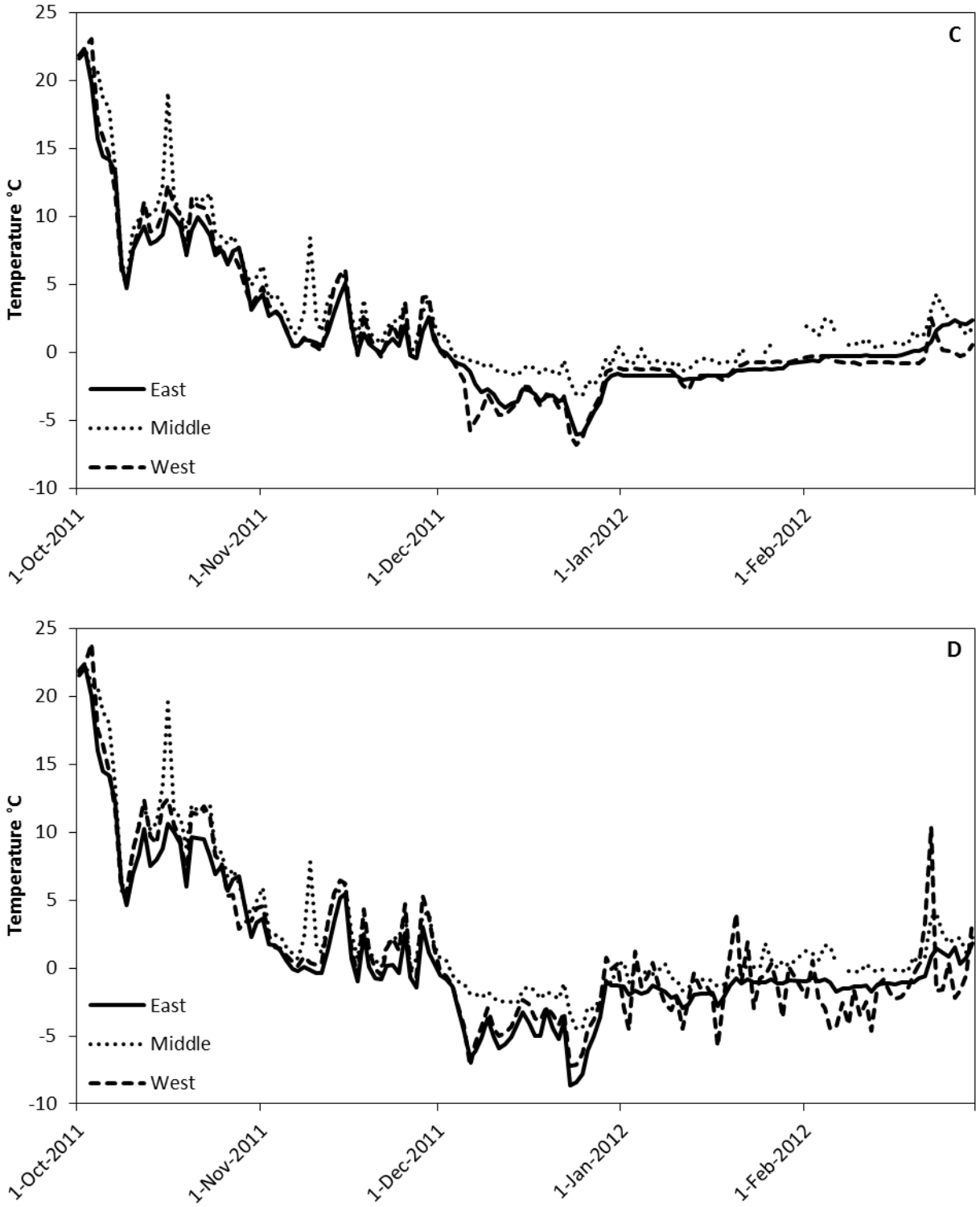


Figure 3.3 continued. Average daily temperature at four locations within each of three cull piles. A-D, 2011 to 2012 temperatures. E-H, 2012 to 2013 temperatures. A, E: 0 cm above soil. B, F: 10 cm above soil. C, G: 20 cm above soil. D, H: 30 cm above soil.

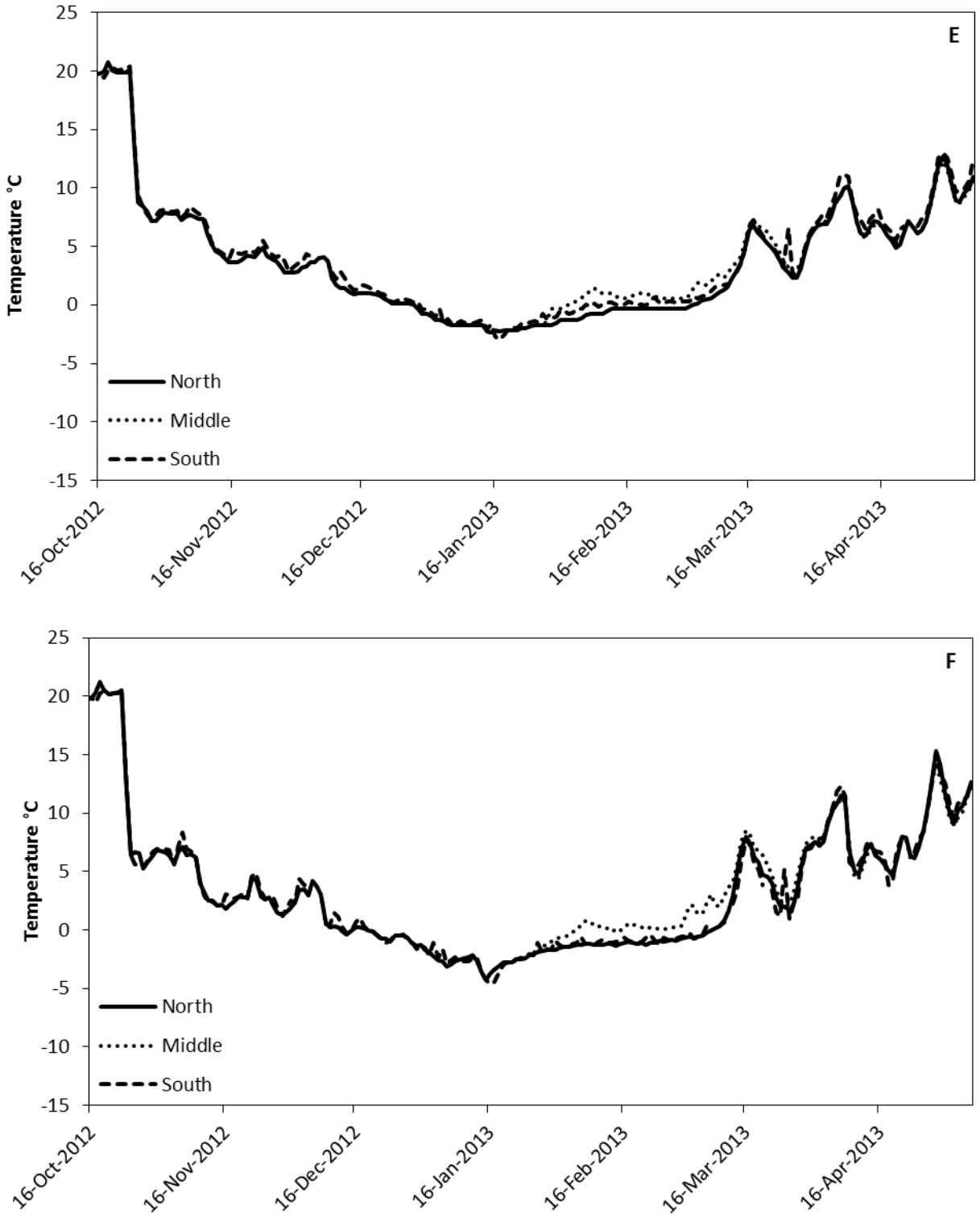
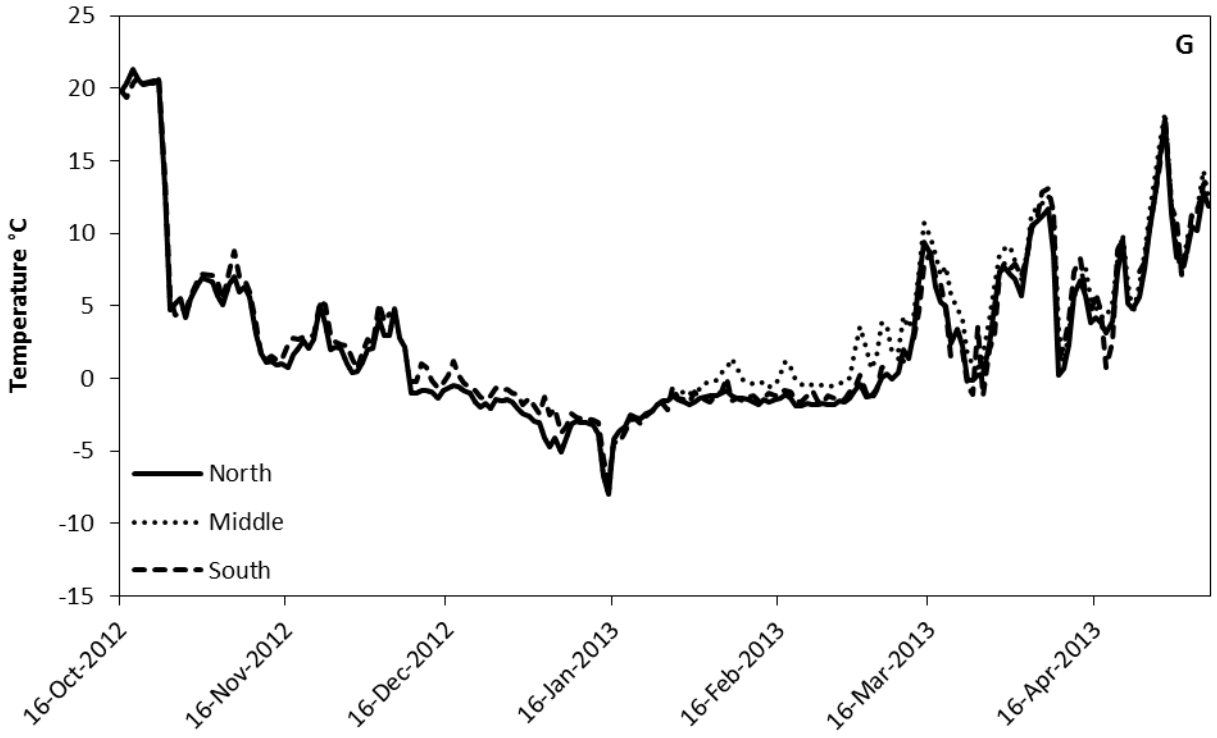


Figure 3.3 continued. Average daily temperature at four locations within each of three cull piles. A-D, 2011 to 2012 temperatures. E-H, 2012 to 2013 temperatures. A, E: 0 cm above soil. B, F: 10 cm above soil. C, G: 20 cm above soil. D, H: 30 cm above soil.



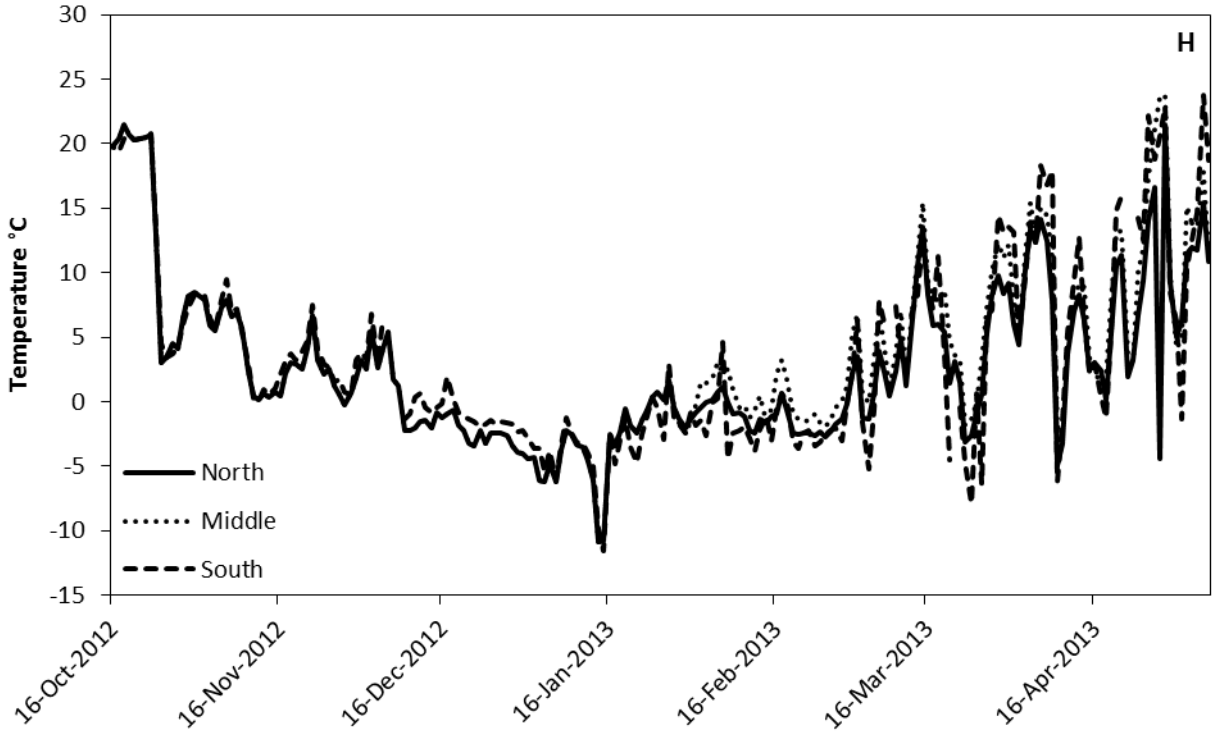


Figure 3.3 continued. Average daily temperature at four locations within each of three cull piles. A-D, 2011 to 2012 temperatures. E-H, 2012 to 2013 temperatures. A, E: 0 cm above soil. B, F: 10 cm above soil. C, G: 20 cm above soil. D, H: 30 cm above soil.

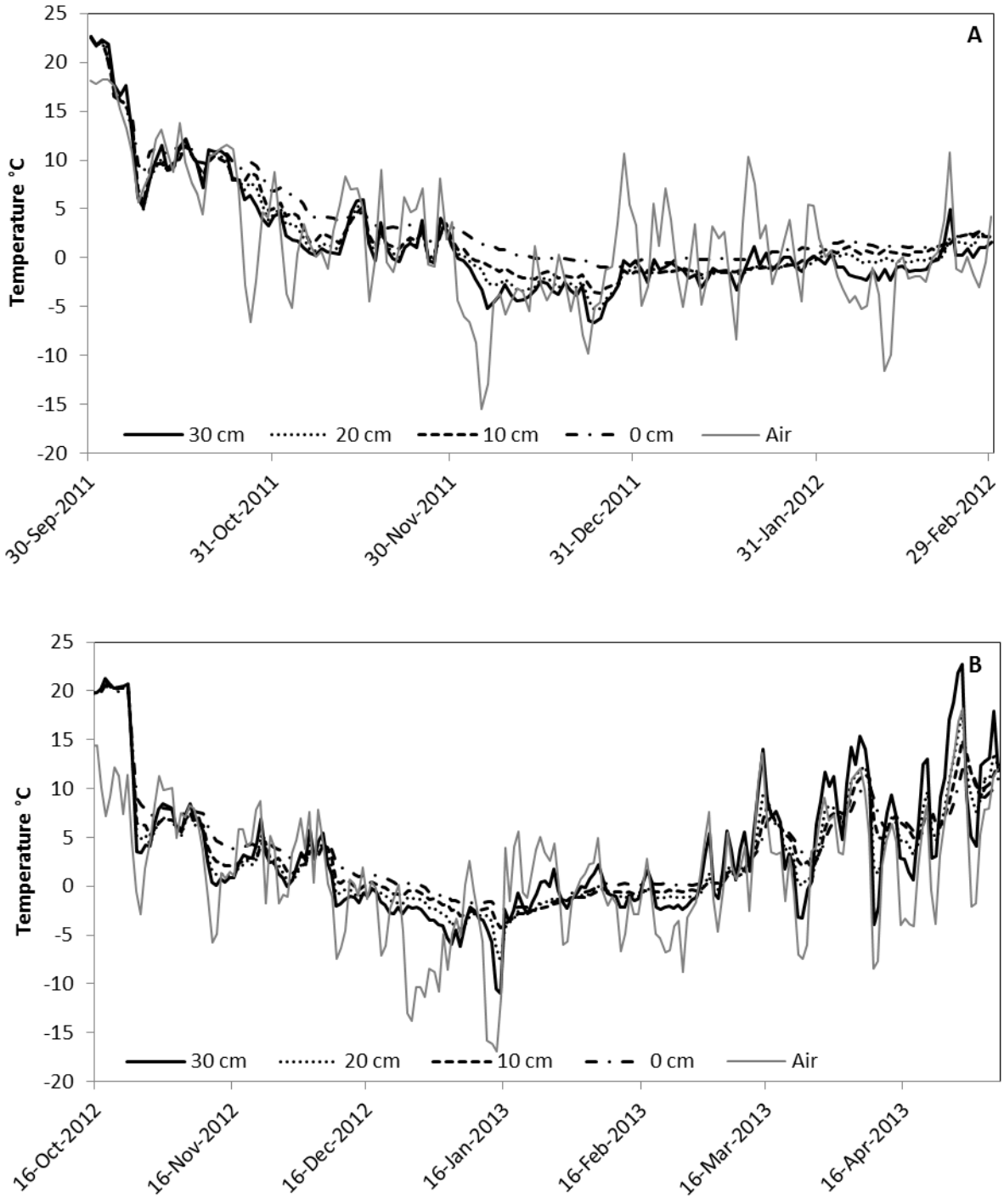


Figure 3.4. Average daily temperature of the three cull piles at each of four locations (0, 10, 20, or 30 cm above the soil) within the three cull piles compared to the average daily air temperature. A: average temperatures for 2011 to 2012; B: average temperatures for 2012 to 2013.

Thrips Activity

In both years, the number of thrips caught on sticky traps decreased as air temperature decreased and increased as air temperature increased (Figure 3.5). Thrips were not trapped during periods when the average temperature remained below 0°C. A single thrips was trapped in January 2012 when the average temperature was above 0°C for several days. Once average temperatures rose above 0°C, thrips began to be caught again despite daily temperatures below 0°C. The average temperature from February to April 2012 was greater than for the same period in 2013, and more thrips were caught during this period in 2012 than in 2013. Thrips numbers further increased after onions were transplanted into the field.

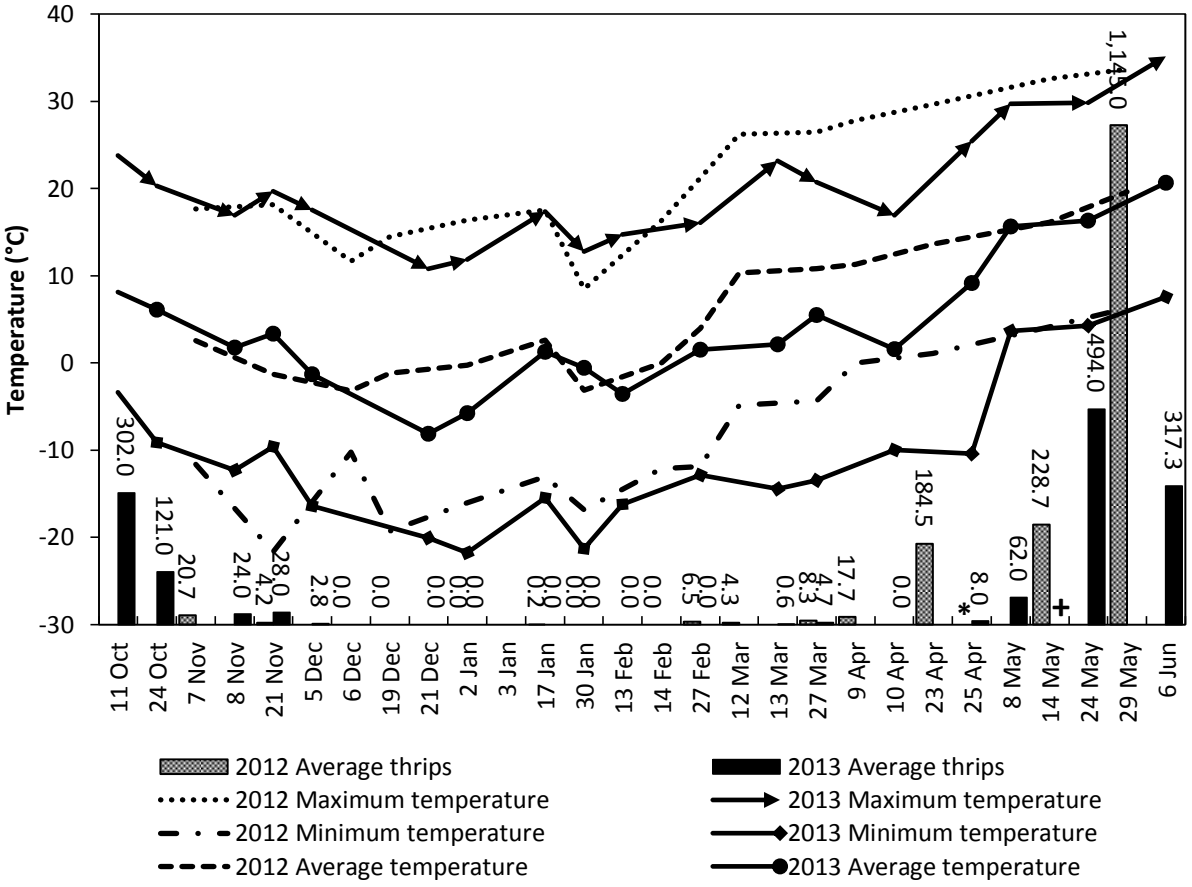


Figure 3.5. Temperatures averaged over the approximately two week intervals that sticky traps were in the field. Thrips numbers are averages over the number of traps remaining in the field at the end of each interval. The 2012 onion planting date is marked with *; the 2013 planting date with +.

Additional plant hosts

Live adult and larval thrips were recovered from 12 different plant species over two winters (Table 3.2). IYSV was detected in field-collected plants of blue mustard, flixweed, prickly lettuce, salsify, smooth brome, sunflower, and winter wheat. The onion plants were not tested as they are a known host of IYSV. IYSV was detected in adult thrips collected from plants of common mallow, dandelion, flixweed, onion, prickly lettuce, and salsify. IYSV was detected in larvae removed from onion, but not in larvae removed from any other plant. Thrips larvae were not found on any of the plants in which IYSV was detected. Adults and larvae collected from the same plant did not always share the same IYSV status; for example, IYSV was detected in adults collected from common mallow in January 2012, but not in the larvae removed from the same plant. Likewise, the IYSV status of the plant and the thrips on the plant was not always identical; for example, IYSV was detected in blue mustard and flixweed (March 2013), but not in the adult thrips associated with those plants. Flixweed and prickly lettuce collected in March 2012 had IYSV and so did the adult thrips. A greater variety of plant species was available later in the season than in January and February.

IYSV was detected in thrips collected from plants in January, February, and March of 2012, while in 2013 it was only detected in thrips in March. IYSV was not detected in plants or thrips collected in April, 2012 from the Hort Farm or from the commercial field in Larimer County; nor was IYSV detected in any of the plants or thrips collected from the Weld County field in 2013.

The primers designed to detect *T. tabaci* and *F. occidentalis* confirmed the morphological identification of *T. tabaci* and *F. occidentalis* (Figure 3.6). The results presented in Figure 3.6 are a representative example, and the additional samples present in the original image have been

removed for simplicity. The thrips represented in Figure 3.6 were removed from common mallow collected in January 2013 from ARDEC. Larvae were separated from adult thrips, and adult thrips were identified and further separated by species. *T. tabaci* and *F. occidentalis* were the only adult thrips species present in this sample. RT-PCR of thrips identified as *T. tabaci* produced an amplicon between 200 to 300 bp (predicted size, 262 bp), and an amplicon of approximately 300 bp (predicted size, 308 bp) was produced from thrips identified as *F. occidentalis*. Both amplicons were produced from the larval sample indicating both species were present as larvae. IYSV was not detected in these thrips samples. No amplification occurred in the negative controls. The primers designed for one species did not produce amplicons when exposed to a different species.

Table 3.2. IYSV status of plants and thrips collected during the winters of 2012 and 2013.

	Plant collected (IYSV status[a])	Location	Number of adult thrips[b] (IYSV status[c])	Number of thrips larvae[b] (IYSV status[c])
Jan. 2012	Common mallow (-)	ARDEC	15 (+)	2 (-)
	Onion (n.t.)	ARDEC	23 (+)	22 (+)
Feb. 2012	Common mallow (-)	ARDEC	6 (+)	3 (-)
	Prickly lettuce (+)	ARDEC	0	0
	Winter wheat (+)	ARDEC	4 (-)	0
Mar. 2012	Bindweed (-)	ARDEC	0	0
	Dandelion (-)	ARDEC	53 (+)	1 (-)
	Flixweed (+)	ARDEC	3 (+)	0
	Prickly lettuce (+)	ARDEC	1 (+)	0
	Redstem filaree[d]	ARDEC	1 (-)	100 (-)
	Salsify (-)	ARDEC	3 (+)	17 (-)
	Smooth brome (+)	ARDEC	1[e]	0
	Sunflower (+)	ARDEC	1[f]	0
	Winter wheat[b]	ARDEC	0	11 (-)
Apr. 2012	Bindweed (-)	Hort Farm	2 (-)	1 (-)
	Bindweed (-)	Larimer	0	6 (-)
	Common mallow (-)	Hort Farm	0	24 (-)
	Dandelion (-)	Hort Farm	2 (-)	33 (-)
	Dandelion (-)	Larimer	2 (-)	17 (-)
	Flixweed (-)	Hort Farm	2 (-)	1 (-)
	Prickly lettuce (-)	Hort Farm	0 (-)	1 (-)
Jan. 2013	Common mallow (-)	ARDEC	23 (-)	7 (-)
	Flixweed (-)	ARDEC	23 (-)	35 (-)
	Flixweed (-)	Weld	2 (-)	0
	Prickly lettuce (-)	ARDEC	5 (-)	11 (-)
	Prickly lettuce (-)	Weld	0	0
Feb. 2013	Blue mustard (-)	Weld	1 (-)	0
	Flixweed (-)	Larimer	10 (-)	9 (-)
	Flixweed (-)	Weld	0	1 (-)
	Prickly lettuce (-)	Weld	0	0
	Salsify (-)	Larimer	2 (+)	0
Mar. 2013	Blue mustard (+)	Larimer	3 (-)	0
	Blue mustard (-)	Weld	0	0
	Common mallow (-)	ARDEC	4 (-)	2 (-)
	Flixweed (-)	ARDEC	13 (-)	7 (-)
	Flixweed (-)	Weld	0	0
	Flixweed (+)	Larimer	36 (-)	0
	Prickly lettuce (-)	Weld	0	0

n.t., not tested. [a]positive status if IYSV was detected in at least one of ten 0.1 g plant leaf tissue samples. [b]the number recovered from all bulked plants of the particular plant species on that collection date. [c]IYSV status of the pooled adult or larval thrips collected from the corresponding plant. [d]samples would not amplify. [e]misplaced, not tested. [f]slide mounted for identification, not tested.

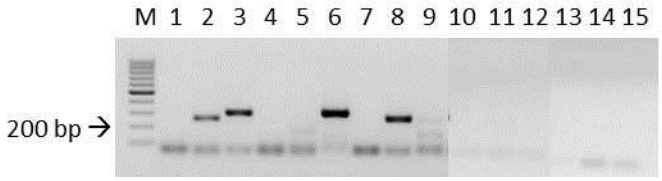


Figure 3.6. Representative agarose gel visualization of amplicons obtained by RT-PCR of RNA extracted from thrips species removed from common mallow. Lanes 1 to 3: mixed larvae; lanes 4-6: adult *F. occidentalis*; lanes 7 to 9, adult *T. tabaci*; lanes 10 to 12: -RT negative control; lanes 13 to 15: water only negative control.
Lane M: 100bp molecular weight marker; lanes 1, 4, 7, 10, 13: IYSV; lanes 2, 5, 8, 11, 14: *T. tabaci*; lanes 3, 6, 9, 12, 15: *F. occidentalis.*

Over the course of two winters, only four thrips species were found on the collected plants (Table 3.3). The most abundant species in these samples were *T. tabaci* and *F. occidentalis*, both of which were present at each location. Most plants harbored more than one species.

Table 3.3. Adult thrips species recovered alive from field collected weed species during 2012 and 2013.

	Location	Plant	Thrips species (number recovered)
Feb. 2012	ARDEC	Winter wheat	*Thrips tabaci* (4)
		Common mallow	*T. tabaci* (4)
			Frankliniella occidentalis (2)
Mar. 2012		Dandelion	*T. tabaci* (54)
			F. occidentalis (3)
			F. fusca[a] (2)
		Salsify	*T. tabaci* (3)
		Flixweed	*T. tabaci* (3)
		Prickly lettuce	*T. tabaci* (1)
		Sunflower	*Microcephalothrips abdominalis*[a] (1)
		Redstem filaree	*F. occidentalis* (1)
Jan. 2013	ARDEC	Common mallow	*T. tabaci* (7)
			F. occidentalis (14)
		Flixweed	*T. tabaci* (16)
			F. occidentalis (8)
		Prickly lettuce	*T. tabaci* (2)
			F. occidentalis (4)
		Flixweed	*T. tabaci* (2)
	Weld	Flixweed	*T. tabaci* (10)
Feb. 2013	Larimer	Salsify	*T. tabaci* (2)
		Common mallow	*T. tabaci* (1)
			F. occidentalis (3)
	ARDEC	Flixweed	*T. tabaci* (10)
			F. occidentalis (2)
		Blue mustard	*T. tabaci* (3)
	Larimer	Flixweed	*T. tabaci* (30)
			F. occidentalis (4)

[a] Specimens were slide mounted for species confirmation, and were not tested for IYSV.

Larval acquisition of IYSV

Thrips feeding (Figure 3.7) and larvae were present on each plant species (common mallow, dandelion, flixweed, prickly lettuce, salsify, and winter wheat) grown from seed in the greenhouse. IYSV was detected by RT-PCR in the adult thrips removed from symptomatic onion plants, thrips larvae removed from salsify (3 of 6 larval samples), and thrips larvae removed from dandelion (1 of 5 larval samples). IYSV was detected in 8 of 15 salsify leaf samples and in 1 of 25 prickly lettuce leaf samples collected from different plants. Dandelion samples did not amplify. IYSV was not detected in common mallow, flixweed, or wheat, or in the thrips larvae removed from those plants.

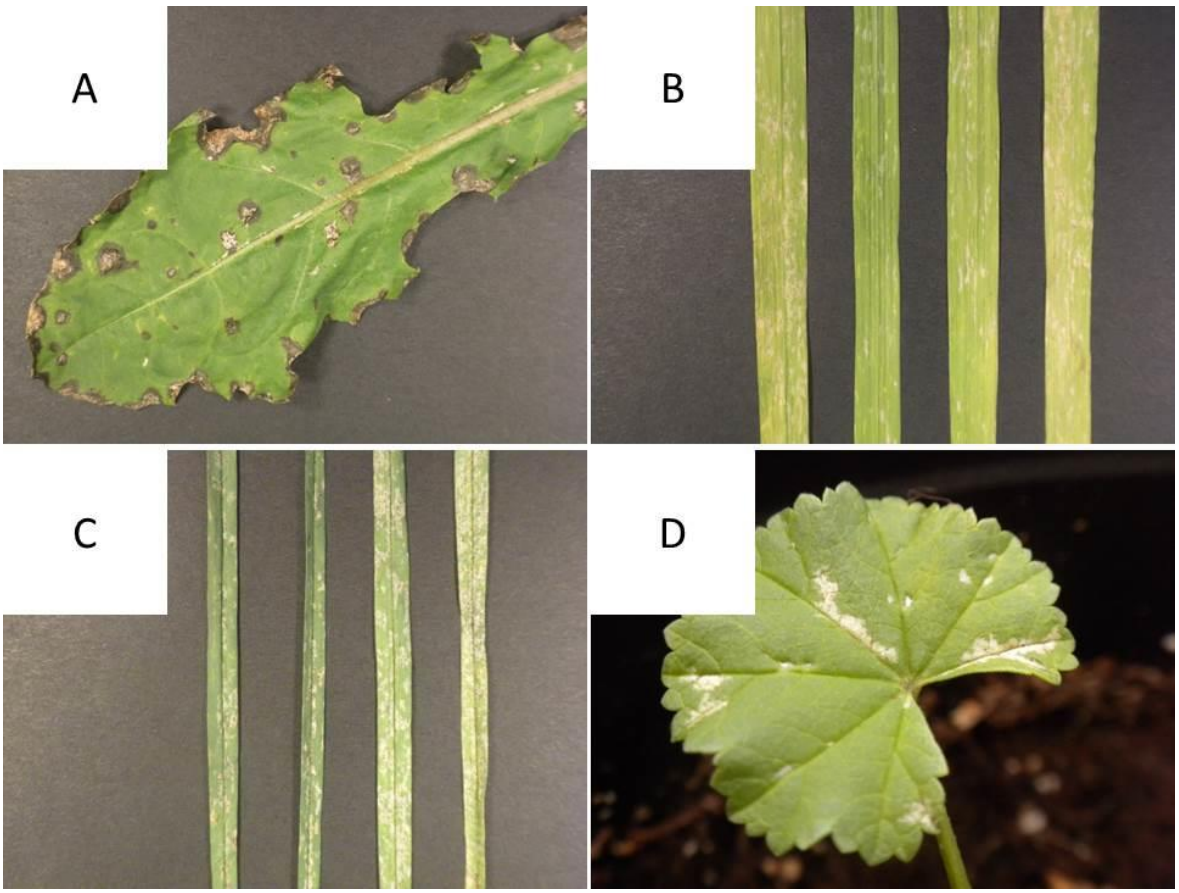


Figure 3.7. Thrips feeding damage and frass on four of the weed species planted in the greenhouse. A: prickly lettuce; B: winter wheat; C: salsify; D: common mallow.

Discussion

Living thrips were found throughout the winter at several locations and on a variety of plants (Table 3.2). *T. tabaci*, the vector of IYSV, was among the species found overwintering on these plants. IYSV was detected in six non-allium plant species and in thrips removed from five non-allium plant species. Of these plants, dandelion (12) and prickly lettuce (23) have been reported as hosts of IYSV, and common mallow and dandelion have been reported to support *T. tabaci* reproduction (26). Blue mustard, flixweed, salsify, sunflower, and winter wheat have not yet been reported as potential hosts of IYSV.

Plants and the thrips associated with them did not always share the same IYSV status, such as the presence of IYSV in thrips removed from field-collected common mallow. IYSV was never detected in common mallow. The discrepancy in IYSV status suggests that IYSV infected onion thrips survive on plants that are either not IYSV hosts or not yet infected, onion thrips may not have acquired IYSV from infected plants during the winter, or the amount of plant material tested was insufficient to detect IYSV.

The results of this study suggest that onion thrips infected with IYSV survive winter months (from last season's onion harvest to the next season's planting) on any available plants. Thrips associated with onions have been shown to survive up to six weeks at 0 to 1°C on a non-preferred food source, although numbers did decrease over time (30). With access to a source of food, female *F. occidentalis* can survive up to 40 days at an average temperature of 0°C (28). During the two winters of this study, the average temperature did not remain below freezing for more than 14 days. *F. occidentalis* will freeze at -26°C (28), however, the lowest air temperature recorded during this study was -21.8° C and it may have been warmer in the plant spaces occupied by thrips. The freezing point of *T. tabaci* has not been reported. Thrips in the field

experience temperature fluctuations which may allow a lifespan beyond those reported under experimental conditions.

Although thrips larvae were found throughout the winter, continual generation of new thrips during winter seems less likely than thrips surviving the season. Edelson and Magaro (7) found that *T. tabaci* development ceased below 11.5°C, and Horsfall and Fenton (11) reported that onion thrips tended to take longer to progress from one life stage to the next at cooler temperatures (20°C compared to 29°C). The larvae collected during this study may have emerged while it was still warm, then were prevented from further development by the low temperatures. Conversely, they may have emerged during brief periods of warmth and remained in the larval stage until temperatures were conducive to further development. The observation of increased adult thrips activity when the average temperature rose above 0°C and the noticeable increase of adult thrips caught on the sticky traps once the average temperature rose above 11.5°C (Figure 3.5) suggests adult thrips already present in the environment may become active at a lower temperature than development occurs. It is also possible that thrips in Colorado have adapted to develop at lower temperatures than thrips in Texas, where the 11.5°C developmental threshold was observed. Insects capable of overwinter survival adapt to local conditions and acclimate themselves to the changing seasons (16). Details about the specific microclimate in which each plant thrips were found are unknown, but these microclimates may also influence survival and development.

It is difficult to say whether thrips acquire IYSV during the winter. Thrips larvae were not present on any of the field collected non-allium plants in which IYSV was detected, and it is unknown if the adult thrips collected for this study acquired IYSV as larvae or as adults. It is generally accepted that *T. tabaci* must acquire IYSV as a larva in order to transmit the virus as an

adult (8). It is reasonable to assume that at least a portion of overwintering adult thrips acquired IYSV as larvae because healthy onion plants became infected with IYSV after exposure to thrips collected in March 2011 from cull piles and from onions that had not yet been plowed under. This suggests the adult thrips were viruliferous. The possibility that onion thrips already present in the greenhouse were responsible for transmitting IYSV to the healthy onion plants, despite the barriers, must also be considered. IYSV was detected in thrips larvae collected from onions in January 2012 suggesting the larvae were overwintering with IYSV that had been acquired when the days were warm, or that they are able to acquire IYSV during the winter.

The third possible explanation for the discrepancy in IYSV status between the plant and associated thrips particularly where IYSV appears in thrips but not in the corresponding plant sample is that IYSV was present in the plant, but not in the particular leaf section that was tested. IYSV is distributed unevenly within onion plants (18, 24) and the same may be true of IYSV distribution within other plant species.

Larvae were found on common mallow, dandelion, flixweed, prickly lettuce, salsify, and winter wheat grown under greenhouse conditions indicating that these plant species can support *T. tabaci* reproduction. Not all of these plants or the larvae associated with them had IYSV. The larval acquisition experiments in the greenhouse show that thrips larvae can acquire IYSV from dandelion and salsify. It is unlikely that the thrips larvae moved from the IYSV infected onions present in the greenhouse onto these weeds grown in isolation chambers. As noted by Hsu et al. (12), *T. tabaci* larvae are not capable of flight and do not travel far from the plant they emerge on. The acquisition efficiency of IYSV from plant to insect may vary by species as IYSV was detected in half of the larval samples collected from salsify, but in only 1 of 5 of the larval samples collected from dandelion. While there is information regarding transmission of IYSV

from *T. tabaci* to plants (13, 14, 19), information is lacking on how efficiently *T. tabaci* acquires IYSV from different plants. It has been shown that some plant species can become systemically infected with IYSV, while others only develop local lesions (1). This difference in virus distribution throughout a plant may affect acquisition by thrips, and it is possible that the IYSV infected plants collected in this study were only locally infected, and the thrips larvae had not fed in a leaf area with IYSV. The possibility of variable acquisition of IYSV by *T. tabaci* is supported by similar work on other plant-thrips-tospovirus complexes. *F. occidentalis* reared on different plant species infected with *Impatiens necrotic spot virus* (INSV) were found to transmit INSV at different efficiencies, indicating differences in efficiency of acquiring the virus as larvae (21). Similarly, *Tomato spotted wilt virus* (TSWV) transmission by *T. tabaci* differed according to the TSWV-infected host plant on which the thrips were reared (3).

The plants collected in this study appear to support IYSV overwintering in two ways: as a direct host of IYSV or as a host for IYSV infected onion thrips. As a winter annual and/or biennial plant (29), common mallow appears to be an indirect green bridge supporting IYSV infected thrips. IYSV was not detected in any of the common mallow samples from the field or greenhouse, but was detected in adult onion thrips removed from some of the field collected common mallow samples. No larval sample collected from the field or greenhouse grown common mallow had acquired IYSV, further indicating this species is not an IYSV host. IYSV infected onion thrips that successfully overwinter on common mallow could move to IYSV susceptible plants once they are available. For this reason, it would be a good idea to control common mallow during the winter.

Dandelion, flixweed, prickly lettuce, salsify, and winter wheat likely play a similar role as common mallow as thrips host plants, but can also serve as a host for IYSV. None of these

plants were consistently IYSV positive or negative in either the field collected samples or in the greenhouse grown plants. For example, IYSV was detected in 8 of 15 greenhouse-grown salsify plant samples, but in none of the field collected plants. Conversely, IYSV was not detected in any of the 15 greenhouse-grown flixweed samples, but was detected in field collected plants.

Larvae removed from dandelions grown under greenhouse conditions had IYSV, but because RT-PCR of the dandelion plant samples did not produce any amplicons, the IYSV status of these plants cannot be confirmed. It can be assumed that the plants had IYSV because the larvae were able to acquire it. Dandelion has been reported as an IYSV host (12) and its perennial nature (29) make it a good green bridge.

Although IYSV was detected in field collected flixweed and adult thrips removed from these plants, it was not detected in any flixweed sample from the greenhouse experiment or in the thrips removed from those plants. As a winter annual (29), flixweed would need to be inoculated with IYSV after germination in the autumn. Cold temperatures inhibiting thrips movement may prevent or reduce inoculation of flixweed under field conditions and is a reasonable explanation of why IYSV was not detected in more of the field collected flixweed samples. However, this does not explain the lack of IYSV in the greenhouse-grown flixweed. Many larvae were observed on the greenhouse-grown flixweed, and larvae were present on flixweed collected from the field. Flixweed may be important in supporting thrips through the winter or in increasing the number of thrips early in the next growing season. This contrasts with the findings of Smith et al. (26), who did not find any thrips larvae on flixweed in New York.

As a biennial or winter annual (29), prickly lettuce is another good candidate as a green bridge. Prickly lettuce has previously been reported as a host of IYSV (23) and the results presented here confirm that. IYSV was found in prickly lettuce and in a single adult onion thrips

collected from that species. Only one prickly lettuce plant had IYSV in the greenhouse, and none of the larvae from that plant or others had IYSV. While it is clear that prickly lettuce can host IYSV and thrips, it is not clear if thrips can acquire IYSV from prickly lettuce since the virus was not detected in any of the larval samples.

IYSV was detected in a greater proportion of greenhouse-grown salsify (8 of 15 plant samples) and in the larvae associated with them (3 out of 6 larval samples) than in the other plant/thrips combinations. This suggests that salsify could be a more important overwintering host of IYSV than the other non-allium plants considered in this study, and that salsify plays a role in the persistence of IYSV in the landscape. Onion thrips are able to reproduce on and acquire IYSV from this plant species. As a biennial (29), salsify could become infected with IYSV during its first year and remain infective during its second year, although it is not known if this occurs. IYSV was not detected in salsify collected from any field location, but was detected in the adult thrips removed from those plants (Table 3.2).

IYSV was detected in only one sample of field collected winter wheat and was not detected in any greenhouse grown wheat samples or in the larvae removed from them. It is unknown what cultivar the field collected winter wheat was, so varietal differences are possible. Winter wheat has not been reported elsewhere as an IYSV host and additional cultivars of winter wheat should be explored to determine if winter wheat is an important additional host of IYSV. Because onion thrips can overwinter on wheat (2, 20) it may serve as a green bridge for the vector, if not the virus, which could be problematic if the overwintering thrips were already infected. The sunflower that IYSV was detected in was a newly sprouted volunteer of a domesticated type and was not present until mid-March. While native sunflowers are common in Colorado, they are not green during the winter and are unlikely to be an important

overwintering site of IYSV or onion thrips, therefore no further investigation of sunflower as a host of IYSV was conducted.

Finding sources of IYSV in the landscape is like looking for a needle in a haystack. IYSV may be more prevalent in the environment than is apparent, but if the right plant wasn't selected, or if the right leaf sampled, it could have been missed. Most of the IYSV positive thrips recovered in this study were adults collected at ARDEC in 2012 when onions were still present in the field. The thrips collected from non-allium plants at ARDEC could have been continually migrating from the onions; however, the sticky traps did not record much adult thrips activity during that time. In addition, *T. tabaci* have been observed to have a preference for onions (6), but will feed on other plants when necessary. It seems unlikely that onion thrips would leave their preferred plant in favor of a less preferred plant. In contrast, onions from the previous growing season had been removed from the Larimer County field in 2013, yet IYSV was present in the thrips collected on salsify from that site. This suggests IYSV can persist in the absence of onions. It is surprising that so few thrips were collected from the plants at the Weld County location, considering the high number of thrips present on the onions during the previous growing season, but the onions had been removed from the field at the end of the growing season.

Thrips activity as monitored by sticky trap collection appeared to follow the temperature trend with fewer thrips caught as the temperature decreased, and more were caught as the temperature increased. Changes in thrips numbers cannot be solely attributed to temperature as available plant matter also varied along with the temperature. With the exception of a single thrips, thrips were not trapped during January and February of either 2012 or 2013. However, live thrips were recovered from plants during the same period. Temperatures may have been too

cold for flight, or the overall number of thrips had dropped so low as to greatly reduce the probability of the traps intercepting any adult thrips. The results of this study make it clear that all thrips do not die during the winter, but that they are present in low numbers with little activity.

Onion cull piles would appear to be a logical place for IYSV and onion thrips to overwinter. Cull piles have been reported as a source of IYSV (12) and have long been considered a source of onion thrips (11). The temperatures near the soil of the cull piles constructed for this study were conducive the survival of other small arthropods. An onion cull pile would seem to provide adequate shelter for onion thrips as the temperature inside remained steady and did not undergo the dramatic changes of the air temperature (Figure 3.4). However, winter sampling of onion cull piles in Ontario, Canada (2) did not yield any onion thrips, and in the work presented here live thrips were not found in the deliberately constructed cull piles, but were found in the field cull piles left from the 2010 growing season. The most reasonable explanation of this result is that many onions in the early 2011 cull piles had started to sprout and this green leaf tissue was where thrips were most often found. The onions in the 2011 to 2012 and 2012 to 2013 cull piles began to decay rather than sprout, hence there was no green tissue. This (along with the results of the non-allium host survey) suggests that onion thrips overwinter survival may be linked to the availability of green plant tissue. Thrips are known to feed on the contents of epidermal, palisade and spongy mesophyll cells, and to develop faster on new, young leaves than on bulbs (15). Frozen onion bulbs may not be an optimal source of nutrition or site for development. Onion cull piles *may* be a source of IYSV if the onions within them sprout, thus providing onion thrips with food and shelter.

The results of this study suggest some strategies that may reduce IYSV and/or thrips. Onions should not be allowed to remain in a field over the winter, as they can support IYSV infected adult and larval *T. tabaci*. Onion culls should also be destroyed in order to prevent volunteers appearing within the cull piles. Hsu et al. (12) found that volunteer onions in cull piles were a source of IYSV, and the results presented here show that volunteers within cull piles can support IYSV infected onion thrips. Green weeds, particularly salsify, should be removed from the vicinity of onion fields. Horsfall and Fenton (11) recommended burning weedy areas along roads, creeks, and windbreaks to eliminate hibernating thrips. However, eradicating all weeds or plants from the vicinity of an onion field may not be practical and may conflict with other IPM goals or practices such as maintaining refugia for beneficial organisms or preventing soil erosion.

This work opens several avenues for further research. It would be useful to know how far from an onion field IYSV infected plants and thrips are, in order to better target weed control. Boyce and Miller (2) found that onion thrips were plentiful in an alfalfa field 45 meters from an onion field, but that there were few onion thrips in an alfalfa field 400 m from the onion field. It would be interesting to know if IYSV followed the same pattern. Likewise, a formal study examining the effectiveness of the control strategies presented above would be useful to onion growers. As discussed earlier, winter wheat should be further evaluated as a potential source of IYSV, especially since it is often grown adjacent to or in rotation with onions in Colorado and elsewhere.

This work did not attempt to determine how competent any of the weed species were to host IYSV, or the efficiency that thrips could acquire IYSV from any of the potential host plants. It would be interesting to know if thrips have oviposited in any of these overwintering plants,

and if there is any relationship between ovipositing and IYSV status of the plant. This information could be useful to prioritize which weeds should be controlled.

There do not appear to be any studies regarding the effect of temperature on the acquisition of IYSV by *T. tabaci* from plants, or of any other plant-tospovirus-thrips complex. Tsumuki et al. (28) found that adult *F. occidentalis* walked less after exposure to -5°C, which prevented them from reaching their food source. The larvae collected in the study presented here were already in direct contact with their food source, but it is possible that the movements required for feeding become inhibited by cold temperatures. It would be interesting to learn if temperature affects a plant's ability to host IYSV, and thrips' ability to acquire the virus.

IYSV infected onion thrips that survive the winter are a likely source of inoculum at the start of the next growing season. Living thrips were found during Colorado winters on a variety of weed species and IYSV was found in both plants and associated thrips during the winter. Cull piles do not appear to be a favorable overwintering site for thrips unless the onions begin to sprout. Thrips activity follows temperature trends, and thrips activity is lowest during the coldest part of the year.

Under greenhouse conditions, *T. tabaci* can complete its life cycle on a number of plants and acquire IYSV from a smaller subset of these plants. To minimize thrips and IYSV overwintering, onions should not be left in the field between growing seasons, cull piles should be destroyed, and weeds should be managed throughout the winter.

LITERATURE CITED

1. Bag, S., and Pappu, H. R. 2009. Symptomatology of *Iris yellow spot virus* in selected indicator hosts. in: Plant Health Progress Plant Management Network.
2. Boyce, K. E., and Miller, L. A. 1953. Overwintering habits of the onion thrips, *Thrips tabaci* Lind. (Thysanoptera: Thripidae), in southwestern Ontario. Annual report of the Entomological Society of Ontario:82-86.
3. Chatzivassiliou, E. K., Peters, D., and Katis, N. I. 2007. The role of weeds in the spread of *Tomato spotted wilt virus* by *Thrips tabaci* (Thysanoptera : Thripidae) in tobacco crops. J. Phytopathol. 155 (11-12):699-705.
4. Cortes, I., Livieratos, I. C., Derks, A., Peters, D., and Kormelink, R. 1998. Molecular and serological characterization of *Iris yellow spot virus*, a new and distinct tospovirus species. Phytopathology 88 (12):1276-1282.
5. Coutts, B. A., McMichael, L. A., Tesoriero, L., Rodoni, B. C., Wilson, C. R., Wilson, A. J., Persley, D. M., and Jones, R. A. C. 2003. *Iris yellow spot virus* found infecting onions in three Australian states. Austral. Plant Pathol. 32 (4):555-557.
6. Doederlein, T. A., and Sites, R. W. 1993. Host-plant preferences of *Frankliniella-occidentalis* and *Thrips-tabaci* (Thysanoptera, Thripidae) for onions and associated weeds on the southern high-plains. J. Econ. Entomol. 86 (6):1706-1713.
7. Edelson, J. V., and Magaro, J. J. 1988. Development of onion thrips, *Thrips-tabaci* (Thysanoptera, Thripidae) Lindeman, as a function of temperature. Southw. Entomol. 13 (3):171-176.
8. Gent, D. H., du Toit, L. J., Fichtner, S. F., Mohan, S. K., Pappu, H. R., and Schwartz, H. F. 2006. *Iris yellow spot virus*: An emerging threat to onion bulb and seed production. Plant Dis. 90 (12):1468-1480.
9. Gent, D. H., Schwartz, H. F., and Khosla, R. 2004. Distribution and incidence of *Iris yellow spot virus* in Colorado and its relation to onion plant population and yield. Plant Dis. 88 (5):446-452.
10. Hoddle MS, M. L., Paris DL. 2012. Thrips of California. CBIT Publishing, Queensland.
11. Horsfall, J. L., and Fenton, F. A. 1922. Onion thrips in Iowa / by J.L. Horsfall and F.A. Fenton. Pp. 56-68 in: Bulletin / Iowa Agricultural Experiment Station; 205 Ames, Iowa : Agricultural Experiment Station, Iowa State College of Agriculture and the Mechanic Arts.
12. Hsu, C. L., Hoepting, C. A., Fuchs, M., Smith, E. A., and Nault, B. A. 2011. Sources of *Iris yellow spot virus* in New York. Plant Dis. 95 (6):735-743.
13. Inoue, T., Murai, T., and Natsuaki, T. 2010. An effective system for detecting *Iris yellow spot virus* transmission by *Thrips tabaci*. Plant Pathol. 59 (3):422-428.
14. Kritzman, A., Lampel, M., Raccah, B., and Gera, A. 2001. Distribution and transmission of *Iris yellow spot virus*. Plant Dis. 85 (8):838-842.
15. Lewis, T., ed. 1997. Thrips as crop pests. CAB International, Wallingford, Oxon, UK.
16. McDonald, J. R., Head, J., Bale, J. S., and Walters, K. F. A. 2000. Cold tolerance, overwintering and establishment potential of *Thrips palmi*. Physiol. Entomol. 25 (2):159-166.

17. Mound, L. A., and Kibby, G. 1998. Thysanoptera: an identification guide. CAB INTERNATIONAL, Wallingford; UK.
18. Nischwitz, C., Mullis, S., Torrance, R., Langston, D., Sparks, A., and Gitaitis, R. 2007. Distribution of *Iris yellow spot virus* in onion leaves. Phytopathology 97 (7):S182-S182.
19. Nischwitz, C., Srinivasan, R., Sundaraj, S., Mullis, S. W., McInnes, B., and Gitaitis, R. D. 2012. Geographical distribution and survival of *Iris yellow spot virus* in spiny sowthistle, *Sonchus asper*, in Georgia. Plant Dis. 96 (8):1165-1171.
20. North, R. C., and Shelton, A. M. 1986. Overwintering of the onion thrips, *Thrips-tabaci* (Thysanoptera, Thripidae), in New-York. Environ. Entomol. 15 (3):695-699.
21. Okuda, M., Fuji, S., Okuda, S., Sako, K., and Iwanami, T. 2010. Evaluation of the potential of thirty two weed species as infection sources of *Impatiens necrotic spot virus*. J. Plant Pathol. 92 (2):357-361.
22. Robene-Soustrade, I., Hostachy, B., Roux-Cuvelier, M., Minatchy, J., Hedont, M., Pallas, R., Couteau, A., Cassam, N., and Wuster, G. 2006. First report of *Iris yellow spot virus* in onion bulb- and seed-production fields in Reunion Island. Plant Pathol. 55 (2):288-288.
23. Sampangi, R. K., Mohan, S. K., and Pappu, H. R. 2007. Identification of new alternative weed hosts for *Iris yellow spot virus* in the pacific northwest. Plant Dis. 91 (12):1683-1683.
24. Schwartz, H. F., Otto, K., Szostek, S., Boateng, C., Cranshaw, W. S., Camper, M. A., and Mahaffey, L. 2008. Thrips and IYSV sources in Colorado onion production systems. Pp. 44-47 in: National Allium Research Conference Proceedings, Savannah, GA.
25. Shirck, F. H. 1951. Hibernation on onion thrips in southern Idaho. J. Econ. Entomol. 44 (6):1020-1021.
26. Smith, E. A., Ditommaso, A., Fuchs, M., Shelton, A. M., and Nault, B. A. 2011. Weed hosts for onion thrips (Thysanoptera: Thripidae) and their potential role in the epidemiology of *Iris yellow spot virus* in an onion ecosystem. Environ. Entomol. 40 (2):194-203.
27. Thompson, J. R., Wetzel, S., Klerks, M. M., Vaskova, D., Schoen, C. D., Spak, J., and Jelkmann, W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria* spp. in combination with a plant mRNA specific internal control. J. Virol. Methods 111 (2):85-93.
28. Tsumuki, H., Ishida, H., Yoshida, H., Sonoda, S., Izumi, Y., and Murai, T. 2007. Cold hardiness of adult western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera : Thripidae). Applied Entomology and Zoology 42 (2):223-229.
29. Weeds of the West. 2004. Western Society of Weed Science in cooperation with the Western United States Land Grant Universities Cooperative Extension Services, Newark, CA.
30. Yokoyama, V. Y., and Miller, G. T. 2000. Response of omnivorous leafroller (Lepidoptera : Tortricidae) and onion thrips (Thysanoptera : Thripidae) to low-temperature storage. J. Econ. Entomol. 93 (3):1031-1034.

CHAPTER 4: REVERSE TRANSCRIPTION QUANTITATIVE REAL-TIME PCR IS USED TO COMPARE THE RELATIVE AMOUNT OF *IRIS YELLOW SPOT VIRUS* BETWEEN TWO ONION CULTIVARS

Introduction

Onions resistant to *Iris yellow spot virus* (IYSV) have not yet been identified (3). Since the emergence of IYSV, several onion cultivar trials (7, 8, 16) and a recent germplasm evaluation (3) have included evaluation of IYSV incidence and severity. Although no onion plants have emerged from the trials as resistant to IYSV, some cultivars appear more tolerant than others.

In the germplasm evaluations conducted by Boateng et al. (3), Advantage (tested as OLYS05N5 prior to commercial release by Crookham Co.), was at the low end of the spectrum for IYSV incidence. Diaz-Montano et al. (7) found that Advantage was more resistant to thrips than other cultivars in their trials, but that no cultivar had resistance to IYSV. When compared to Granero, Advantage had a lower IYSV severity rating and yielded more super colossal and colossal onions (16). Cultivar trials have shown that Granero is susceptible to IYSV (8, 16). For the work presented here, Advantage was chosen as an onion cultivar with more resistance to IYSV, and Granero was chosen as an IYSV susceptible cultivar.

Real-time PCR can be used to quantify the number of copies of a specific DNA sequence, however, as IYSV is an RNA virus (4) it must first be reverse transcribed to cDNA before detection by PCR. Reverse transcription real-time PCR has been shown to detect IYSV from onion samples (15), and to detect several viruses infecting sweet potatoes (12). Reverse transcription quantitative real-time PCR has also been used to compare relative amounts of virus between resistant and susceptible plants (2).

The objective of this work was to use reverse transcription quantitative real-time PCR (RT-qPCR) to determine if there were differences in the relative amount of IYSV between the susceptible onion cultivar, Granero, and the more resistant cultivar, Advantage, which could be related to the difference in susceptibility. In order to achieve this objective, a plasmid containing a portion of the IYSV nucleoprotein gene (IYSV N-gene) was constructed. Standard curves were constructed with known quantities of the constructed plasmid which allowed the relative amount of IYSV within each sample to be calculated.

Materials and Methods

Sampling method

Three leaves displaying IYSV symptoms were removed from each of the onion cultivars Advantage and Granero. These onions were grown in the onion research field at CSU-ARDEC near Fort Collins, Colorado during 2013. The leaves were rinsed, blotted dry, and slit lengthwise to lay flat. A borer was used to punch out circular samples of approximately 0.7 cm diameter. Samples encompassed the middle of the lesion, the lesion tip, and areas 1, 2, and 3 cm from the tip of the lesion (Figure 4.1). Sampling away from the lesion followed the direction that had the fewest lesions in the area, and therefore was either toward the leaf tip or leaf base. Sample weights ranged from 10 to 33 mg (Table 4.1). Samples were frozen and stored at -80°C until RNA extraction approximately two months later.

RNA extraction and cDNA synthesis

Total RNA was extracted using the Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO) with the 750 µl binding solution option according to the manufacturer's instructions.

Total RNA was measured from a 2.0 µl aliquot of the extracted RNA using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). Eight microliter aliquots of RNA were treated with DNase I according to the manufacturer's instructions (Fermentas, Glen Burnie, MD). cDNA was synthesized from approximately 90 ng DNase I treated total RNA using M-MLV reverse-transcriptase according to the manufacturer's instructions for use with random primers (Invitrogen, Carlsbad, CA).

RT-PCR conditions

Reverse-transcriptase PCR (RT-PCR) was conducted for each sample to confirm the presence or absence of IYSV. RT-PCR reactions were performed on a MJ Research PTC-200 thermocycler (Waltham, MA) with an initial step of 94° C for 3 min; followed by 40 repeats of the three step sequence of 94° C for 45 sec, 55° C for 30 sec, 72° C for 90 sec; and a final step of 72° C for 10 min. Each 20 µl reaction consisted of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.15 mM dNTP mix, 1 unit Taq DNA polymerase (New England Biolabs, Ipswich, MA), 9 ng cDNA and molecular biology reagent grade water (Sigma-Aldrich, St. Louis, MO) sufficient to bring each reaction to 20 µl. The IYSV nucleoprotein gene was amplified by RT-PCR with primers designed by Coutts et al. (6), and primers specific to plant NADH dehydrogenase ND2 subunit (18) were used as a control. Reaction products were run in a 1% agarose gel (Bio-Rad Laboratories, Inc., Hercules, CA) stained with SYBRSafe (Invitrogen, Carlsbad, CA) in 1X TAE buffer (Bio-Rad Laboratories, Inc., Hercules, CA), and visualized upon exposure to UV light. A 100bp molecular weight marker (Fermentas, Glen Burnie, MD) was included on each gel.

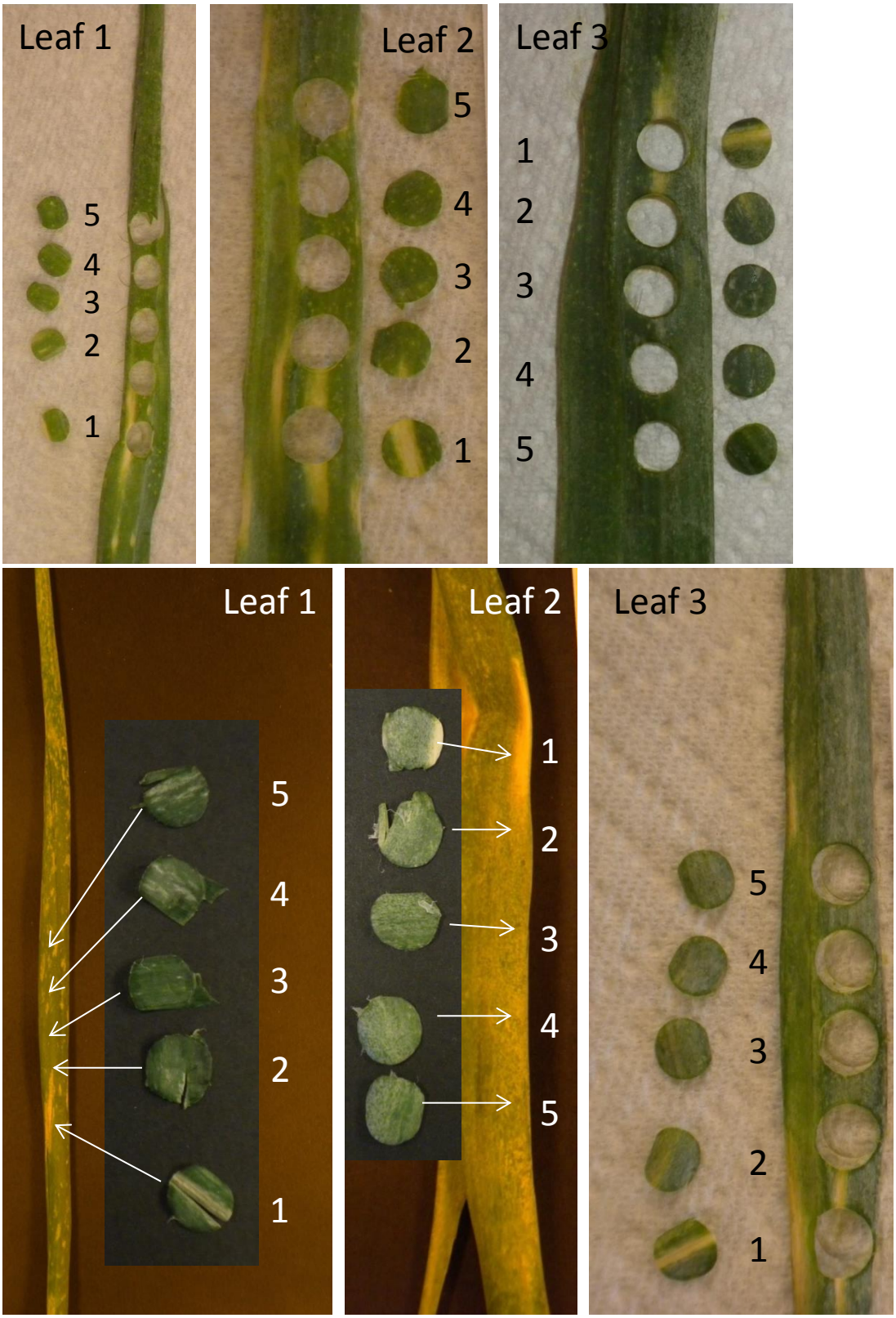


Figure 4.1. Top, Advantage leaves, Bottom, Granero leaves. 1: mid lesion; 2: lesion tip; 3: 1 cm from lesion; 4: 2 cm from lesion; 5: 3 cm from lesion.

Real-time PCR standard curve and primer design

A cDNA sample from an onion plant already known to be positive for IYSV was amplified by RT-PCR and the PCR product was run on a 1% agarose gel. Two 792 bp bands were excised from the gel, together yielding 44.5 ng/µl DNA upon purification with Qiaquick Gel Extraction kit (Qiagen, Valencia, CA) according to manufacturer's instructions and ligated into the pGEM-T Easy vector (Promega Corporation, Madison, WI) using the Fermentas rapid ligation kit (Fermentas, Glen Burnie, MD). The vector was heat shock transformed into chemically competent *Escherichia coli*, strain DH5α, and transformed colonies were visible when grown on selective media as described in the instruction manual for the pGEM-T Easy vector. Five ml of 4 transformed colonies were grown separately and plasmids were purified from culture using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Plasmid purification of the four selected transformed colonies yielded between 200 to 300 ng/µl plasmid DNA. The plasmid was digested with EcoRI and viewed on a 1% agarose gel to verify that the plasmid contained an insert of expected molecular weight. EcoR1 digestion of the purified plasmids yielded three fragments of expected molecular weight (3015, 605, and 187 bp) based on the plasmid and insert sequences. The standard curve used to detect the IYSV N-gene consisted of 10-fold serial dilutions of the purified plasmid from 1.0 ng/reaction to 1.0 $x10^{-6}$ ng/reaction.

Real time PCR primers were designed with PrimerQuest software (IDT, Coralville, IA) specific to the plasmid insert. Primers were also designed to a reference gene, *Allium cepa* cytochrome oxidase subunit 2 gene (GenBank accession no. GU253305), to verify that each RT reaction produced a similar amount of cDNA. Several primer pairs were tested and the pair that had the most efficient amplification and a slope in the target range was selected for the plasmid insert and for the reference gene.

The primer pair, 5'-AAATCTGCGGGCTTCCTCTGGTAA-3' and 5'-AAAGGAAGAGCAGCTGCAGCAAAG-3' amplified the targeted region of the IYSV N-gene 148 bp long from both the plasmid insert and plant samples with an average efficiency of 96%. Excellent replication of the points on the IYSV N-gene standard curve occurred with as little as 1.0 femtogram of starting material. The average slope of three standard curves was -3.428 (R^2 = 0.992), which falls within the acceptable range of -3.15 to -3.51. The dissociation curve displayed a single peak, indicating the presence of only one PCR product. Samples that were known to be IYSV positive by RT-PCR crossed the threshold between 19 to 31 cycles. The triplicate reactions were nearly identical.

The primer pair, 5'-GGGAGCGAGAAGTATGGGATATGT-3' and 5'-TGCGTAATGGATGACCCTCGAAAC-3' amplified the reference gene in the plant samples with an average efficiency of 97.2%. The amplicon was 102 bp long. The average slope of three standard curves was -3.44 (R^2 = 0.98), which falls within the acceptable range. The standard curve used with the reference gene primers consisted of a dilution series from 1.0 ng/reaction to 1.0 x10^{-3} ng/reaction of pooled cDNA. The dissociation curve displayed a single peak, indicating the presence of only one PCR product. Amplification of the control region occurred in all samples, other than negative controls. Amplification curves of the control region were tightly grouped indicating each reaction contained the same amount of starting cDNA.

Real time PCR conditions

RT-qPCR reactions to select primer pairs were performed on a Bio-Rad iCycleriQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the following conditions: 95°C for 3 min; 40 repeats of the two step sequence of 95°C for 10 sec., 60°C for 45

sec.; 95°C for 1 min; 55°C for 1 min. A dissociation curve was included at the conclusion of the 40 repeats to verify the amplification of only one target sequence. Each 25 µl reaction consisted of 12.5 µl 2x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 1.0 µl of a 2.5 µM mixture of the forward and reverse primers, 6.5 µl water and 5.0 µl cDNA diluted to 6.0 $x10^{-2}$ ng/µl. Non-template controls (water) were included for each standard curve and a no reverse transcriptase (-RT) negative control was included with each group of samples. All reactions were run in triplicate.

RT-qPCR reactions of the two onion cultivars were performed on a BioRad CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using the following conditions: 95°C for 10 min; 40 repeats of the two step sequence of 95°C for 15 sec., 60°C for 60 sec.; 95°C for 1 min; 55°C for 1 min. A dissociation curve was included at the conclusion of the 40 repeats to verify the amplification of only one target sequence. Each 25 µl reaction consisted of 12.5 µl FastStart Universal SYBR Green Master (ROX) (F. Hoffmann-La Roche AG, Basel, Switzerland), 1.0 µl of a 2.5 µM mixture of the forward and reverse primers, 0.5 µl $MgCl_2$, 6.0 µl water and 5.0 µl cDNA diluted to 5.0 $x10^{-2}$ ng/µl. Non-template controls (water) were included for each standard curve and a no reverse transcriptase (-RT) negative control was included with each group of samples. All reactions were run in triplicate.

Data analysis

RT-qPCR reaction efficiency was determined from the standard curves and provided by the software associated with the real-time PCR machines. Amplification of a single RT-qPCR product was verified by a single peak in the melting curve. The amount of amplicon was log transformed and analyzed with the MIXED procedure in SAS version 9.3 (SAS Institute Inc,

Cary, NC). Significant differences of the mean amount of amplicon between cultivars, between locations, and between location by cultivar were determined with Tukey's adjustment to the differences of least square means, α=0.05.

Results

RNA extraction

The leaf disks yielded RNA concentrations from 12.65 to 63.66 ng/µl (Table 4.1). In order for each sample to contain the same amount of starting material, the lowest concentration of total RNA limited the amount of cDNA that could be made. The optimal $A_{260/280}$ value for RNA is within the range of 1.8 to 2.2 (11); 18 of the 30 RNA extractions were outside the optimal range.

Table 4.1. Total RNA yield and purity of samples from two onion cultivars displaying IYSV symptoms.

Advantage					Granero				
Leaf	Location[a]	Weight[b] (mg)	Yield (ng/µl)	Purity (A_{260}/A_{280})	Leaf	Location	Weight (mg)	Yield (ng/µl)	Purity (A_{260}/A_{280})
1	1	12	20.00	2.26	1	1	12	25.72	2.30
	2	13	28.19	2.25		2	13	24.87	2.54
	3	17	24.38	2.35		3	13	15.45	2.27
	4	16	50.53	2.06		4	12	16.27	2.44
	5	18	14.65	2.64		5	10	16.60	2.32
2	1	15	22.43	2.08	2	1	13	32.33	2.14
	2	17	31.10	2.12		2	19	30.30	2.09
	3	15	24.80	2.03		3	17	16.84	2.01
	4	14	20.96	2.17		4	23	12.65	1.97
	5	15	14.33	1.94		5	30	49.57	2.18
3	1	18	25.77	2.29	3	1	14	22.91	2.10
	2	19	48.80	2.26		2	12	34.63	2.31
	3	26	50.90	2.24		3	13	27.05	2.35
	4	32	63.66	2.27		4	12	13.56	2.22
	5	33	47.10	2.28		5	12	16.95	2.49

[a]Location 1: mid-lesion; location 2: lesion tip; location 3: 1 cm from lesion tip; location 4: 2 cm from lesion tip; location 5: 3 cm from lesion tip. [b]Weight of the leaf disk before RNA extraction.

RT-PCR

IYSV was detected in each of the Advantage and Granero samples (Figure 4.2). One sample (lane 29) had evaporated during the PCR and appears negative on the gel, however, the subsequent RT-qPCR reactions verify that IYSV was present in that sample. There was no amplification in the –RT controls (indicating genomic DNA was not present), or in the water only control (indicating no contamination). Differences in band intensity can be observed on the gel with a trend of decreasing band intensity as the distance from the lesion increases.

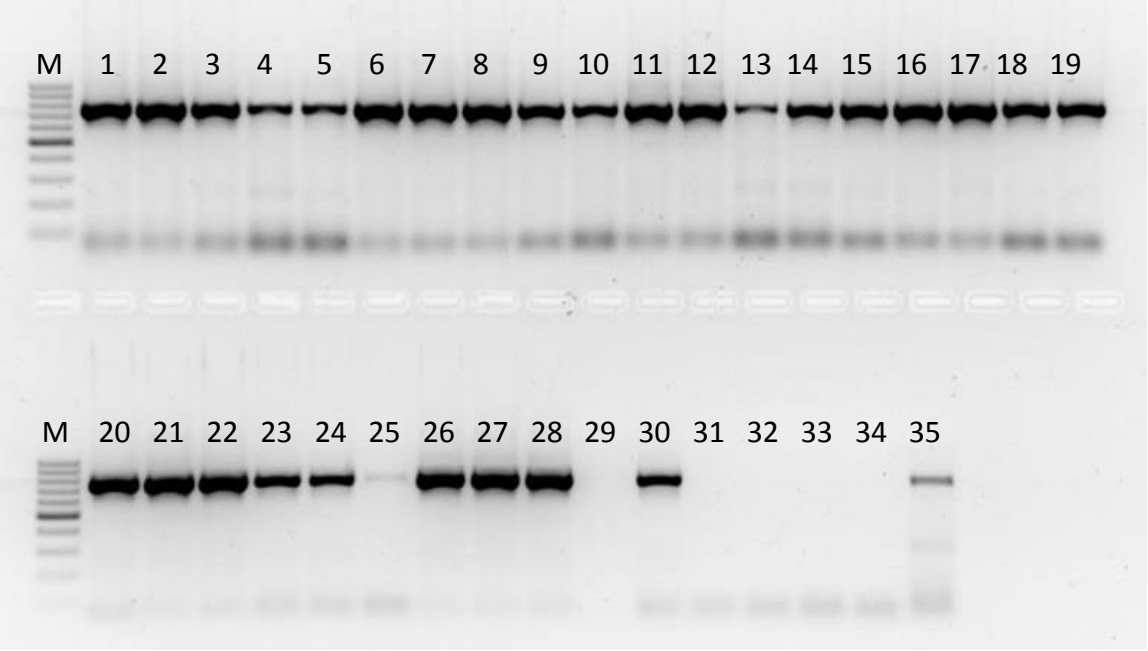


Figure 4.2. IYSV was detected by RT-PCR in the Advantage and Granero samples. Lanes 1-5: Advantage leaf 1; lanes 6-10: Advantage leaf 3; lanes 11-15: Advantage leaf 2; lanes 16-20: Granero leaf 2; lanes 21-25: Granero leaf 1; lanes 26-30: Granero leaf 3. Each leaf follows the order: mid-lesion, lesion-tip, 1, 2, 3 cm from lesion tip. Lanes 31 and 32: -RT control; lanes 33 and 34: NTC; lane 35: positive control. The reaction represented by lane 29 had evaporated during the PCR.

RT-qPCR

The RT-qPCR reactions of the leaf disk cDNA were not as efficient as the initial reactions that were performed when selecting primers. When using onion leaf cDNA the average efficiency of the reference gene reactions was 85%, and was 76% for the IYSV N-gene

reactions. The average slope and correlation coefficient (R^2) values for the two genes are shown in Figure 4.3. The dissociation curve for each gene displayed a single peak, indicating the presence of only one PCR product in the respective reactions. No amplification occurred in any of the –RT or NTC reactions.

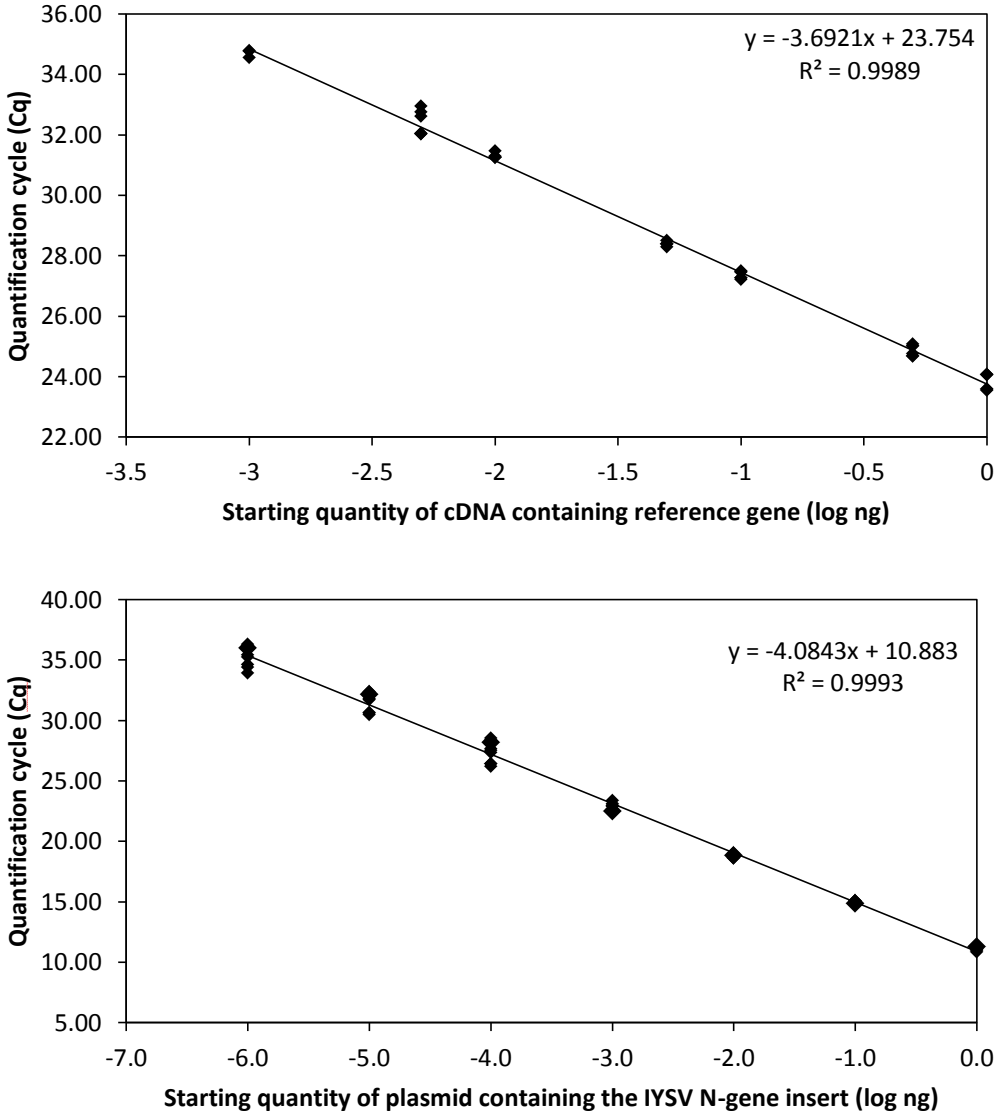
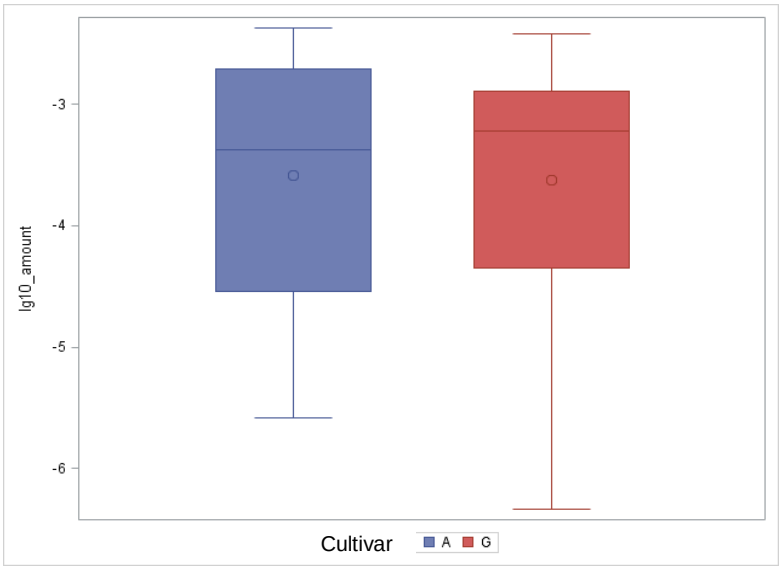
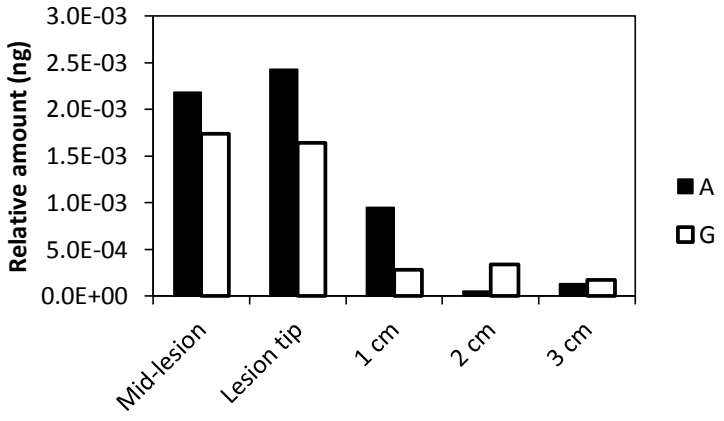


Figure 4.3. Standard curves of the RT-qPCR reactions for the reference gene (top) and IYSV N-gene (bottom). The top curve shows two runs plotted together, with a total of six individual reactions per each starting quantity. The bottom curve shows three runs plotted together, with a total of 9 individual reactions per each starting quantity. The reference gene standard curve ranged from 1.0 ng/reaction to 1.0 x 10^{-3} ng/reaction. The IYSV N-gene standard curve ranged from 1.0 ng/reaction to 1.0 x 10^{-6} ng/reaction. Slope and correlation coefficient (R^2) for each curve are presented as an average of the runs.

No significant differences in the mean amount of reference gene amplicon were found between the cultivars Advantage and Granero ($F= 0.03$, $p= 0.8638$), locations ($F=0.23$, $p= 0.9152$), or locations by cultivar ($F= 1.29$, $p=0.3162$).

There were no significant differences in the mean amount of IYSV N-gene amplicon between the two onion cultivars ($F= 0.02$, $p= 0.9078$), or between locations by cultivar ($F=0.41$, $p=0.8003$) (Figure 4.4, top and middle). There was a significant difference between locations ($F=7.34$, $p=0.0015$). In particular, the differences were between the lesion and distal samples (Figure 4.4, bottom). The amount of IYSV appeared to decrease as the distance from the lesion increased. There were statistically significant differences between mid-lesion and 2 cm from the lesion ($t= -3.16$, $p= 0.0419$); between mid-lesion and 3 cm from the lesion ($t=-4.21$, $p= 0.0052$); between the lesion tip and 2 cm from the lesion ($t= -3.22$, $p=0.0369$); and between the lesion tip and 3 cm from the lesion ($t=-4.27$, $p=0.0045$).





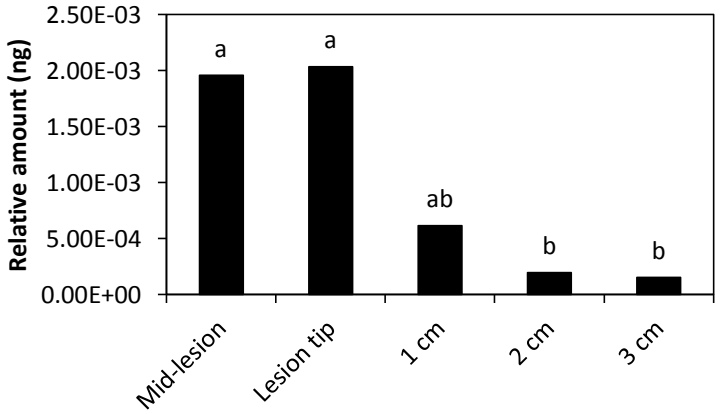


Figure 4.4. Comparisons of the mean relative amount (ng) of IYSV N-gene amplicon in onion cultivars Advantage (A) and Granero (G). Top: The mean amount of IYSV N-gene amplicon between the two onion cultivars was not significantly different (α= 0.05, F= 0.02, p= 0.9078). Middle: The mean amount of IYSV N-gene amplicon between the two onion cultivars at each location was not significantly different (α= 0.05F=0.41, p=0.8003). Bottom: There are significant differences of the mean amount of IYSV N-gene amplicon between the five locations along the leaf. The distances were measured from the lesion tip. Bars labeled with the same letters are not significantly different from each other.

Discussion

The leaf disk samples used here were 3 to 10 times below the 100 mg plant material recommended to be used in the RNA extraction kit, which explains the relatively low yield of RNA from each sample. In addition, the samples were ground by hand with a pestle which yields variable cell lysis between samples. Several samples' $A_{260/280}$ values fell outside the accepted range of purity which was likely caused by the presence of proteins, phenol, or other plant metabolites in solution with the RNA (11). In some cases these impurities can affect the results of RT-qPCR. However, these problems can be avoided if the amplicon is small and by comparing relative amounts of amplicon (9), as is the case of the work presented here.

The differences in band intensity observed on the gel (Figure 4.2) give some indication that a different amount of IYSV was present in each sample. The trend of decreasing band intensity with increasing distance from the lesion is also apparent in the results of the RT-qPCR reactions (Figure 4.4).

Although the Roche master mix produced standard curves within the acceptable range when first tested with the plasmid, it did not perform as well in the subsequent reactions, or as well as the mix purchased from BioRad. The differences in amplification efficiency were most likely due to using different reagents (19). Decreased amplification efficiency may cause the amount of amplicon to be underestimated. However, as relative amounts were compared, the decreased efficiency should not affect the overall results. The lack of any statistically significant differences among the mean amount of reference gene amplicon shows that each reaction started with the same amount of genetic material and that the differences in amplification of the IYSV N-gene are actual differences and not introduced through any errors.

From the results presented here, Advantage and Granero appear to be equally susceptible to IYSV, but field trials (3, 7, 8, 16) suggest differences in sensitivity to the disease or perhaps even a degree of tolerance in Advantage. The lack of difference in the relative amount of IYSV between the two onion cultivars suggests that the better performance of Advantage in the field (3, 16) cannot be attributed to a mechanism to limit viral movement or destroy the virus. Advantage may possess other physiological traits that allow expression of desirable agronomic characteristics while under stress.

Although differences in relative amount of IYSV were not found between the two cultivars, the differences in IYSV amount between locations along the leaf show that IYSV is not equally distributed throughout the onion leaves. It has been demonstrated by DAS-ELISA that IYSV is unevenly distributed throughout onion (13, 14) and leek (17) plants. The results of this study show that IYSV is present outside the lesions, but not as abundant. If the amount of IYSV surrounding the lesions was below the threshold of detection by DAS-ELISA, it would appear that IYSV was absent from areas where it was actually present.

Smith et al. (17) found a patchy distribution of IYSV in the leaves of asymptomatic leek plants, while samples from the stems, scapes, basal plates, and roots were consistently negative. When IYSV distribution in onion plants was examined by Kritzman et al. (13) it also was not found in bulb or root tissue. When IYSV was detected in the younger leek leaves (17), it was concentrated near the top and middle and rarely at the base of the leaf. Smith et al. (17) concluded that because IYSV was not detected in basal plates or young leaves, it does not move systemically through the phloem but rather from cell to cell. The IYSV genome contains a gene that is expected to code for a movement protein (5) so some amount of cell-to-cell movement would not be surprising. It may take a long time for IYSV to travel to and enter phloem tissues,

if it does that at all. A more meticulous dissection of the leaves may show just how far and in what direction the virus spreads. Further work is needed to determine exactly which cells IYSV occupies over the course of an infection, as many systemically infecting plant viruses are not uniformly distributed throughout the host (10).

It has been proposed that the patchy distribution of IYSV is the result of multiple point inoculations by thrips during their feeding activity (13, 17). Thrips feed on plant epidermal and mesophyll tissues (1) and viral movement through these cells can be slow (10). Because thrips had obviously been feeding on the sampled areas (the feeding can be observed as silvery flecks in Figure 4.1), it is impossible to tell from these results whether the virus present outside the lesion got there on its own through cell-to-cell movement, or if it was deposited by viruliferous thrips while feeding. Questions about IYSV movement in onion plants and its accumulation over time will be difficult to answer until a reliable method for mechanical inoculation is developed.

Mapping pathogen concentration in plants is often undertaken to improve sampling protocols and to increase the likelihood of detecting the target pathogen. These results suggest that a positive detection of IYSV will occur from leaf tissue samples that contain a lesion. If asymptomatic onion plants are to be tested, areas showing thrips damage should be chosen.

Although no difference in the relative amount of IYSV was found between the two onion cultivars, the RT-qPCR method presented here may prove useful in screening other cultivars where resistance to IYSV is suspected. Similar RT-qPCR approaches can be applied to further studies of IYSV, including detection of IYSV in asymptomatic plants and the study of genes involved in host resistance or tolerance to IYSV or thrips.

LITERATURE CITED

1. Ananthakrishnan, T. N. 1980. Thrips. Pp. 158-161 in: Vectors of Plant Pathogens, K. F. Harris and K. Maramorosch, eds. Academic Press, New York.
2. Balaji, B., Bucholtz, D. B., and Anderson, J. M. 2003. *Barley yellow dwarf virus* and *Cereal yellow dwarf virus* quantification by real-time polymerase chain reaction in resistant and susceptible plants. Phytopathology 93 (11):1386-1392.
3. Boateng, C. O., Schwartz, H. F., Havey, M. J., and Otto, K. 2014. Evaluation of onion germplasm for resistance to Iris yellow spot (*Iris yellow spot virus*) and onion thrips, *Thrips tabaci*. Southw. Entomol. 39 (2):237-260.
4. Cortes, I., Livieratos, I. C., Derks, A., Peters, D., and Kormelink, R. 1998. Molecular and serological characterization of *Iris yellow spot virus*, a new and distinct tospovirus species. Phytopathology 88 (12):1276-1282.
5. Cortez, I., Aires, A., Pereira, A. M., Goldbach, R., Peters, D., and Kormelink, R. 2002. Genetic organisation of *Iris yellow spot virus* M RNA: indications for functional homology between the G((C)) glycoproteins of tospoviruses and animal-infecting bunyaviruses. Arch. Virol. 147 (12):2313-2325.
6. Coutts, B. A., McMichael, L. A., Tesoriero, L., Rodoni, B. C., Wilson, C. R., Wilson, A. J., Persley, D. M., and Jones, R. A. C. 2003. *Iris yellow spot virus* found infecting onions in three Australian states. Austral. Plant Pathol. 32 (4):555-557.
7. Diaz-Montano, J., Fuchs, M., Nault, B. A., and Shelton, A. M. 2010. Evaluation of onion cultivars for resistance to onion thrips (Thysanoptera: Thripidae) and *Iris yellow spot virus*. J. Econ. Entomol. 103 (3):925-937.
8. du Toit, L. J., and Pelter, G. Q. 2005. Susceptibility of storage onion cultivars to iris yellow spot in the Columbia Basin of Washington, 2004. Biological & Cultural Tests 20 (V006).
9. Fleige, S., and Pfaffl, M. W. 2006. RNA integrity and the effect on the real-time qRT-PCR performance. Molecular Aspects of Medicine 27 (2/3):126-139.
10. Hull, R. 2002. Matthews' Plant Virology. Academic Press, San Diego, Calif.
11. Inc., T. F. S. 2010. Nucleic Acid. p. 11 in: Thermo Scientific NanoDrop Spectrophotometers Thermo Fisher Scientific Inc. http://www.nanodrop.com/ND1/NucleicAcid-Booklet.html.
12. Kokkinos, C. D., and Clark, C. A. 2006. Real-time PCR assays for detection and quantification of sweetpotato viruses. Plant Dis. 90 (6):783-788.
13. Kritzman, A., Lampel, M., Raccah, B., and Gera, A. 2001. Distribution and transmission of *Iris yellow spot virus*. Plant Dis. 85 (8):838-842.
14. Nischwitz, C., Mullis, S., Torrance, R., Langston, D., Sparks, A., and Gitaitis, R. 2007. Distribution of *Iris yellow spot virus* in onion leaves. Phytopathology 97 (7):S182-S182.

15. Pappu, H. R., Rosales, I. M., and Druffel, K. L. 2008. Serological and molecular assays for rapid and sensitive detection of *Iris yellow spot virus* infection of bulb and seed onion crops. Plant Dis. 92 (4):588-594.
16. Shock, C. C., Feibert, E., Jensen, L., Mohan, S. K., and Saunders, L. D. 2008. Onion variety response to *Iris yellow spot virus*. HortTech. 18 (3):539-544.
17. Smith, T. N., Wylie, S. J., Coutts, B. A., and Jones, R. A. C. 2006. Localized distribution of *Iris yellow spot virus* within leeks and its reliable large-scale detection. Plant Dis. 90 (6):729-733.
18. Thompson, J. R., Wetzel, S., Klerks, M. M., Vaskova, D., Schoen, C. D., Spak, J., and Jelkmann, W. 2003. Multiplex RT-PCR detection of four aphid-borne strawberry viruses in *Fragaria* spp. in combination with a plant mRNA specific internal control. J. Virol. Methods 111 (2):85-93.
19. Wolffs, P., Grage, H., Hagberg, O., and Radstrom, P. 2004. Impact of DNA polymerases and their buffer systems on quantitative real-time PCR. J. Clin. Microbiol. 42 (1):408-411.

CONCLUSION

There is still no evidence that *Iris yellow spot virus* (IYSV) is seed transmitted, and because of this it is unlikely that onion (*Allium cepa* L.) seed is a source of IYSV inoculum. IYSV can be detected in the seed coat, and that should be kept in mind for future seed assays. It remains to be determined if IYSV infection of the mother plant affects the subsequent seeds (e.g., size, quality, viability).

IYSV was detected in several weed species (*Malva neglecta* Wallr. (common mallow), *Taraxacum officinale* Weber in Wiggers (dandelion), *Descurainia sophia* (L.) Webb. Ex Prantl (flixweed), *Lactuca serriola* L. (prickly lettuce), and *Tragopogon dubius* Scop. (salsify)), and in onion thrips (*Thrips tabaci* Lind.) during two Colorado winters. Greenhouse studies demonstrated that thrips can reproduce on these weed species and acquire IYSV from some of them. IYSV was more often detected in greenhouse grown salsify plants and in the thrips larvae developing on them than in any of the other weeds or thrips larvae. It would be useful to determine the distance from an onion field at which IYSV infected thrips and weeds can no longer be found. This could provide insight as to how feasible weed control might be in reducing or delaying IYSV outbreaks.

Temperatures in Colorado did not fall low enough during the winter to completely eradicate thrips populations. Although thrips numbers and activity are greatly decreased during the winter, some IYSV infected onion thrips survive on green plants in sheltered locations. Even if the plant is not an IYSV host, it contributes to thrips survival and may be considered an indirect green bridge. Onion cull piles were not an important site for thrips overwintering once decay set in. Volunteer onions and onions left standing in the field did harbor IYSV infected

thrips over the winter. It would be interesting to elucidate if volunteer onions have overwintered with IYSV, or if the plants are inoculated by onion thrips once growth resumes. In Colorado, winter wheat is often planted adjacent to onions, in rotation with onions, or as a companion crop to onion. Thrips and IYSV were detected from field collected winter wheat samples, but the winter wheat grown under greenhouse conditions did not become infected with IYSV. The role of winter wheat as a green bridge for IYSV and onion thrips needs further investigation.

No onion cultivar has yet been identified with resistance to IYSV, however, some cultivars are more tolerant to the virus than other. An RT-qPCR assay was developed to compare the relative amount of IYSV between an IYSV susceptible onion cultivar, Granero, and a tolerant cultivar, Advantage. There were no differences in the relative amount of IYSV between the two cultivars. Applying the RT-qPCR assay to additional onion cultivars would be interesting and may aid the search for resistance or be helpful in understanding cultivar tolerance to IYSV. It has been established that IYSV is unevenly distributed within onion leaves. This assay revealed a gradual decrease in the amount of IYSV as distance from the lesion increased. The assay should also prove useful in further investigations of IYSV behavior in onion plants, and help fine tune sampling for diagnostic procedures.

It is hoped that the results of this work prove useful to onion growers as part of their integrated pest management programs, and to researchers investigating the behavior of onion thrips and IYSV in onions.

APPENDIX 1: INDUCTION OF ONION BOLTING

Two experiments were performed to induce onions to bolt with the longer term goal of obtaining additional seeds to test for IYSV.

For the first experiment, onion sets were placed in a household chest freezer (approximately -16°C) for 1, 4, or 8 hours. Two bags of 80 sets of the cultivars Ebenezer, Red Weathersfield, and Yellow Rock underwent each treatment. Two bags of each cultivar were left untreated. After treatment the sets were planted into the field at ARDEC in late April, 2012. Sets that did not survive the treatments (mushy from the freeze-thaw process) were discarded before planting, and the number of sets per treatment that were planted varied. The remaining sets were split into two reps, with the exception of 8 hour treated Ebenezer and Red Weathersfield. Too few of these sets survived the treatment to plant two reps.

The plants that grew from the sets remained small and did not develop more than 6 to 7 leaves. The numbers of plants that grew and bolted are listed in Table A1.1. Only 9 scapes had developed by June 26, 2012. The plants were in poor condition and no further data was recorded after this time. The Red Weathersfield sets appeared to survive the freeze treatments better than the other two cultivars.

Table A1.1 The number of plants and scapes that grew from freeze treated onion sets.

	Ebenezer		Red Weathersfield		Yellow Rock	
	Plants[a]	Scapes[b]	Plants	Scapes	Plants	Scapes
Untreated	46	1	143	2	95	-
1h	65	-	141	-	55	-
4h	34	1	103	3	40	1
8h	9	-	21	-	25	1

[a]The plants were counted on June 11, 2012. [b]The scapes were counted on June 26, 2012.

Sets are typically stored at warm temperatures (27-28°C) to prevent bolting; long term storage at 7-12°C can predispose larger onion sets to bolting (1). It would appear that -16°C is too cold for onion sets because many of the sets froze and became mushy upon thawing. It would be interesting to evaluate these short-term treatments at warmer temperatures.

In the second experiment, 50 onion bulbs each of cultivars Belmar, Cometa, and Granero were harvested from the field on September 20, 2012. The bulbs were stored for approximately three months in the workroom at ARDEC, then planted into pots in a greenhouse on January 4, 2013. Bulbs that began to decay during storage were discarded. Table A1.2 shows the number of bulbs planted, and the number that subsequently grew and bolted by March 19, 2013. The percentage of plants that bolted was calculated from the number of plants that grew.

Table A1.2. The number of onion bulbs that grew and bolted under greenhouse conditions.

Cultlivar	Planted	Grew (%)	Bolted (%)[a]
Belmar	50	46 (92)	27 (58)
Cometa	47	22 (47)	10 (46)
Granero	44	39 (88)	21 (54)

[a] Calculated from the number of plants that grew.

More plants developed from the Belmar and Granero bulbs than from the Cometa bulbs. Approximately half the plants that grew developed scapes. Although the onion plants bolted and flowered, seeds did not form. If this method were to be used to produce seeds, the appropriate pollinator, such as honeybees, would need to be introduced. Storing the bulbs at a temperature within the range of 4.5 to 14°C for the same time period would likely increase the number of plants that bolted (1) within six months of field harvest of the bulbs.

LITERATURE CITED

1. Brewster, J. L. 2008. Onions and other vegetable alliums. CABI, Wallingford, UK.

APPENDIX 2: COMPARISON OF DAS-ELISA AND RT-PCR ON FROZEN AND DRIED ONION SCAPE TISSUE

The following work was undertaken to determine an appropriate storage and detection method of IYSV in onion scapes. These results were then applied to test the scapes described in Chapter 2.

Five IYSV symptomatic scapes of the cultivars Solid Gold and Pentium, and four IYSV symptomatic scapes of cultivar Red Defender were collected from ARDEC on August 31, 2010. Each scape was rinsed and blotted dry. Two samples were removed from each scape; one sample contained a lesion and surrounding green tissue and the other sample consisted of an asymptomatic area distal to the lesion. Each sample was quartered as shown in Figure A2.1. Each quarter was either frozen at -80°C or dried at room temperature before being tested for IYSV by RT-PCR or DAS-ELISA (both techniques are described in Chapter 2) approximately four months later. Samples from three of the Solid Gold scapes intended for the freezer were accidentally left at room temperature overnight before freezing. Results were analyzed with the freq procedure in SAS version 9.3 (SAS Institute Inc, Cary, NC).

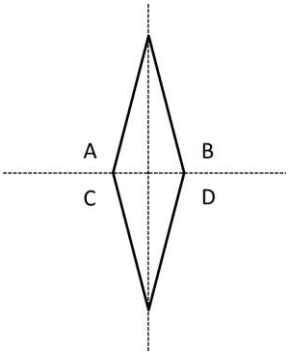


Figure A2.1. Each sample removed from the onion scapes was quartered. Portion A was frozen before testing by RT-PCR. Portion B was dried before testing by RT-PCR. Portion C was frozen before testing by DAS-ELISA. Portion D was dried before testing by DAS-ELISA.

The DAS-ELISA and RT-PCR results are summarized in Table A2.1. No statistically significant differences were found among the samples containing a lesion. The method used to preserve or to test the samples containing a lesion did not influence the results. There was a significant difference when comparing the results of DAS-ELISA and RT-PCR on samples removed from the distal region (p= 0.009). IYSV was detected in more samples when RT-PCR was used. In addition, when IYSV detection by DAS-ELISA and RT-PCR is compared from the frozen samples, a statistically significant difference exists (p=0.039). Again, IYSV is detected more frequently by RT-PCR than by DAS-ELISA in frozen samples from the distal region. No other significant differences were found among the results of the samples removed from the area distal to the lesion.

Table A2.1. IYSV status as determined by DAS-ELISA or RT-PCR of frozen and dried onion leaf tissue.

	DAS-ELISA				RT-PCR			
	Lesion		Distal		Lesion		Distal	
	Dried	Frozen	Dried	Frozen	Dried	Frozen	Dried	Frozen
SG1	+	+	-	+	+	+	+	+
SG2	+	+	-	-	+	+	+	+
SG3	+	+	-	-	+	+	-	-
SG4	+	+	+	+	-	-	+	+
SG5	+	+	-	-	-	+	+	+
P1	-	+	+	+	+	+	-	+
P2	+	+	-	-	+	+	+	+
P3	+	+	-	-	+	+	+	-
P4	-	-	+	+	+	+	+	+
P5	+	+	-	-	-	+	-	+
RD1	+	+	+	+	-	+	+	+
RD2	+	+	+	+	+	+	+	+
RD3	+	+	-	-	+	+	+	+
RD4	+	+	+	+	+	+	+	+

SG: Solid Gold; P: Pentium; RD: Red Defender

The results indicate that using DAS-ELISA to detect IYSV from dried scapes is adequate, so long as the sample includes a lesion. The results of the distal region suggest that RT-PCR is more sensitive than DAS-ELISA. It is also possible that the IYSV genome is present at the distal

location before the coat proteins are expressed. When testing asymptomatic plants for IYSV, RT-PCR is the better option.

APPENDIX 3: GENERATING CLEAN THRIPS COLONIES

This appendix has been adapted from (4).

Inoue et al. (2) showed no significant differences in the larval mortality of *Iris yellow spot virus* (IYSV)-infected and uninfected *Thrips tabaci*, however it would be interesting to determine whether IYSV infected and uninfected *T. tabaci* respond differently to various environmental conditions. Before those comparative studies can be undertaken, reliable access to colonies of IYSV infected and non-infected thrips is required. Initially, onions with no apparent symptoms of IYSV were collected from the field with the intent to rear the thrips from those onions as potentially IYSV-free thrips. Unfortunately, these field collected plants consistently tested positive for IYSV by DAS-ELISA (described in Chapter 2), and the thrips feeding on them were assumed to also be infected. Although onion thrips prefer onion, they will use other plants as hosts (1, 3). As far as we know IYSV is not transmitted to offspring, but must be acquired during thrips feeding on infected plant material (5). If a plant isn't a host for IYSV, the virus won't infect that plant as thrips feed on it. If the plant does not get infected, then subsequent generations of thrips that feed on that same plant should have no source of IYSV to acquire.

Five pots each of radish (Alta Globe cultivar) and onion (grown from Gurney's yellow onion sets) were planted with 4 plants/pot and physically isolated from external sources of thrips with a plastic and mesh cylinder placed over the plants (Figure A3.1). Before adding thrips from symptomatic onion plants, a leaf tissue sample from each of the 4 plants in a pot was pooled and tested by RT-PCR (described in Chapter 2) for the absence of IYSV. A sample of 34 thrips (adult and juvenile) was removed from a single symptomatic onion plant and 12 thrips were

placed onto radishes in a single pot, 12 were placed onto onions in a single pot, and the remaining 10 were tested for IYSV by RT-PCR. Samples of 34 thrips were removed and transferred from a total of 5 symptomatic onion plants, and IYSV presence in symptomatic leaves was verified by RT-PCR.

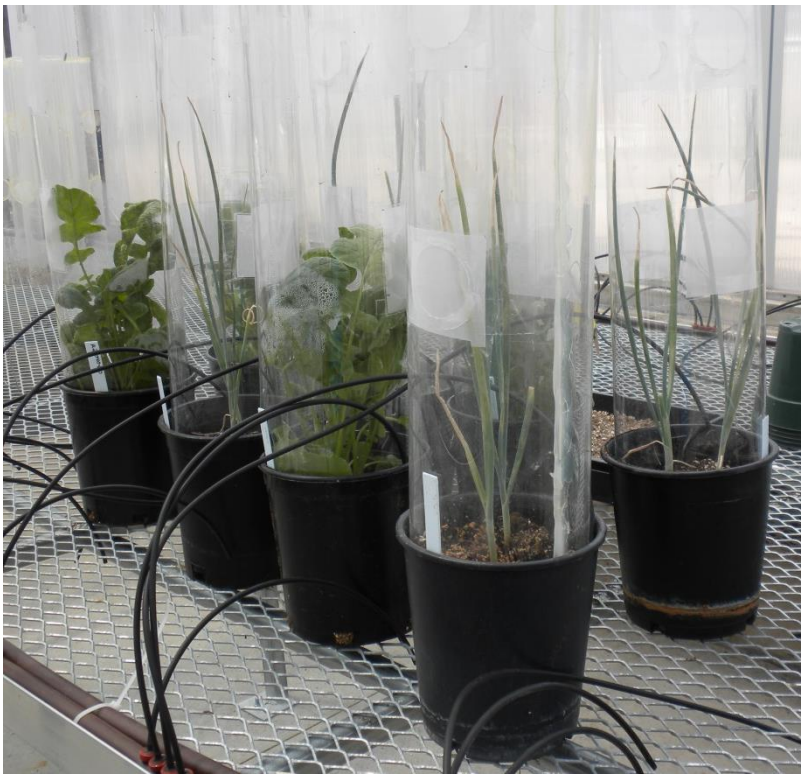


Figure A3.1. Barriers placed over onion and radish plants.

At 2 week intervals for 12 weeks, a portion of a leaf from each of 4 plants was removed and pooled by pot before testing for IYSV by RT-PCR. To increase the chance of detecting IYSV, tissue samples with thrips damage were preferentially selected. Five thrips (adult and larval) were collected from each pot and pooled by pot on the same day. Thrips were not collected during week two as insufficient time had passed to produce the next generation and insect numbers were too low.

RT-PCR results are summarized in Table A3.1. The symptomatic source plants (not shown) and onion thrips removed from them and transferred to the onion and radish plants were positive for IYSV. The onion and radish plants were negative for IYSV when the infected thrips were initially transferred to them. IYSV was detected in onion plants by week two and remained in the plants. IYSV was not detected in the radish plants during the 12 week period.

Thrips collected from IYSV positive onion plants remained positive for IYSV over the 12 weeks. Thrips collected from radish plants were negative by week 4 and remained IYSV-free over the 12 weeks. During week 9, thrips were transferred from old to new plants of the same species. IYSV was detected in three of the four thrips samples collected at week 10 from radish. This is very likely a contamination event due to collecting IYSV infected thrips from onions before collecting uninfected thrips from the radish plants. Another possibility is that the adult thrips in these samples were from the original IYSV infected thrips placed on the plants at the start of the experiment. IYSV was not detected in radish reared thrips during week 12, with the exception of a mislabeled sample that may have actually been the onion reared thrips.

Higher thrips numbers were generally observed on onion. For future work where large numbers of clean thrips are needed, the best strategy may be to rear thrips on radish for 4 to 6 weeks, then transfer clean thrips larvae to onion plants to increase their numbers. Results from onion pot 5 indicate this may be a good strategy, as plants in this pot did not become infected with IYSV, nor was IYSV detected in the thrips collected from that pot.

Table A3.1. Summary of RT-PCR results for the presence (+) or absence (-) of IYSV.

	Onion										Radish									
	Plants					Thrips (5/sample)					Plants					Thrips (5/sample)				
Pot	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Week 0	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
2	+	-	+	+	-	*	*	*	*	*	-	-	-	-	-	+	-	*	*	*
4	+	+	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
6	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-
10	-	-	+	+	X	+	+	+	+	X	-	-	-	-	X	+	+	-	+	X
12	+	-	+	+	X	+	-[a]	+	+	X	-	-	-	-	X	-	+[a]	-	-	X

IYSV presence indicated by +; IYSV absence indicated by -. *: Samples not collected. X: Pots no longer available. [a]: Mislabeled sample indicated. Thrips samples consisted of adults and larvae. Plant samples consisted of one leaf tissue sample per plant pooled by pot.

LITERATURE CITED

1. Doederlein, T. A., and Sites, R. W. 1993. Host-plant preferences of *Frankliniella-occidentalis* and *Thrips-tabaci* (Thysanoptera, Thripidae) for onions and associated weeds on the southern high-plains. J. Econ. Entomol. 86 (6):1706-1713.
2. Inoue, T., Murai, T., and Natsuaki, T. 2010. An effective system for detecting *Iris yellow spot virus* transmission by *Thrips tabaci*. Plant Pathol. 59 (3):422-428.
3. Sakimura, K. 1932. Life history of *Thrips tabaci* L. on *Emilia sagitata* and its host plant range in Hawaii. J. Econ. Entomol. 25:884-891.
4. Szostek S. and Schwartz. H. F. 2010. Generating clean thrips colonies and quantifying IYSV in onion. in: National Allium Research Conference, Reno, NV.
5. Whitfield, A. E., Ullman, D. E., and German, T. L. 2005. Tospovirus-thrips interactions. Annual Review of Phytopathology 43:459-489.

APPENDIX 4: VOLUNTEER ONIONS AND ASSOCIATED THRIPS

To determine if IYSV first appears in volunteer onions or in onion thrips, volunteer onions were collected from a research field planted with onions at the Agricultural Research Development and Education Center (ARDEC) approximately 10 miles (16km) northeast of Fort Collins, Colorado. Ten plants were randomly selected and collected every two weeks from late May to early July 2010. Plastic 3.79 liter resealable bags were quickly placed over the leaves and necks of the onion plants before the onions were disconnected from the soil in order to minimize loss of thrips. The first ten onions were completely removed from the soil and the whole plant was closed inside the plastic bag, however, soil from the roots interfered with locating thrips. In order to reduce interference from soil, the necks of subsequent onions were cut at the soil line and only the leaves and neck were enclosed in the plastic bags. Bagged plant material was placed at 4°C from one to several hours to chill the thrips and facilitate their recovery for evaluation.

Individual leaves were removed from each plant by slicing them with a razor blade. Adult and larval thrips were counted and removed from the plants with a damp fine tipped (#1, white bristle) artist's paintbrush, placed in 1.5 ml microcentrifuge tubes, and kept on ice until storage at -80°C. Plants were numbered 1 to 10 and the tubes of thrips were labeled to correspond with the plant the thrips were removed from. With the exception of the first ten plants, any additional thrips found in the plastic bags were removed and added to the appropriate microcentrifuge tube. After the thrips were removed from the leaves, the leaves were returned to 4°C until they were tested for IYSV by DAS-ELISA (described in Chapter 2).

Three leaves (oldest, newest, and one of intermediate age) were removed from each plant. Leaves displaying IYSV symptoms were preferentially selected. A 0.2g segment of each leaf was cut from the top, middle, and bottom of the leaf and tested for IYSV by DAS-ELISA. Positive plants had at least one sample with a reading more than twice the background level. The thrips collected from the volunteer onion plants were tested for IYSV with RT-PCR (described in Chapter 2).

IYSV was not detected in volunteer onions or in thrips removed from the onions until early June, 2010 when IYSV was detected in both the plants and thrips (Table A4.1). IYSV was detected in both symptomatic and asymptomatic plants. IYSV status of a particular plant and the thrips removed from it were not always in agreement. It is interesting that IYSV was not detected in any of the July plant samples, but was detected in all but one of the thrips populations removed from the plants. The total number of thrips increased over time, and most volunteer onions supported a mixed population of thrips species, primarily *T. tabaci* and *F. occidentalis*.

Volunteer onions have been considered a source for IYSV (1, 2), but it is still unclear if IYSV overwinters in the onion, or if the onion becomes reinoculated during thrips feeding once it sprouts. The results of the IYSV status of volunteer onions and their associated thrips investigated here did not clarify the situation. IYSV was first detected in both volunteers and their thrips on the same collection date (Table A4.1). In some cases, IYSV appears in thrips but not in the corresponding volunteer; due to the uneven distribution of IYSV within onion plants (3, 4) it is possible that IYSV was present in the plant, but not in the particular leaf section that was tested. It is also possible that the volunteer was not infected. In other cases, IYSV was detected in the plant but not in the thrips associated with the plant. Again, it is not clear if IYSV

overwintered in the plant and the thrips had not yet acquired the virus, or if the plant had been inoculated by thrips that had since moved on.

Table A4.1. IYSV status of volunteer onions and thrips associated with them collected at ARDEC.

	IYSV status of volunteer	Symptoms	Thrips/plant and IYSV status	Thrips species[a]
26 May 2010	1 (-)	No	1 (-)	*T. tabaci*
	2 (-)	No	2 (-)	*T. tabaci*
	3 (-)	No	0	
	4 (-)	No	0	
	5 (-)	No	0	
	6 (-)	No	0	
	7 (-)	No	3 (-)	*T. tabaci*
	8 (-)	No	0	
	9 (-)	No	0	
	10 (-)	No	7 (-)	*T. tabaci*
9 June 2010	1 (-)	Maybe	10 (-)	
	2 (-)	No	6 (-)	*T. tabaci, F. occidentalis*
	3 (-)	No	26 (+)	*T. tabaci, F. occidentalis*
	4 (-)	No	33 (-)	*T. tabaci, F. occidentalis*
	5 (+)	Yes	6 (+)	*T. tabaci, F. occidentalis*
	6 (-)	No	32 (-)	*T. tabaci, F. occidentalis*
	7 (-)	No	5 (-)	*T. tabaci, F. occidentalis*
	8 (-)	No	3 (-)	*T. tabaci*
	9 (-)	No	37 (-)	*T. tabaci, F. occidentalis*
	10 (+)	Yes	5 (+)	*T. tabaci, F. occidentalis*
23 June 2010	1 (+)	No	40 (+)	*T. tabaci, F. occidentalis*
	2 (+)	No	11 (-)	*T. tabaci, F. occidentalis*
	3 (+)	No	15 (-)	*T. tabaci, F. occidentalis*
	4 (-)	No	7 (-)	*T. tabaci, F. occidentalis*
	5 (-)	No	14 (-)	*T. tabaci, F. occidentalis*
	6 (-)	No	208 (-)	*T. tabaci, F. occidentalis*
	7 (-)	No	103 (-)	*T. tabaci*
	8 (+)	No	19 (+)	*T. tabaci*
	9 (-)	No	7 (-)	*T. tabaci*
	10 (+)	No	137 (+)	*T. tabaci*
9 July 2010[b]	1 (-)	No	105 (+)	*T. tabaci, F. occidentalis*
	2 (-)	No	25 (-)	*T. tabaci, F. occidentalis*
	3 (-)	No	206 (+)	*T. tabaci, F. occidentalis*
	4 (-)	No	91 (+)	*T. tabaci, F. occidentalis*
	5 (-)	Maybe	123 (+)	*T. tabaci, F. occidentalis*
	6 (-)	No	64 (+)	*T. tabaci, F. occidentalis*
	7 (-)	No	172 (+)	*T. tabaci, F. occidentalis*
	8 (-)	Maybe	123 (+)	*T. tabaci, F. occidentalis*
	9 (-)	No	47 (+)	*T. tabaci, F. occidentalis*
	10 (-)	No	30 (+)	*T. tabaci, F. occidentalis*

[a] Species determined by RT-PCR; *T.* = *Thrips*, *F.* = *Frankliniella*. Other species may have been present, but primers were not developed for any additional species. [b]Most volunteers in the field at this time had bolted. The collected samples had not bolted.

It is interesting that IYSV was detected in plants before symptoms were present. Unfortunately, these results do not give any indication of the length of time between IYSV infection and appearance of symptoms.

In order to determine whether IYSV overwinters in onion bulbs, future work could include growing out bulbs from infected plants in the absence of thrips and testing the new plant tissue for IYSV. However, if IYSV does not overwinter in the bulbs, or if it occurs at a low frequency, the number of bulbs that would need to be tested is very large. The far greater challenge would be ensuring the plants remained free of thrips during the grow-out.

LITERATURE CITED

1. Gent, D. H., Schwartz, H. F., and Khosla, R. 2004. Distribution and incidence of *Iris yellow spot virus* in Colorado and its relation to onion plant population and yield. Plant Dis. 88 (5):446-452.
2. Hsu, C. L., Hoepting, C. A., Fuchs, M., Smith, E. A., and Nault, B. A. 2011. Sources of *Iris yellow spot virus* in New York. Plant Dis. 95 (6):735-743.
3. Nischwitz, C., Mullis, S., Torrance, R., Langston, D., Sparks, A., and Gitaitis, R. 2007. Distribution of *Iris yellow spot virus* in onion leaves. Phytopath. 97 (7):S182-S182.
4. Schwartz, H. F., Otto, K., Szostek, S., Boateng, C., Cranshaw, W. S., Camper, M. A., and Mahaffey, L. 2008. Thrips and IYSV sources in Colorado onion production systems. Pp. 44-47 in: National Allium Research Conference Proceedings, Savannah, GA.

APPENDIX 5: EFFECTIVENESS OF A THRIPS LURE AND A SEEDCORN MAGGOT LURE TO ATTRACT TARGET INSECTS IN AN ONION FIELD

This report was initially prepared for: Dr. Jan Meneley of AgBio Development, Inc., Westminster, CO and has been reformatted for inclusion in this dissertation.

Introduction

Thrips are an important pest of many crops, including onions, and thrips damage to crops can be compounded when the thrips also vector plant pathogens. Onions can suffer yield loss due to *Iris yellow spot virus* (IYSV) which is vectored by the onion thrips, *Thrips tabaci*. Controlling *T. tabaci* is currently the best strategy for preventing IYSV in onions (2). In order to control thrips at the right time, the field must be monitored for the initial presence of thrips.

Earlier studies (1, 4) have shown that a variety of chemical compounds are attractive to thrips and that using a chemical attractant in combination with a water trap in onion fields is more effective at trapping thrips than a water trap alone. In our 2010 study, a thrips attractant lure was evaluated against a yellow sticky card to assess the potential benefit of the lure attracting more thrips than the card alone. Lures were also used in combination with water traps for the same purpose.

Seedcorn maggot can also be a pest of onion, and lures have been shown to be effective at attracting the adults to sticky cards (3).

Two separate lures for thrips (Thrips attractant, P178-lure) and seedcorn and onion maggots (SOM) (*Delia platura*/antigua, P316-lure) manufactured by ChemTica International, S.A. were provided by AgBio Development, Inc. (courtesy of J. Meneley). These lures were

attached to yellow sticky cards and placed in an onion field at CSU-ARDEC near Fort Collins, Colorado in 2010 to determine if they attract more target insects than a yellow sticky card alone. The number of thrips on onion plants surrounding the traps was also monitored for any secondary effect the lures might have. Thrips were also collected in water traps to further compare the efficacy of the lure and for future work investigating species composition.

Materials and Methods

Sticky cards

Yellow sticky cards (34 x 10 cm) were placed in the onion field alone or with one of a thrips lure or SOM lure. The yellow sticky cards were wrapped sticky side out over the end of a short (30 cm) stake and stapled in place (Figure A5.1). The top of the sticky card was 19.5-24 cm above the soil. Lures used in conjunction with sticky cards were stapled to the side edge of the sticky card. A sticky card alone, one with a thrips lure, or one with a SOM lure were placed in a straight line 7.6 meters apart. One set of this arrangement was placed in beds planted with onion sets (cultivar Solid Gold), and a second set was placed in beds of seeded onion (cultivar Belmar). This was repeated three times; once each in June, July and August of 2010.

Thrips were counted on ten plants (four in the same bed and six in the two beds on either side of the stake) when the stakes were initially placed in the field and again two days later. Sticky cards and lures were left in the field for three days. In June, the number of insects on the sticky cards was counted every day for three days, but in July and August the insects were counted once at the end of three days. Final counts of insects were done in the lab aided by a dissecting microscope in July and August. Insect counts were placed in the categories of thrips,

large flies, small flies, and other. Large flies included all flies longer than 2 mm and small flies included all flies smaller than 2 mm.

The ratio of thrips on the control to lure sticky card was calculated for each of the three months, and the mean ratio was compared to 1 (no difference) using Student's t-test, $\alpha=0.05$. The mean ratio of large flies caught on the traps was analyzed the same way. The mean number of thrips on plants surrounding the sticky cards before and two days after the cards and lures were placed were also compared using Student's t-test, $\alpha=0.05$.

Figure A5.1. Sticky trap with lure and water trap.

Water traps

In July and August, the sticky cards were removed after three days and replaced with a water trap (Figure XYZ.1). Water traps were placed within centimeters of the sticky card locations. The water traps were constructed by attaching a small yellow one liter bucket to the top of a short stake. The buckets were the same apparent shade of yellow as the sticky cards. Lures were removed from the sticky cards and attached to the inside edge of the buckets. A small amount of detergent was added to the water in the buckets to break the surface tension of the water. Insects were collected over a 24 hour period, then removed and stored in 70% ethanol at 4°C. Thrips were separated from the collected insects and counted.

The ratio of thrips collected in the control to lure water traps was calculated for July and August, and the mean ratio was compared to 1 (no difference) using Student's t-test, α=0.05.

Results

Sticky cards

In both the seeded onions and onion sets, thrips numbers peaked in July while no clear pattern emerged for either the large or small flies (Tables A5.1 and A5.2). In the seeded onions, the number of thrips caught on the control sticky cards exceeded that of the sticky cards with the thrips lure attached to it, with the exception of August. In the onion sets, higher numbers of thrips were caught on the sticky cards with thrips lures than on the control, with the exception of August. The results of the t-test (Table A5.3) on the thrips ratios averaged over the three months indicate no significant difference existed in the number of thrips caught on sticky cards with or without the lure in either the seeded onions or the onions sets.

Table A5.1. Insects captured on sticky traps placed in an area of Belmar onions grown from seed.

Seeded Onions	**Control**			**Thrips Lure**			**Seedcorn Maggot Lure**		
	June	**July**	**August**	**June**	**July**	**August**	**June**	**July**	**August**
Thrips	491	3730	2316	386	2706	2763	305	2971	1799
Large Flies	36	40	46	62	51	107	29	56	35
Small Flies	161	210	150	292	195	196	187	330	169
Other	39	162	54	25	167	100	82	143	58

Table A5.2. Insects captured on sticky traps placed in an area of Solid Gold onions grown from sets.

Onion Sets	**Control**			**Thrips Lure**			**Seedcorn Maggot Lure**		
	June	**July**	**August**	**June**	**July**	**August**	**June**	**July**	**August**
Thrips	399	3297	1929	581	5490	1536	358	2743	- *
Large Flies	15	30	38	39	49	158	37	51	-
Small Flies	158	246	171	459	358	231	459	241	-
Other	22	126	53	32	268	132	27	158	-

*additional lures were unavailable for August sampling

Table A5.3. Ratio of number of thrips on control sticky cards to sticky cards with thrips lure.

Seeded Onions			Onion Sets		
June	**July**	**August**	**June**	**July**	**August**
1.27	1.38	0.84	0.69	0.6	1.26
p-value: 0.4282			p-value: 0.5360		

The results of the t-test on the large fly ratios averaged over the three months (Table A5.4) indicate no significant difference existed in the number of large flies caught with or without the lure.

The ratio of all flies caught on the control vs lure was also calculated for each of the three months in each of the areas of the field (not shown). The results of the t-test on the large fly ratios averaged over the three months (p-value: 0.1970) indicate no significant difference existed in the number of flies caught with or without the lure.

Table A5.4. Ratio of number of large flies on control sticky cards to sticky cards with SOM lure.

Seeded Onions			Onion Sets		
June	**July**	**August**	**June**	**July**	**August**
1.24	0.71	1.34	0.4	0.59	-
p-value: 0.6603			p-value: 0.1144		

Thrips on plants

Tables A5.5 and A5.6 show the number of thrips counted on each of 10 plants surrounding the sticky card sites before and two days after placing the cards in the field. With the exception of the plants surrounding the control sticky card in the seeded onions in June (p-value 0.0381, α=0.05), there were no significant differences in the average number of thrips per plant surrounding the sticky cards (α=0.05) two days after the traps were placed in the field. When

averaged across the three months, there was no significant change in the number of thrips on the plants two days after placing the traps in the field.

Table A5.5. Number of thrips on plants before and two days after placing sticky cards and lures in a seeded onion bed.

Seeded Onions, Cultivar Belmar												
	June				**July**				**August**			
	Control		**Lure**		**Control**		**Lure**		**Control**		**Lure**	
Site	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	2	3	2	2	7	25	26	39	9	1	32	65
2	0	1	1	1	12	11	24	22	6	2	0	103
3	0	0	1	0	11	24	18	67	34	1	84	103
4	0	1	0	4	19	17	16	57	11	6	91	23
5	2	4	1	1	14	4	22	45	91	0	16	0
6	1	2	0	1	22	28	28	8	6	0	5	46
7	0	0	0	0	18	21	49	17	63	4	56	1
8	0	4	0	1	17	12	53	14	18	17	31	6
9	1	5	0	0	11	17	47	53	34	25	52	62
10	2	2	0	3	18	20	19	28	8	73	28	53
ave	0.8	2.2	0.5	1.3	14.9	17.9	30.2	35.0	28.0	12.9	39.5	46.2
std dev:	0.92	1.75	0.71	1.34	4.63	7.31	13.98	20.17	28.64	22.70	30.96	38.63

Table A5.6. Number of thrips on plants before and two days after placing sticky cards and lures in an onion set bed.

Onion Sets, Cultivar Solid Gold												
	June				**July**				**August**			
	Control		**Lure**		**Control**		**Lure**		**Control**		**Lure**	
Site	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	3	3	48	43	6	20	32	17	3	91	7	26
2	19	14	0	2	38	83	29	17	0	91	28	36
3	0	3	5	0	89	29	11	12	2	103	22	17
4	34	2	60	25	18	23	19	22	78	0	5	16
5	6	3	13	11	14	67	38	58	63	6	18	8
6	7	8	3	3	67	26	36	27	9	85	3	8
7	4	3	1	29	22	16	15	11	34	39	2	2
8	10	2	21	1	24	37	19	22	57	0	6	50
9	15	2	21	2	68	19	4	9	62	10	5	6
10	20	10	22	0	97	47	18	50	58	2	5	25
ave:	11.8	5.0	19.4	11.6	44.3	36.7	22.1	24.5	36.6	42.7	10.1	19.4
std dev:	10.33	4.19	20.30	15.31	33.13	22.48	11.20	16.61	30.49	44.50	9.10	15.08

Water Traps

Table A5.7 shows the number of thrips collected in the water traps during a 24 hour period. In both the seeded onions and onion sets, more thrips were collected in August than in July. In the seeded onions there was a significant difference in the number of thrips caught between the control water trap and the water trap with the thrips lure (Table A5.8, p-value: 0.0031, α=0.05). The difference in the number of thrips caught in the onion sets was not significant (Table A5.8).

There was no significant difference in the number of thrips caught between the control water traps and the water traps with the SOM lure.

Table A5.7. Thrips captured in water traps placed in an area of Belmar onions grown from seed and Solid Gold onions grown from sets.

	Control		**Thrips Lure**		**Seedcorn Maggot Lure**	
Seeded Onions	**July**	**August**	**July**	**August**	**July**	**August**
Total Thrips	65	478	151	1126	64	219
Onion Sets						
Total Thrips	80	201	190	368	125	- *

*additional lures were unavailable for August sampling.

Table A5.8. Ratio of number of thrips caught in control water traps to water traps with thrips lure.

Seeded Onions		**Onion Sets**	
July	**August**	**July**	**August**
0.43	0.42	0.42	0.55
p-value: 0.0031		p-value: 0.0767	

Discussion

Sticky cards

When Kuhar et al. (3) used SOM lures (also manufactured by ChemTica), they found that the sticky traps associated with lures significantly attracted more flies than sticky traps alone. Results reported here show no significant difference between the number of flies caught with or

without the SOM lure. Several differences in experimental design could account for these conflicting results. Their sample sizes were larger, their experiment spanned a much longer time (up to 61 days in one case), and field locations were in different parts of the country.

This experiment reported here lasted only three days and it is possible the results would have been different over a longer period. Sticky cards and lures were limited, so only one of each type of trap at a time was placed out in each section of the field. While this allowed us to collect insects during three months, we could not generate an average number of insects per type of trap per time point. Replicates of each trap distributed throughout the field at each time point (5 of each at a time for example) would make our results more meaningful.

Because the seeded onions and onion sets were at different developmental stages, their corresponding insect counts were kept separate. If the experiment were conducted in a field of uniform development there would be more reps to compare. It is also possible that the different lures and controls were placed too close together and interfered with each other. Repeating this with more replicates and greater spacing between lures and controls would be interesting.

Thrips on plants

While there were no significant differences in the number of thrips on onion plants surrounding the sticky cards after the cards and lures were placed in the field, it does appear that thrips were not necessarily evenly distributed within the field. When averaged over the three months, the data suggests no difference in the number of thrips at the site of the control sticky card compared to the site of the sticky card with the thrips lure in the seeded onions before setting out the cards and lure (p-value: 0.1378, α=0.05). Thrips counts taken two days later suggest that the number of thrips on the plants had changed such that the number of thrips on

plants surrounding the control was no longer equal to the number of thrips on plants surrounding the lure (p-value: 0.0080, α=0.05). In this instance plants surrounding the lure had a greater number of thrips on them than did the control plants. The opposite occurred in the onion sets, with an unequal distribution of thrips on the plants before placing the sticky cards and lures in the field (p-value: 0.0262, α=0.05), then no significant difference was noted in the number of thrips on the plants at the two sticky card sites two days later (p-value: 0.1515, α=0.05). This may indicate that thrips were moving throughout the field. Again, a larger sample size should give a more meaningful result.

Water traps

If the thrips counts from both the seeded onions and onion sets are combined, the results indicate a significant difference in the number of thrips caught in the water traps with the lures as compared to the control (p-value: 0.0004, α=0.05). Repeating the experiment with more traps per 24 hour period should lend more robust support to these results. It would also be interesting to place water traps in the field earlier in the season and more frequently to get a better idea when exactly thrips arrive and can vector IYSV.

The thrips lures appear to be more effective when combined with the water trap than with the sticky cards. Thrips caught in the water traps remain intact and can be recovered and identified. This may be useful if specific thrips species need to be identified before determining appropriate pest control actions or in studies of thrips species composition and IYSV studies.

Repeating these experiments with more replicates, greater spacing between lures and controls, and with earlier and more frequent time points would be interesting for future

investigations into the value and potential application of these lures for use in onion integrated pest management programs.

LITERATURE CITED

1. Davidson, M. M., Butler, R. C., and Teulon, D. A. J. 2009. Pyridine compounds increase thrips (Thysanoptera: Thripidae) trap capture in an onion crop. J. Econ. Entomol. 102 (4):1468-1471.
2. Gent, D. H., du Toit, L. J., Fichtner, S. F., Mohan, S. K., Pappu, H. R., and Schwartz, H. F. 2006. *Iris yellow spot virus*: An emerging threat to onion bulb and seed production. Plant Dis. 90 (12):1468-1480.
3. Kuhar, T. P., Hutchison, W. D., Whalen, J., Riley, D. G., Meneley, J. C., Doughty, H. B., Burkness, E. C., and Wold-Burkness, S. J. . 2006. Field evaluation of a novel lure for trapping seedcorn maggot adults. Plant Health Progress. doi: 10.1094/PHP-2006-0606-01-BR
4. Teulon, D. A. J., Davidson, M. M., Hedderley, D. I., James, D. E., Fletcher, C. D., Larsen, L., Green, V. C., and Perry, N. B. 2007. 4-pyridyl carbonyl and related compounds as thrips lures: Effectiveness for onion thrips and new zealand flower thrips in field experiments. J. Agric. Food Chem. 55 (15):6198-6205.

APPENDIX 6: REVERSE TRANSCRIPTION-LOOP MEDIATED ISOTHERMAL AMPLIFICATION OF *IRIS YELLOW SPOT VIRUS*

Introduction

Iris yellow spot virus (IYSV) is typically detected with double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) or reverse transcription polymerase chain reaction (RT-PCR), both of which are time consuming and require specialized equipment. In addition, RT-PCR is costly; and reagents for 100 RT-PCR reactions can cost approximately $615.

Notomi et al. (2) developed loop mediated isothermal amplification (LAMP), a DNA amplification technique that occurs under isothermal conditions, uses four to six primers, and a strand displacing DNA polymerase. The multiple primers confer greater specificity, and the strand displacing polymerase allows for rapid amplification. Eiken Chemical Co., Ltd. (Tokyo, Japan) produced an animation of the process which can be viewed at: http://loopamp.eiken.co.jp/e/lamp/anim.html.

LAMP reactions can be modified by adding reverse transcriptase to amplify RNA (RT-LAMP), and by adding fluorescent dyes to detect amplicons under UV light (2). These modifications have been applied to detect several plant RNA viruses including *Banana bract mosaic virus* (3), *Southern rice black-streaked dwarf virus* (5), and *Zucchini yellow mosaic virus* (1). RT-LAMP has the potential to be a sensitive, rapid, and cost effective diagnostic test that uses very little specialized equipment. The objective of this work was to investigate the use of an RT-LAMP assay that could detect IYSV directly from onion tissue with minimal equipment.

Materials and Methods

Four primers were designed with PrimerExplorer V3 at http://primerexplorer.jp/e/v3_manual/index.html, and were synthesized by IDT (Coralville, IA). The two outer primers were F3: 5'CTGGCAAGTCTTGACAGC 3', and B3: 5' GGAGTGCATTTAGTCAGGATC 3'. The two inner primers were FIP: 5' TGCTCATAAGTTGAGAATCTGCTTTTTTACTCTTCCTTTAGCTTATTTCCA 3' and BIP: 5' AGTTGCAAGAGTCATGGCAGTTTTTACAGTTTCATCAAAGATCTTCT 3'.

Reaction conditions were optimized using a BioRad CFX Connect Real-Time System (Bio-Rad, Hercules, CA), before investigating the use of simpler equipment. SybrSafe dye (Invitrogen, Carlsbad, CA) was added to the reactions at a final concentration of 1X for amplification to be detected by the machine. The machine was programmed with different time and temperature cycles. Forty cycles of 30 s at 64°C, 90 s at 65°C produced the best amplification. Melt curves were included with each run.

Initial reactions were prepared with RNA where the IYSV status of samples was previously determined by RT-PCR (the RT-PCR procedure is described in Chapter 2). Reactions were typically prepared in duplicate. Amounts and concentrations of $MgSO_4$, dNTP mix, DTT, Bst, MMLV-RT, and RNase inhibitor were varied before settling on the mixture in Table A6.1. Dilution series from 150 to 1 ng RNA per reaction and from 1 to 1 x 10^{-6} ng RNA per reaction were prepared.

Once the reaction mixture was optimized using the BioRad CFX Connect Real-Time System, the reactions were attempted using a MJ Research PTC-200 thermocycler (Waltham, MA). Results were viewed on a 1.5% agarose gel (Bio-Rad Laboratories, Inc., Hercules, CA) stained with SYBRSafe (Invitrogen, Carlsbad, CA) in 1X TAE buffer (Bio-Rad Laboratories,

Inc., Hercules, CA) and visualized upon exposure to UV light. A 100bp molecular weight marker (Fermentas, Glen Burnie, MD) was included on each gel. RNA, cDNA, and aliquots of a simple onion leaf extract were used as templates for amplification. Onion leaf extracts were prepared in 1.5 ml microcentrifuge tubes by macerating a 0.01 g section of onion leaf displaying IYSV symptoms in 1 ml molecular biology grade water.

Efforts to prevent contamination included preparing the reactions in a PCR hood (in a different lab) that had been exposed to UV light and wiped down with 10% bleach or 70% ethanol, using pipettes wiped with 10% bleach or 70% ethanol before and after use, using a separate set of pipettes for preparing reactions and loading gels, using filter tips, overlaying each reaction with mineral oil, using dedicated reagents, wearing fresh gloves before preparing reactions, and taking care not to touch surfaces that could come in contact with the reagents.

Table A6.1. Reagent list for RT-LAMP of IYSV.

Reagent (manufacturer)	Stock concentration	Working concentration or amount	Amount in 25 µl reaction
FIP/BIP primer mix	10 µM	1.6 µM	4 µl
Betaine (Sigma-Aldrich, St. Louis, MO)	5 M	0.8 M	4 µl
Isothermal amplification buffer (New England Biolabs, Ipswich, MA)	10X	1X	2.5 µl
DTT (Invitrogen, Carlsbad, CA)	0.1 M	10 mM	2.5 µl
Molecular biology grade H_2O (Sigma-Aldrich, St. Louis, MO)			variable[a]
dNTP mix (Fermentas, Glen Burnie, MD)	10 mM	0.4 mM	1 µl
$MgSO_4$(Sigma-Aldrich, St. Louis, MO)	100 mM	4 mM	1 µl
Bst 2.0 DNA polymerase (New England Biolabs, Ipswich, MA)	8000 U/ml	8 U	1 µl
F3/B3 primer mix	10 µM	0.2 µM	0.5 µl
MMLV-RT (Invitrogen, Carlsbad, CA)	200 U/µl	100 U	0.5 µl
RNase inhibitor (Fermentas, Glen Burnie, MD)	40 U/µl	20 U	0.5 µl

[a]Water was added to bring the final reaction volume to 25 µl, and varied according to the amount of template added.

Results and Discussion

When using the BioRad CFX Connect Real-Time System, noticeable amplification occurred with as little as 0.1 ng RNA per reaction. Single melt peaks appeared with each

reaction, and melt peaks were typically within 0.5°C of each other. The duplicate reactions did not always behave the same. Frequently, one would amplify and the other would not. RT-LAMP results did not always agree with RT-PCR results. Usually the discrepancy was that amplification would occur by RT-LAMP and not by RT-PCR.

Amplification from a simple onion leaf extract

Direct amplification from onion leaves was achieved (Figures A6.1 and A6.2). Reactions were prepared with 5 µl plant extract (prepared from a 0.01 g section of onion leaf displaying IYSV symptoms macerated in 1 ml molecular biology grade water) and held at 65°C for 90, 120, and 150 minutes in a MJ Research PTC-200 thermocycler (Waltham, MA). Amplification appeared strongest and most consistent when the reactions were held at 65°C for 150 minutes (Figure A6.1), so that time period was selected for subsequent reactions.

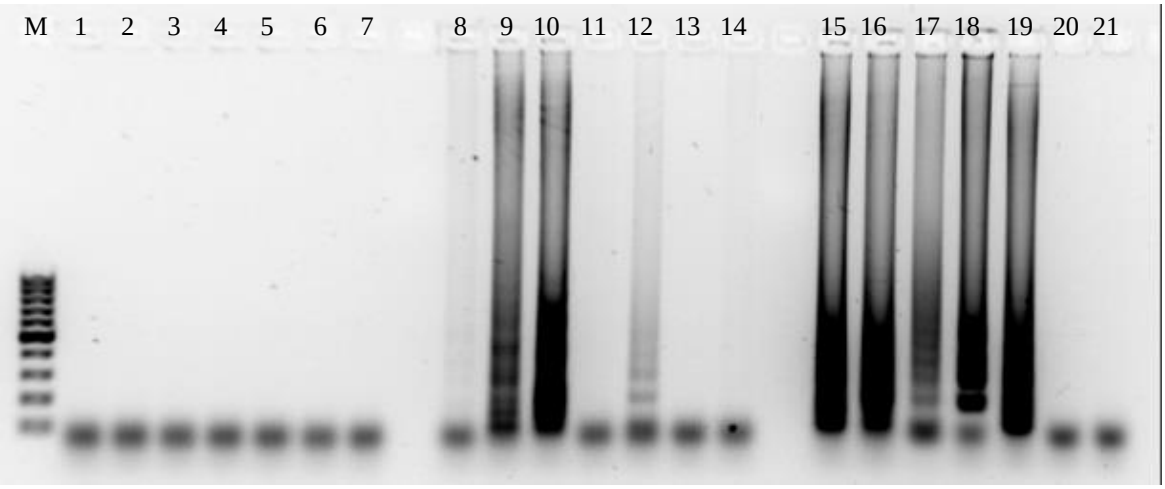


Figure A6.1. RT-LAMP of IYSV after 90 (lanes 1 to 7), 120 (lanes 8 to 14), and 150 (lanes 15 to 21) minutes at 65°C. M: 100 bp molecular weight marker. Lanes 1 to 5, 8 to 12, and 15 to 19: 5µl of 0.01g IYSV symptomatic onion leaf macerated in 1ml water; lanes 6 and 7, 13 and 14, 20 and 21: NTC.

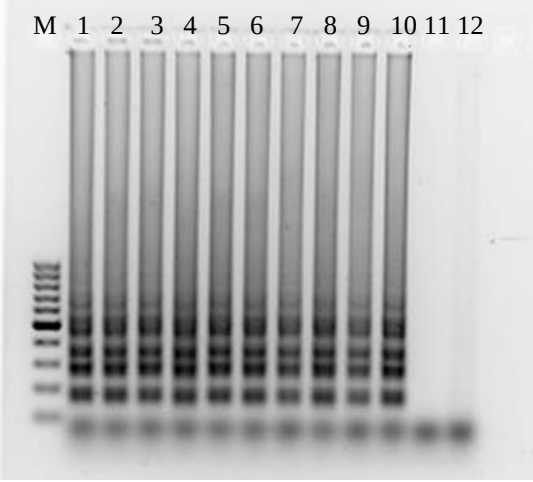


Figure A6.2. Amplification directly from plant tissue. 0.01g IYSV symptomatic onion leaf macerated in 1ml water. 5µl extract per reaction, 150 minutes at 65°C. M, 100 bp molecular weight marker. Lanes 1-10, reactions prepared with aliquots from the same extract; lanes 11 and 12 NTC.

Dyes

Adding SybrSafe (at a final concentration of 1X) to the reactions worked well to detect amplification when using the BioRad CFX Connect Real-Time System, but did not work well for viewing amplification visually upon exposure to UV light. Other reagents fluoresced when exposed to UV light and it was impossible to discern if amplification had occurred. Adding 2 µl PicoGreen (Invitrogen, Carlsbad, CA) to each reaction tube after the reaction was complete looked promising. Exposing the tubes to 395 nm UV light produced a green color in tubes with amplification (Figure A6.3). Reactions that had no amplicons remained pale orange. The green color corresponded to the amplification shown in lanes 1 to 4 of the gel in Figure A6.3. It was difficult to discern green from orange if amplification was weak, or if contamination occurred.

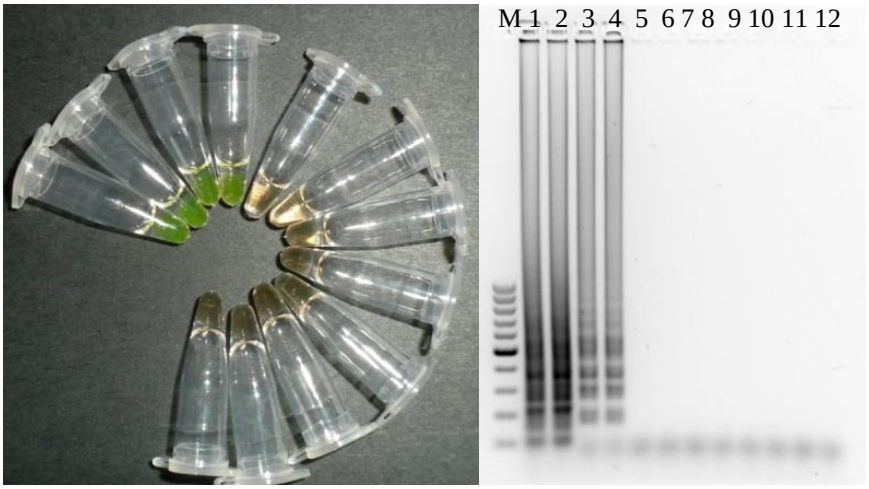


Figure A6.3. Amplification from an extract prepared from an onion leaf with IYSV symptoms. Left: PicoGreen was added to each tube. Tubes with the green color indicate amplification occurred. Tubes with no amplification remained pale orange. Right: The amplification shown in lanes 1 to 4 correspond to the green tubes on the left. M: 100 bp molecular weight marker; lanes 1 to 4: extract prepared from an onion leaf displaying IYSV symptoms; lanes 5 to 10: extracts prepared from IYSV- free onion leaves; lanes 11 and 12: NTC.

Unexpected results

It appeared that amplifying IYSV directly from onion leaf tissue with this method was feasible. However, amplification frequently occurred in reactions prepared with extracts of onion leaves known to be free of IYSV (i.e., they had been grown from seed in the absence of thrips and tested by DAS-ELISA). The pattern of bands visible on the gel sometimes varied, especially when amplification occurred from plant samples known to be free of IYSV (Figure A6.4). It is not known if this amplification was caused by contamination, primer-primer interactions, or by primers binding to genomic DNA of the onion, or of an unknown endophyte or epiphyte.

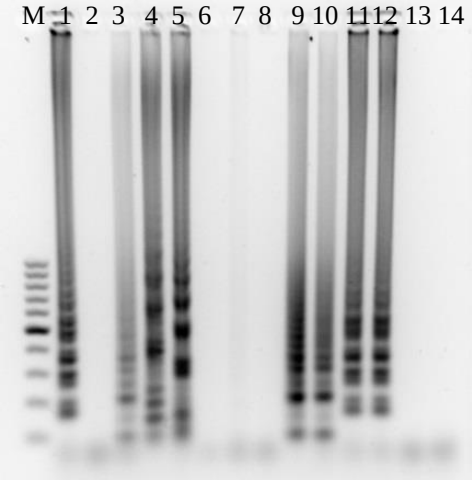


Figure A6.4. Amplification from IYSV-free onion leaf extracts. Unexpected amplification occurred in some of these reactions, and several different banding patterns were produced. M: 100 bp molecular weight marker. Lanes 1 to 5: red onion cultivar; lanes 6 to 10: white onion cultivar; lanes 11 and 12: positive control; lanes 13 and 14: NTC.

Variability between reactions prepared from the same extract also occurred (Figure A6.5), which could possibly be explained by differences in enzyme efficiency or heterogeneity within the plant extracts.

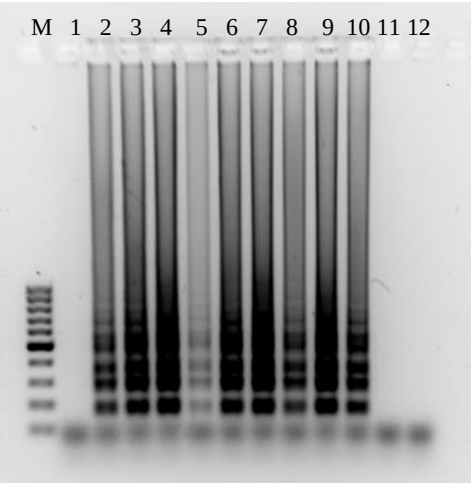


Figure A6.5. Variability between reactions made with aliquots from the same symptomatic onion leaf extract. M: 100 bp molecular weight marker; lanes 1 to 10: onion leaf extracts; lanes 11 and 12: NTC.

8 µl aliquots of onion leaf extract were treated with 1 to 5 µl DNase. If the amplification in the uninfected samples was caused by non-target amplification of genomic DNA, the DNase

treatments should have degraded any DNA present in the sample. No difference was observed between aliquots treated with DNase and those left untreated.

Passing the plant extract through a 0.22 µm filter did not eliminate amplification from IYSV free samples. Onion plant extracts were prepared in a buffer described by Thomson and Dietzgen (4), however, no amplification occurred from extracts prepared in that buffer. Either the buffer inhibited the RT-LAMP reactions, or it destroyed the RNA.

Contamination

Despite precautions taken to prevent contamination, it still occurred in about half the runs. Amplicons would be detected in non-template controls (NTC) and in samples known to be free of IYSV. Contamination appeared to be a random event. Figure A6.7 shows ten NTC reactions aliquoted from a single master mix. Because amplification occurred in only one of the ten aliquots, contaminated reagents can be ruled out. It is interesting that contamination did not occur in any of the RT-qPCRs (Chapter 4) which are supposed to be very sensitive to contamination. If RT-LAMP is more sensitive to contamination than other PCR methods, it may not be practical as a simple diagnostic method.

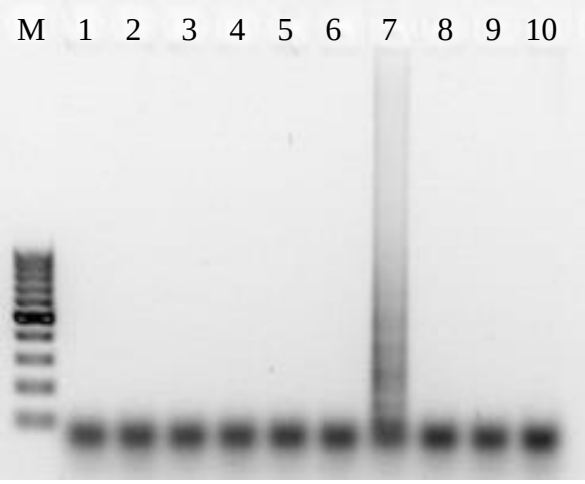


Figure A6.7. Seemingly random contamination in lane 7. M: 100 bp molecular weight marker. Lanes 1 to 10: aliquots from a single master mix with no template.

Conclusions

RT-LAMP did detect IYSV from a simple extract prepared from symptomatic onion leaf tissue, and amplification was detected by adding PicoGreen and observing the color change. However, the amplification that occurred from IYSV free extracts could be interpreted as a false positive result. In addition, reactions were easily contaminated. Amplification (or lack of amplification in the case of IYSV free samples) was not satisfactorily reliable or reproducible to recommend this method as a diagnostic technique. This is unfortunate, as the complete process only took about four hours, as compared to over 11 hours to complete the typical RT-PCR process, and cost approximately one quarter that of RT-PCR.

LITERATURE CITED

1. Kuan, C. P., Deng, T. C., Huang, H. C., Chi, H. H., and Lu, Y. L. 2014. Use of reverse transcription loop-mediated isothermal amplification for the detection of *Zucchini yellow mosaic virus*. J. Phytopathol. 162 (4):238-244.
2. Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28 (12):i-vii.
3. Siljo, A., and Bhat, A. I. 2014. Reverse transcription loop-mediated isothermal amplification assay for rapid and sensitive detection of *Banana bract mosaic virus* in cardamom (*Elettaria cardamomum*). Eur. J. Plant Pathol. 138 (2):209-214.
4. Thomson, D., and Dietzgen, R. G. 1995. Detection of DNA and RNA plant-viruses by PCR and RT-PCR using a rapid virus release protocol without tissue homogenization. J. Virol. Methods 54 (2-3):85-95.
5. Zhou, T., Du, L. L., Fan, Y. J., and Zhou, Y. J. 2012. Reverse transcription loop-mediated isothermal amplification of RNA for sensitive and rapid detection of *Southern rice black-streaked dwarf virus*. J. Virol. Methods 180 (1-2):91-95.