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Temporal and Localized Distribution of *Iris Yellow Spot Virus* within Tissues of Infected Onion Plants

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Abstract. Passage of *Iris yellow spot virus*, *Tospovirus* sp., in its vector, as well as its spatial and temporal distribution in an onion (*Allium cepa* L.) field, have been described. However, little is known of the distribution of the virus within onion hosts. The temporal dynamics of *Iris yellow spot virus* titer in leaves of 'Colorado 6' and 'Talon' onion cultivars in a greenhouse were investigated with or without thrips by using double antibody sandwich enzyme-linked immuno-sorbent assay. There was no significant difference in titer between the two cultivars (P = 0.1224); however, titer was significantly greater with thrips than without (P < 0.0001). Plant leaves, divided into top, middle, and base sections, showed that virus titer significantly increased from 1.813 and 1.854 optical densities in top and middle, respectively, to 2.065 in base sections. Leaves of field-infected plants were categorized by age into older, intermediate, and younger leaves. In pre-bulb (6- to 8-leaf-stage) plants, *Iris yellow spot virus* was most frequently detected in intermediate leaves; however, there was no significant difference in virus titer between leaf age categories. In post-bulb (11+ leaf-stage) plants, frequency of virus detection was almost the same in older and intermediate leaves (P = 0.7217) but significantly less in younger leaves. Virus titer was greater in younger, then intermediate, and older leaves, with optical densities of 2.778, 2.649, and 2.349, respectively. Virus was not detected in dead leaves, bulb scales, basal plates, or roots. Information on *Iris yellow spot virus* distribution in host tissues is vital in epidemiological studies, evaluations of germplasm, and quarantine control of this tospovirus.

Introduction

Following its first identification on onion (*Allium cepa* L.) in Brazil in 1981, *Iris yellow spot virus* (family Bunyaviridae, genus *Tospovirus*) has become a worldwide threat to the economic production of food and ornamental plants (Pappu et al. 2009). In the U.S., the virus was first confirmed on onion in the Pacific Northwest in 1989, and has spread to several onion-producing states (Gent et al. 2006). On onion plant scapes, symptoms of *Iris yellow spot virus* appear as chlorotic or necrotic, straw-colored to white, dry, elongate or spindle-shaped lesions frequently more numerous in mid- to lower portions of the scape. Some lesions have an island of green tissue in the center of the necrotic tissue. As more lesions develop and increase in size, they coalesce, often completely girdling the scape. On infected leaves, straw-colored, lenticular-shaped lesions with green centers or alternating

rings of green and straw-colored tissues appear. No symptoms or virus particles have been consistently associated with bulbs (Gent et al. 2006, Pappu et al. 2008).

Edible allium crops and some cut flower and potted ornamental species including alstroemeria (*Alstroemeria* sp.), chrysanthemum (*Chrysanthemum* sp.), iris (*Iris hollandica* L.), and lisianthus (*Eustoma grandiflorum* (Raf.) Shinn.) are the most economically important hosts of *Iris yellow spot virus* (FERA 2007). Infections result in loss of quantity and/or quality of produce depending on the host species. Infections at early stages of crop growth often result in stunting or death, leading to considerable yield loss; however, infections at later stages may still cause significant losses in yield and quality of produce (Smith et al. 2006, Pappu et al. 2008). Among all hosts, onion crops have been particularly affected. In bulb onion, significant loss occurs from reduced bulb size, significantly reducing the percentage of colossal- and jumbo-grade bulbs in susceptible cultivars. Overall yield losses of 1-10% or more are frequently reported in Colorado (Schwartz et al. 2002, Gent et al. 2004a, 2006). On individual farms, yield losses range from undetectable to 100% (Gent and Schwartz 2008). In seed onion production, infected scapes often lodge, leading to umbel rot and loss, which in turn leads to significant seed loss (Pozzer et al. 1999; Crowe and Pappu 2005; Gent et al. 2006, 2007; du Toit et al. 2007).

Several species of thrips transmit tospoviruses in a circulative propagative way. *Iris yellow spot virus* is known to be transmitted primarily by the polyphagous onion thrips, *Thrips tabaci* L. However, limited transmission by tobacco thrips, *Frankliniella fusca* L., has recently been confirmed in Georgia (Srinivasan et al. 2012). First- or second-instar larvae acquire the virus by feeding on infected plants, and become viruliferous and infective for the rest of their lives (Moritz et al. 2004, Whitfield et al. 2005, Hogenhout et al. 2008). Nineteen tospoviruses have been described globally (Fauquet et al. 2005, Tsompana and Moyer 2008). However, *Tomato spotted wilt virus* is the most studied species, and most of the tissue or cellular interactions between tospoviruses and their vectors are described for it and one of its major vectors, western flower thrips, *Frankliniella occidentalis* P. (Ullman et al. 2002, Whitfield et al. 2005, Pappu et al. 2009). Such studies have led to the description of the passage and replication of *Tomato spotted wilt virus* (as it applies to the other tospoviruses) in its vector (Whitfield et al. 2005, Hogenhout et al. 2008). Spatial and temporal distribution of *Iris yellow spot virus* in an onion field has been described (Gent et al. 2004a, Schwartz et al. 2010), but little is known of the distribution of the virus within plant hosts. This is because of relatively recent description (Tsompana and Moyer 2008, Pappu et al. 2009) and lack of indicator hosts, coupled with lack of an efficient mechanical inoculation procedure to infect hosts (Bag and Pappu 2009, Srinivasan et al. 2011). Unlike *Tomato spotted wilt virus* that has numerous indicator hosts for mechanical inoculation-mediated vector-*Tomato spotted wilt virus*-host interaction studies, *Iris yellow spot virus* has a narrow host range, and mechanical transmission in hosts is difficult (Bag and Pappu 2009). Also, the within-plant distribution of *Iris yellow spot virus* cannot be inferred from the within-plant distribution of *Tomato spotted wilt virus* because *Tomato spotted wilt virus* causes systemic infections whereas *Iris yellow spot virus* moves systemically less readily and tends to remain localized (Smith et al. 2006, Pappu et al. 2009). In leek (*Allium porrum* L.), *Iris yellow spot virus* was localized in patches of infection mainly in the middle and top subsections of unfurled leaves, but infrequently in the bases. *Iris yellow spot virus* was not detected in furled leaves, basal plates, or roots (Smith et al. 2006). In onion, virus was detected in all segments of infected leaves

although the distribution was not uniform. Also, higher concentrations were found consistently in internal leaves and in leaf segments close to the bulb. Just as in leeks, no virus was detected in bulbs or roots of infected onion plants (Kritzman et al. 2001).

The objectives of this study were to determine the distribution and build-up of *Iris yellow spot virus* in infected leaves of tolerant and susceptible onion cultivars, and its distribution in tissues of naturally infected onion plants from the field. Because it is a yield-limiting factor, researchers are concerned with identifying and incorporating plant resistance as an important component of the integrated disease management strategy for *Iris yellow spot virus* and/or onion thrips (e.g., Boateng 2012). Onion cultivars with resistance to onion thrips have been identified (Jones et al. 1935, Brar et al. 1993, Diaz-Montano et al. 2010, 2011). Currently, no onion cultivar is resistant to *Iris yellow spot virus* (Diaz-Montano et al. 2010); however, studies by du Toit and Pelter (2005), Shock et al. (2008), and Boateng (2012) indicated some cultivars exhibit some tolerance to the virus. In this study, Colorado 6 and Talon were used as tolerant and susceptible onion cultivars, respectively, in greenhouse experiments. Colorado 6 was chosen for its tolerance to *Iris yellow spot virus* based on less incidence of disease in cultivar evaluations during 2003 and 2004 in Colorado (Gent et al. 2004b, Schwartz et al. 2004). Talon was chosen based on high incidence of disease in commercial fields in 2005 and 2006 (Schwartz et al. 2010) and during later experiments in Colorado.

Materials and Methods

Establishment and Maintenance of Colonies of Potentially Viruliferous Onion Thrips. *Iris yellow spot virus*-infected and symptomatic onion plants with associated onion thrips were collected from an onion field at the Colorado State University Agricultural Research, Extension and Education Center (ARDEC) near Fort Collins in northern Colorado. Plants were given Arabic numbers, planted in pots, and kept in thrips-proof Bug Dorms (Bug Dorm Store, Megaview Inc., Taichung, Taiwan) at 25-30^{o}C, 80-90% relative humidity, and a photoperiod of 14:10 light:dark hours in a greenhouse. The associated potentially viruliferous onion thrips were maintained on the plants and used for thrips-mediated inoculation of onion plants in the experiment. Infection status of all the plants in the colony was assessed symptomatically and verified by double antibody sandwich enzyme-linked immuno-sorbent assay (DAS ELISA) (Gent et al. 2004a) each month. Dead plants were replaced with new onion seedlings as required to maintain the colony. Presence of *Iris yellow spot virus* in onion thrips in the colony was verified by reverse transcription polymerase chain reaction (RT-PCR) each month. Two onion thrips larvae/adults were collected from each plant in the colony. Onion thrips from the same plant were combined as one sample and identified by plant number before RT-PCR was used.

Temporal Dynamics of *Iris Yellow Spot Virus* Titer in Infected Onion Plants. Seeds of the *Iris yellow spot virus*-field tolerant Colorado 6 and IYSV-susceptible Talon onion cultivars were planted in soil-less mixture (Fafard's Professional Custom Mix Formula, Conrad Fafard, Inc., Agawam, MA) in nursery trays at the Colorado State University greenhouse facility at Fort Collins. After emergence and 3 weeks of growth, seedlings were thinned to retain those at the same growth stage and of equal height and size. After 3 weeks of further growth, two seedlings were transferred into the same soil-less mixture in each 2-liter pot

(one plant was used for a study not reported here), and pots were kept in 75 x 75 x 115-cm Bug Dorms with 34 x 9 cm^2 mesh and a 680-µm mesh aperture. Irrigation, with attached liquid fertilization applied as needed to maintain adequate plant growth, was provided by an automated drip system (Orbit Irrigation Products, Inc., Bountiful, UT) that supplied 150 ml of water every other day.

Treatments, consisting of healthy check, *Iris yellow spot virus* only and thrips+*Iris yellow spot virus*, were allocated to three Bug Dorms, and 10 pots of each cultivar were randomly placed in each Bug Dorm. This arrangement ensured that plants of the two cultivars were subjected to the same environmental conditions and pest/pathogen pressure within a Bug Dorm. Five potentially viruliferous larvae and adult thrips were transferred from the colony onto leaves of each plant in the Bug Dorms of *Iris yellow spot virus* only and thrips+*Iris yellow spot virus* treatments. However, with the *Iris yellow spot virus* only treatment, thrips were transferred 5 days earlier. This provided sufficient time for virus transmission. After successful virus transmission as confirmed by DAS ELISA, the thrips were killed by application of a combination of Movento (active ingredient spirotetramat; Bayer CropScience, Research Triangle Park, NC), Radiant (active ingredient spinetoram; Dow AgroSciences LLC, Indianapolis, IN), and Regent (active ingredient fipronil; BASF Corp, Research Triangle Park, NC) insecticides at recommended application rates of 0.39 and 0.63 ml per liter for Movento and Radiant, respectively. Regent (pending registration for use on onion) was applied at 0.39 ml per liter. *Iris yellow spot virus* only treatment was started at 66 days, while thrips+*Iris yellow spot virus* treatment was started at 71 days after sowing. The experiment was in a randomized complete block design with two replications.

Six plants per cultivar per treatment were selected every other week starting at 75 until 145 days after sowing for a total of six sampling dates and used for serological detection of *Iris yellow spot virus*. Samples were taken only from the selected plants throughout the experiment. The four remaining plants were used for an experiment not reported here. At 66 days after sowing (start of the *Iris yellow spot virus* only treatment), plants were at the 6-leaf developmental stage. Thus, all sampled leaves were present at the time of inoculation, and leaves that emerged post-inoculation were not sampled. During sampling, the oldest leaf of each selected plant was excised at the junction of the leaf blade and sheath (Fig. 1). Entire leaves were divided into top, middle, and base sections, and each section was further divided into 0.3-g subsamples for testing by DAS ELISA.

***Iris Yellow Spot Virus* Distribution among Leaves of Naturally Infected Onion Plants.** In 2010, 21 *Iris yellow spot virus*-infected and symptomatic transplanted onion plants at 6- to 8-leaf stage (pre-bulb) (Fig. 2A) and 20 plants at 11 and older leaf stage (post-bulb) (Fig. 2B) of susceptible cultivar 'Charismatic' (H. F. Schwartz unpublished data) were sampled from an onion research field at ARDEC. The field was furrow irrigated and weeds were controlled, but no insecticide was applied. Leaves of pre-bulb plants were gently peeled back all the way to the basal plate such that the leaf included the furled (sheath) portion (Fig. 1). Plants were separated into bulb scales (dead outermost leaves surrounding the bulb region), leaves (the blade (unfurled) and sheath portions), basal plates, and roots. On post-bulb plants, only leaf blades were tested; no sheath, bulb scales, basal plate, bulb, or roots were included. As in a preliminary experiment, the tissues were consistently negative for the virus. Two, 1.0-g leaf blade and one, 1.0-g sheath (pre-bulb plants only) tissue segments were sampled from each leaf for *Iris yellow spot virus* detection. On symptomatic leaves, samples were taken from

healthy tissues surrounding the necrotic regions, while leaf base tissues close to the bulb were sampled (Kritzman et al. 2001) in non-symptomatic leaves. Basal plate and root tissues were divided into 0.5-g samples.

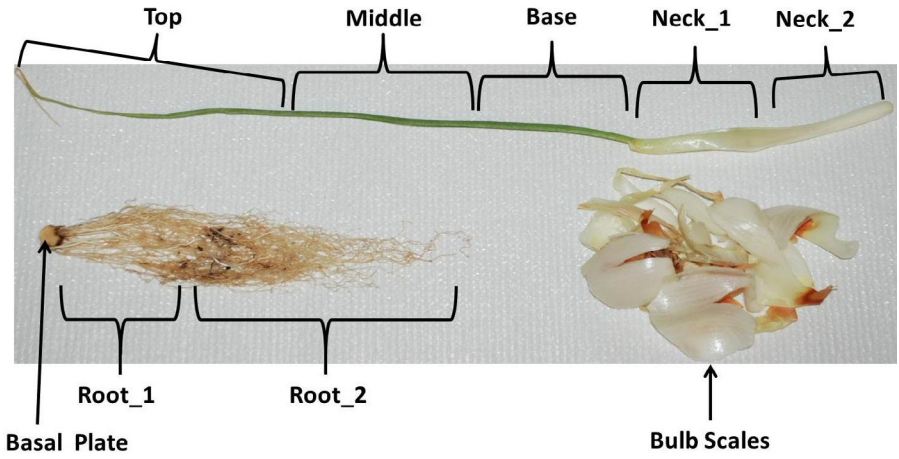

Fig. 1. Pictorial representation of an onion leaf, bulb scales, basal plate, and roots. Leaf was divided into leaf blade (top, middle, and base) and sheath (neck_1 and neck_2) sections; and roots into root_1 and root_2 sections.

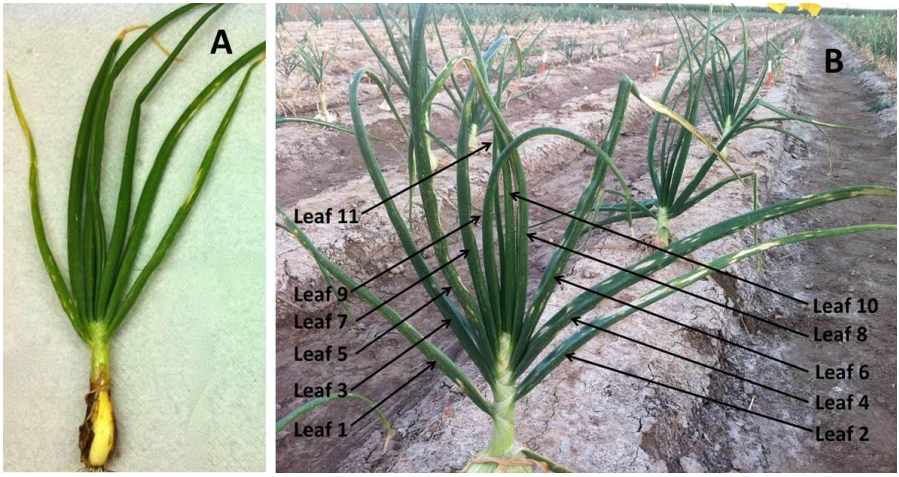

Fig. 2. Onion plants showing symptoms of *Iris yellow spot virus* infection in a field near Fort Collins in northern Colorado. **A**: Pre-bulb onion plant. **B**: Post-bulb onion plant. Leaf age was indicated by leaf numbers in which Leaf 1 was the oldest leaf and Leaf 11 the youngest leaf.

***Iris Yellow Spot Virus* Distribution within Tissues of Naturally Infected Onion Plants.** Sixty symptomatic pre-bulb transplanted onion plants of susceptible cultivar 'Granero' (H. F. Schwartz unpublished data) were sampled during the 2011 growing season from an onion field at the same location as in 2010. Plants were separated into the same sections as in 2010, however, leaf blades were divided into top, middle, and base sections; sheath segments into neck_1 and neck_2; and root segment into root_1 and root_2 (Fig. 1). One, 1.0-g sample was taken from each leaf segment, while basal plates and roots were divided into 0.5-g samples for serological testing for *Iris yellow spot virus*.

Using DAS ELISA for Serological Detection and Quantification of *Iris Yellow Spot Virus* Titer. Plant samples were frozen in liquid nitrogen and ground to a powder with a mortar and pestle (Gent et al. 2004a), after which an Agdia DAS ELISA kit following manufacturer protocol (Agdia Inc., Elkhart, IN) was used for serology of the ground samples. The ELx 800 Universal Micro-plate Reader (Bio-Tek Instruments Inc., Winooski, VT) was used to read absorbance values (optical densities) at 405 nm. Tissues were considered positive for *Iris yellow spot virus* if the absorbance values were equal to or greater than two times the values of healthy negative checks.

Virus Detection Using Reverse Transcription Polymerase Chain Reaction. Virus was also verified by using RT-PCR. Total RNA was extracted from leaf and onion thrips tissues using a Spectrum Plant Total RNA Kit (Sigma Life Science, Sigma-Aldrich Co., St. Louis, MO) and following manufacturer's instructions. After extraction, RNA yield was quantified with a NanoDrop 1000 spectrophotometer v. 3.7.0 (Thermo Fisher Scientific LLC., Milwaukee, WI) and concentrations equalized in all samples before RT-PCR. Reverse transcription for complementary DNA (cDNA) synthesis was done using M-MLV RT from Invitrogen (Life Technologies, Carlsbad, CA) and reactions were done using the 48 x 0.2 ml reaction module of Peltier Thermal Cycler (PTC) 200 DNA Engine (M J Research Inc., Waltham, MA). First, 1 µl 10 mM dNTP mixture and 1 µl random hexamers were mixed with 10 µl of the extracted RNA and incubated at 65^{o}C for 5 minutes. Then 4, 2, and 1 µl of 5X First-Strand Buffer, 0.1 M DTT, and RNase Out, respectively, were added and processed at 37^{o}C for 2 minutes, after which 1 µl M-MLV reverse transcriptase was added and processed at 25^{o}C for 10 minutes, 37^{o}C for 50 minutes, and 70^{o}C for 15 minutes to complete cDNA synthesis. The cDNA synthesis was followed by PCR on the *Iris yellow spot virus* nucleocapsid (N) gene using Au-R (5'-CTTGGAGGGATTCTTGGGTTTAG-3') and Au-F (5'-AGGGTAAAAGCTTCAGAAATCGAGA-3') as the reverse and forward primer sets, respectively, that amplify a 792 bp fragment of the N gene. Nad2 primer sets (nad2-F (5'-GGACTCCTGACGTATACGAATTATC-3') and nad2-R (5'-AAACAACGCTTGTAAGGAGTCC-3')) were used as check primers in the PCR reaction. PCR was done in 20 µl reactions consisting of 2 µl of 20 ng cDNA, 0.4 µl mM dNTP mix, 1.2 µl 50 mM $MgCl_2$, 0.5 µl of each primer, 2 µl of 10X PCR Reaction Buffer, and 13.4 µl of nuclease-free water. The PTC-200 DNA Engine was configured to do the PCR reaction at an initial step of 94^{o}C for 3 minutes followed by denaturation at 94^{o}C for 45 seconds, annealing at 55^{o}C for 30 seconds, and extension at 72^{o}C for 90 seconds. Denaturation, annealing, and extension were repeated 40 times, followed by a final extension step at 72^{o}C for 10 minutes.

The final PCR products were analyzed by 1% agarose gel electrophoresis in Tris/Acetic Acid/EDTA (TAE) buffer stained with ethidium bromide. The Power Pac 300 power supply (Bio-Rad Laboratories Inc., Philadelphia, PA) at 75 V for 30-40

minutes was used for electrophoresis. The GeneRuler 100 bp Plus DNA ladder (Fermentas Inc., Glen Burnie, MD) was used as a size standard. DNA bands were visualized using the Gel Logic 100 High Performance UV trans-illuminator (UVP LLC, Upland, CA) and photographed using the Kodak 1D v. 3.6.3 imaging system (Kodak Scientific Imaging Systems, New Haven, CT).

Statistical Analysis. For the greenhouse experiments, the mixed procedure in SAS (v. 9.3, SAS Institute, Cary, NC) was used for analysis. The response variable was ln transformed optical density. Fixed effects were cultivar, treatment, days after sowing, leaf section, and all possible interactions. There were no significant interactions between leaf section and other factors, so the interaction terms were removed from the model. A random effect for plant (nested within treatment and cultivar) was included in the model to account for repeated measures on plants (across days after sowing and sections). The model allowed for different estimated variance at different days after sowing points. Significant differences between cultivar, treatment, and leaf section means were determined using Tukey-Kramer adjustments at 0.05 probability. With the 2010 field data, *Iris yellow spot virus* titer distribution among leaves of pre-bulb and post-bulb plants was analyzed using the mixed procedure in SAS with ln transformed optical density as the response variable. No statistical analysis was done for sheath, bulb scale, basal plate, or root tissues (of pre-bulb plants) because none of the tissues (except for the sheath of Leaf_1) was positive for the virus. Logistic regression used the glimmix procedure in SAS to determine the frequency of virus detection. The binary response variable was virus infection (present or absent). With the 2011 field data, the distribution of *Iris yellow spot virus* among leaves and between leaf sections was analyzed using the mixed procedure in SAS. The response variable was ln transformed optical density. Fixed effects were leaf age, section (top, middle, base, and neck_1), and all interactions. A random effect for plant was included in the model to account for repeated measures. The model allowed for different estimated variance for different leaf sections. As in 2010, bulb scale, basal plate, neck_2, and root tissues were negative for the virus and not included in the statistical analysis. Pair-wise comparisons were calculated and Tukey-Kramer adjustment at $P \leq 0.05$ was applied. Logistic regression used the glimmix procedure in SAS. The binary response variable was virus infection status. Model terms were leaf age and section (top, middle, base, and neck_1), leaf age*section interaction, and plant. In 2010 and 2011, leaves were categorized by age into older, intermediate, and younger groups to determine virus distribution and titer among the age categories.

Results

Temporal Dynamics of *Iris Yellow Spot Virus* Titer in Infected Onion Plants. There was a significant treatment difference ($P < 0.0001$) but no significant cultivar difference ($P = 0.1224$). There was a significant day after sowing difference ($P < 0.0001$) as well as significant day after sowing*cultivar ($P < 0.0001$) and day after sowing*treatment ($P < 0.0001$) interactions. There was no significant cultivar*treatment interaction ($P = 0.1604$), however, there was a significant day after sowing*cultivar*treatment interaction ($P < 0.0001$). In the interactions, there was no significant cultivar difference on all days after sowing for all treatments ($P > 0.05$) except at 89 and 117 days (Fig. 3). At 89 days after sowing, virus titer in thrips+*Iris yellow spot virus* and *Iris yellow spot virus* only was significantly greater in Colorado 6 than Talon, with P values of 0.0288 and 0.0146, respectively. At 117

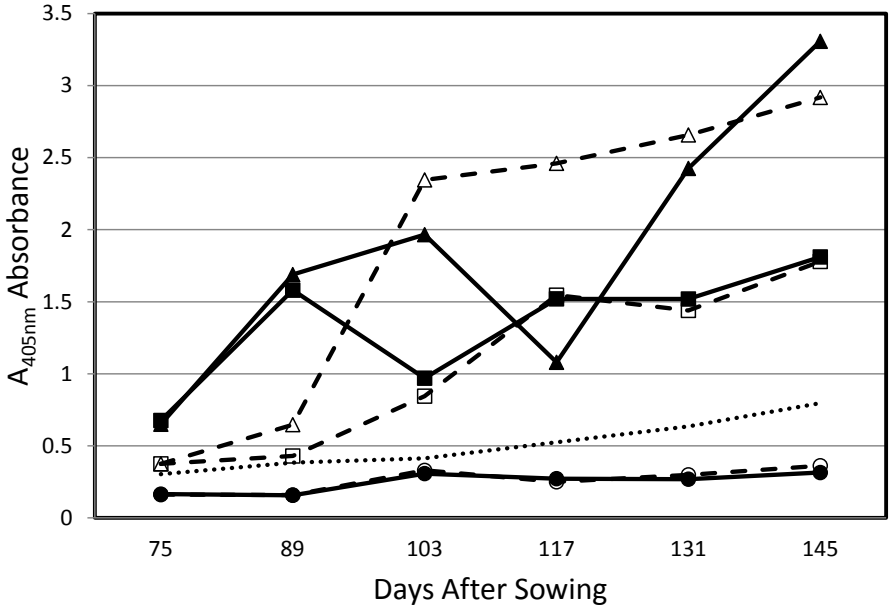

Fig. 3. Graphical representation of the titer of *Iris yellow spot virus* in leaf tissues of Colorado 6 (solid lines and closed symbols) and Talon (broken lines and open symbols) onion cultivars with three treatments; healthy check (●/○), *Iris yellow spot virus* only (■/□), and thrips+*Iris yellow spot virus* (▲/△) as determined by DAS ELISA. Samples were positive (round dot line) for *Iris yellow spot virus* if the absorbance value at 405 nm was equal or greater than 2X that of a negative check.

days after sowing, virus titer in thrips+*Iris yellow spot virus* was significantly greater in Talon than Colorado 6 ($P < 0.0001$), however, there was no significant difference between the two cultivars in *Iris yellow spot virus* only ($P = 0.6208$). In Colorado 6, virus titer was significantly greater in thrips+*Iris yellow spot virus* than in *Iris yellow spot virus* only from 103 days after sowing ($P = 0.0022$) to the end of the study ($P < 0.0001$) except at 117 days, at which time there was no significant difference between the two treatments ($P = 0.7598$). Similar to Colorado 6, virus titer in thrips+*Iris yellow spot virus* in Talon was consistently and significantly greater than in *Iris yellow spot virus* only from 103 days after sowing ($P = 0.0194$) to the end of the study period ($P < 0.0001$). In Colorado 6 and Talon, both thrips+*Iris yellow spot virus* and *Iris yellow spot virus* only reached and maintained positive virus status from 75 days after sowing to the end of the study.

The three leaf sections were significantly different; however, all interactions involving leaf section were not significantly different. Virus titer was significantly greater in base (optical density = 2.065) than middle (optical density = 1.813) ($P = 0.0003$) or top (optical density = 1.854) ($P = 0.0002$) sections, however, there was no significant difference between middle and top sections ($P = 0.9662$). In general, virus titer increased in all treatments, leaf sections, and cultivars over time (Fig. 3).

Distribution of *Iris Yellow Spot Virus* in Leaves of Naturally Infected Onion Plants. For pre-bulb plants, there was no significant difference in virus titer between the different leaves in 2010 (P = 0.5527), but in 2011, there were significant differences in titer between the leaves (P < 0.0001) (Table 1). Virus titer increased with leaf age until Leaf 3 and then was less in younger leaves in 2011. The frequency of virus detection followed a similar trend in 2010 and 2011 in which frequency increased from Leaf 1 to 5 and then was less in younger leaves. However, in 2010, there were no significant differences in detection frequency (P = 0.1140) between the leaves. Frequencies were significantly different in 2011 (P < 0.0001), and increased from 55% in Leaf 1 to 90% in Leaf 5 and then decreased.

Although distribution of *Iris yellow spot virus* among the three leaf-age categories indicated a similar trend in which virus titer was greater in younger, then older and intermediate leaves in 2010 and 2011, there was no significant difference between the categories, with P values of 0.4466 and 0.4353, respectively, in 2010 and 2011 (Table 1). Similarly, frequency of detection followed the same trend in both years in which frequency was significantly greater in intermediate than older or younger leaves but not significantly different between older and younger leaves.

Iris yellow spot virus was not detected in all bulb scale, basal plate, or root tissues in 2010 and 2011. Virus was not detected in the sheath tissues of all leaves in 2010, except for Leaf 1 with 57% detection. In 2011, *Iris yellow spot virus* was not detected in neck_2 but was detected in several neck_1 tissues.

Table 1. Distribution of *Iris Yellow Spot Virus* as Determined by DAS ELISA in Leaves of Naturally Infected Pre-bulb Onion Plants from a Field in Colorado in 2010 and 2011

	Optical density (A405 nm)[w]		Frequency of IYSV detection (%)		Number of plants tested	
Leaf age[t]	2010	2011	2010	2011	2010	2011
[x]1	0.998a	0.750a	48a	55a	21	60
[x]2	1.211a	0.855ab	52a	57a	21	60
[x]3	1.463a	1.259c	62a	85bc	21	60
[y]4	1.495a	1.156bcd	81a	77b	21	60
[y]5	1.110a	1.049abcd	81a	90c	21	60
[y]6	1.223a	0.911abd	57a	68ab	21	60
[z]7	1.124a	0.839ab	57a	67ab	21	51
[z]8	1.071a	1.083abcd	45a	54a	20	26
Older	1.224a	1.008a	54a	66a	63	180
Intermediate	1.276a	1.046a	73b	78b	63	180
Younger	1.098a	0.902a	51a	62a	41	77

[t]Leaf age from oldest (Leaf 1) to youngest (Leaf 8). Optical density values were ln transformed before use in statistical analysis, and means were separated by Tukey-Kramer tests at $P \leq 0.05$. Leaves with the same letter(s) in a column are not significantly different. For frequency of *Iris yellow spot virus* detection, means were separated by Chi square tests in which means with the same letter(s) in a column are not significantly different at $P \leq 0.05$. Leaves were categorized into three groups in which x, y, and z represented older, intermediate, and younger leaves, respectively. Category means were separated by Tukey-Kramer and Chi square tests for optical density and detection frequency, respectively. Categories with the same letter(s) in a column are not significantly different at $P \leq 0.05$.

In post-bulb plants, *Iris yellow spot virus* titer and detection frequency were significantly different among the leaves, with $P < 0.0001$ and 0.0003, respectively. However, there was no apparent trend in titer or frequency of detection (Table 2). With the three leaf-age categories, virus titer was significantly greater in younger than older leaves ($P = 0.0095$), but the two categories were not significantly different from that in intermediate leaves. Titer was greater in younger, then intermediate, and older leaves, with optical densities of 2.778, 2.649, and 2.349, respectively. Frequency of virus detection was almost the same in older and intermediate leaves ($P = 0.7217$) but frequencies in both categories were significantly greater than in younger leaves.

Distribution of *Iris Yellow Spot Virus* in Leaf Sections of Naturally Infected Pre-bulb Onion Plants. Among the different leaf sections, virus titer increased from top, to base, middle, and neck_1, with optical densities of 0.881, 1.010, 1.034, and 1.184, respectively. However, there was no significant difference between the leaf sections ($P = 0.9039$); instead, there were significant leaf age*section interactions ($P = 0.0024$). In the interactions (Fig. 4), there were no significant section differences in Leaves 1, 4, 5, 7, and 8. In Leaf 2, virus titer was significantly greater in neck_1 than top sections ($P = 0.0025$), but all other interactions

Table 2. Distribution of *Iris Yellow Spot Virus* in Leaves as Determined by DAS ELISA of Naturally Infected Post-bulb Onion Plants from a Field in Colorado in 2010

Leaf age[t]	Optical density ($A_{405\ nm}$)[w]	Frequency of IYSV detection (%)	Number of plants tested
[x]1	2.020c	95a	20
[x]2	2.120bc	95a	20
[x]3	2.314abc	65b	20
[x]4	2.478abc	95a	20
[x]5	2.857abc	85ab	20
[y]6	2.526abc	100a	20
[y]7	2.966ab	100a	20
[y]8	2.996ab	90ac	20
[y]9	1.871c	65b	20
[z]10	2.351abc	70bc	20
[z]11	3.225a	70bc	20
[z]12	3.101ab	67bc	18
[z]13	2.372abc	60b	15
Older	2.349a	87a	100
Intermediate	2.649ab	89a	80
Younger	2.778b	67b	73

[t]Leaf age from oldest (Leaf 1) to youngest (Leaf 13). Optical density values were ln transformed before use in the statistical analysis, and means were separated by Tukey-Kramer tests at $P \leq 0.05$. Leaves with the same letter(s) in a column are not significantly different. For frequency of *Iris yellow spot virus* detection, means were separated by Chi square tests in which means with the same letter(s) in a column are not significantly different at $P \leq 0.05$. Leaves were categorized into three groups in which x, y, and z represented older, intermediate, and younger leaves, respectively. Category means were separated by Tukey-Kramer and Chi square tests for optical density and detection frequency, respectively. Categories with the same letter(s) in a column are not significantly different at $P \leq 0.05$.

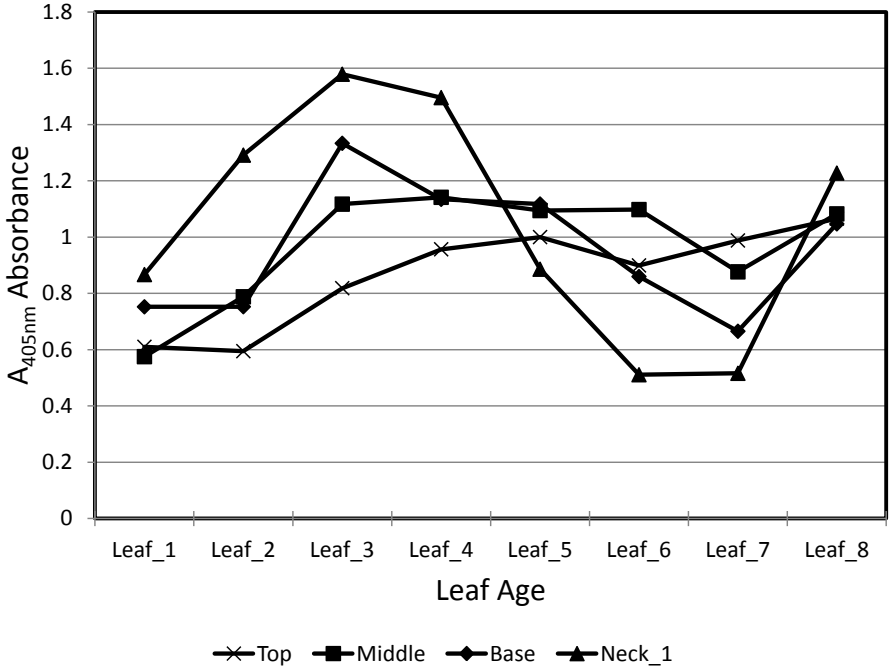

Fig. 4. Graphical representation of *Iris yellow spot virus* titer in leaf tissues as determined by DAS ELISA of naturally infected pre-bulb plants from a field in Colorado in 2011. Samples were positive for *Iris yellow spot virus* if the absorbance value at 405 nm was equal or greater than 2X that of a negative check. The graph represents only samples positive for *Iris yellow spot virus*. All non-green plant tissues tested negative for *Iris yellow spot virus* and were not shown.

were not significantly different. In Leaf 3, virus titer in top sections was significantly less than in base (P = 0.0318) or neck_1 (P = 0.0036) sections; no other interactions were significantly different. Virus titers in middle sections were significantly greater than in neck_1 (P = 0.0180) in Leaf 6; all other interactions were not significantly different.

The frequency of virus detection was not significantly different among the leaves for middle (P = 0.4918), base (P = 0.4406), or neck_1 (P = 0.3125) sections. For top sections, frequency increased with leaf age such that Leaf 1 had the least and Leaves 7 and 8 the most (Table 3). For the three leaf categories, detection frequency was not significantly different for middle (P = 0.1169) and base (P = 0.2589) sections. For top sections, frequency was not significantly different between older and intermediate leaves but both were significantly less than in younger leaves. For neck_1, frequency was significantly greater in older than intermediate or younger leaves. Overall, the average frequencies between the leaf sections was significantly different (P = 0.0018), and decreased from 32% in leaf tops to 24, 25, and 19% in middle, base, and neck_1 tissues, respectively.

Table 3. Frequency of *Iris Yellow Spot Virus* Detection in Leaf Sections as Determined by DAS ELISA of Naturally Infected Pre-bulb Onion Plants from a Field in Colorado in 2011

	Frequency of IYSV detection (%)				Number of plants
Leaf age[t]	Top	Middle	Base	Neck_1	tested
[x]1	17a	19a	32a	20a	60
[x]2	26a	25a	24a	24a	60
[x]3	20a	25a	28a	26a	60
[y]4	24a	28a	28a	18a	60
[y]5	27ac	29a	31a	13a	60
[y]6	30ac	32a	18a	12a	60
[z]7	50b	21a	17a	12a	51
[z]8	47bc	13a	20a	20a	26
Older	21a	23a	28a	23a	180
Intermediate	26a	29a	26a	14b	180
Younger	49b	18a	18a	16ab	77
Average	32a	24b	25b	19c	-

[t]Leaf age from oldest (Leaf 1) to youngest (Leaf 8). Frequency means were separated by Chi square tests in which means with the same letter(s) in a column are not significantly different at $P \leq 0.05$. Leaves were categorized into three groups in which x, y, and z represented older, intermediate, and younger leaves, respectively. Category means were separated by Chi square tests in which categories with the same letter(s) in a column are not significantly different at $P \leq 0.05$. Leaf section averages were separated by Chi square tests in which sections with the same letter(s) are not significantly different at $P \leq 0.05$.

Isolated/patchy detections (Table 4) in only one section per leaf (single) occurred 62% in top, 4% in middle, 22% in base, and 12% in neck_1 sections. Detection in two adjacent sections (double) was greatest in top-middle, followed by base-neck_1, and middle-base sections with frequencies of 42, 33, and 25%, respectively. Top-middle-base sections had 74% triple detections and middle-base-neck_1 sections had 26%. Detections with at least one negative section between two positive sections (spaced detections) occurred 17 times and *Iris yellow spot virus* was detected in all sections (entire leaf) of 103 leaves.

Discussion

Plant resistance is an important component in integrated management of plant pathogens. In infections, virus replication rapidly increases and then subsides in host plants. Although this occurs in all plant materials, tolerant hosts are better able than susceptible hosts to capitalize on the process to reduce virus (Maule et al. 2000). This phenomenon might explain the increase and decrease of virus titer in Colorado 6, while titer in Talon continued to rise throughout the study (Fig. 3). This feature, however, did not result in significant differences in titer between the two cultivars. At 89 days after sowing, leaves of Talon were larger than leaves of Colorado 6. At 117 days after sowing, the sampled leaves of Colorado 6 were much bigger than those of Talon in the thrips+*Iris yellow spot virus* treatment. Differences in titer:weight ratio might explain the observed titer differences between the two cultivars at those dates. The loss of the field tolerance of Colorado 6 in the

Table 4. *Iris Yellow Spot Virus* Detection in Isolated Sections in Leaves as Determined by DAS ELISA of Naturally Infected Pre-bulb Onion Plants from a Field in Colorado in 2011

	Single				Double			Triple			
Leaf age[t]	T	M	B	N	TM	MB	BN	TMB	MBN	SD	EL
[x]1	0	0	1	3	0	0	9	8	0	0	9
[x]2	5	0	2	7	3	5	2	5	2	2	22
[x]3	1	0	2	2	3	4	12	1	5	4	23
[y]4	0	0	5	0	4	2	3	15	7	5	12
[y]5	4	2	9	0	8	3	0	11	4	3	11
[y]6	19	2	0	0	11	4	0	9	0	2	11
[z]7	25	0	0	0	4	2	0	3	0	1	10
[z]8	8	0	3	0	0	0	0	0	0	0	5
Total	62	4	22	12	33	20	26	52	18	17	103
Frequency (%)	62	4	22	12	42	25	33	74	26	-	-

[t]Leaf age from oldest (Leaf 1) to youngest (Leaf 8). Leaves were categorized into older (x), intermediate (y), and younger (z) groups. T, M, B, and N represent top, middle, base, and neck_1 leaf sections, respectively. Single, double, and triple indicate the number of times *Iris yellow spot virus* was detected once, twice, or three times in adjacent leaf section(s). SD = spaced-detection (number of *Iris yellow spot virus* detections with at least one negative section between positive sections). EL = entire leaf (*Iris yellow spot virus* detection in all leaf sections, excluding neck_2 sections).

study was manifested in virus titer and also yield. This was because an experiment ancillary to the one reported here investigated the effects of the treatments on yield of onion bulbs. Results indicated that while there were significant treatment differences, there was no significant yield difference between Colorado 6 and Talon during the 2 years of the experiment (Boateng 2012). As mentioned, sources of resistance to the *Iris yellow spot virus* pathosystem are yet to be identified and use of Colorado 6 was because of tolerance to *Iris yellow spot virus* in Colorado. Its field tolerance might have been lost in this study because of the enclosed and high pathogen pressure of the study. The overwhelmingly significant difference in virus titer between thrips+*Iris yellow spot virus* and *Iris yellow spot virus* only in both cultivars throughout the study cycle (Fig. 3) is a further emphasis on the indispensability of vector control in integrated management of vector-borne diseases, especially in this pathosystem in which the virus is transmitted and disseminated solely by the vector. The amount of *Iris yellow spot virus* was greatest in both cultivars at 145 days after sowing (the last sampling day) indicating that virus replication continued as long as a susceptible host and physiologically active tissues were available. This was also observed in field plants in which virus titer was greater in post-bulb than pre-bulb plants (Tables 1 and 2).

Unlike most tospoviruses, *Iris yellow spot virus* causes localized infection in which the virus moves less systemically (Bag et al. 2009, Pappu et al. 2009). If systemic movement is expected, virus titer in one leaf section would progressively decrease while another leaf section increased during the same time (Ueki and Citovsky 2006). Plant viruses are biotrophic pathogens and it would be expected that as onion leaves age and senesce from leaf tips toward bases, virus titer should accordingly move ahead of the senescing front. This should result in less titer in

leaf tops and increases in other leaf sections, especially during the latter part of the season. In the *Iris yellow spot virus*-onion thrips-onion pathosystem, intimate association of the virus with thrips vectors is the main way virus is introduced and dispersed. Onion thrips reside in deep, tight folds at leaf bases to escape predators. This habit has been attributed as a basic association for inner leaves and leaf bases having greatest *Iris yellow spot virus* titers (Kritzman et al. 2001, Cranshaw 2008). Thus, virus build up in these sites is a result of replication in the vector and multiple inoculations via vector feeding as well as *in planta* replication. Cell-to-cell movement through plasmodesmata rather than through the phloem might explain some of the virus movement in infected plants. However, this movement is limited because only 8-10 cells (1 mm) can be travelled in a day (Agrios 2005).

Fig. 3 depicts the temporal phenomenon of *Iris yellow spot virus* titer and distribution, while Fig. 4 is a demonstration of within-tissue dynamics at a moment in time during the season. It represents virus distribution on a single sampling date that might occur on Fig. 3 under field conditions. In all leaves tested, neck_1 contained some green pigmentation (chlorophyll) (Fig. 1). Because mesophyll cells contain plant chlorophyll (Campbell and Reece 2008) and onion thrips feed on mesophyll cell contents (Dai et al. 2009, Ueki and Citovsky 2006), the *Iris yellow spot virus*-positive status of neck_1 could be caused by direct virion deposition resulting from vector feeding in the section. The fact that all non-green tissues tested negative suggested onion thrips feed only on chlorophyll-containing cells. The rare occurrence or complete absence of *Iris yellow spot virus* in onion bulbs, basal plates, and roots could also be explained by lack of vector feeding in these tissues. This also explains the *Iris yellow spot virus*-positive status of the neck region of Leaf 1 in pre-bulb plants in 2010. Early-season leaves of pre-bulb plants are usually shorter with smaller sheath sections than later-season leaves. This usually resulted in the entire sheath section being used for virus detection. In bigger leaves, sheath samples were taken more toward the basal plate (chlorophyll-free sections) and explains why *Iris yellow spot virus* was not detected in the sheaths of Leaves 2-8 in 2010. This observation prompted us to divide sheath segments into two sections in 2011.

The absence of virus in dead leaves and bulb scales could be caused by the biotrophic pathogenicity of viruses because they require live host cells to provide the physiological machinery for replication. If infection occurred early in the season, it is conceivable the dead leaves and scales were once inhabited by virus. An efficient system would be required to move virus from old dying leaves to younger ones to prevent virus perishing with dying tissues. It is conceivable that in pre-bulb plants, there was no significant difference in virus titer between the three leaf age groups (Table 1). As mentioned, *Iris yellow spot virus* localization in plants is a function of viruliferous vector feeding and the timing of incidence. Thus, all leaves of young plants were fresh and succulent enough to presumably attract almost equal vector feeding. In post-bulb plants, however, younger leaves would be the most preferred feeding sites for the vector because older leaves would be too fibrous or senescing (Brewster 1994). This feeding habit resulted in the observed significant difference in titer between older and younger leaf categories in advanced plants. Results are consistent with that of Kritzman et al. (2001) who found most virus titers in leaf segments close to onion bulbs and in inner (younger) leaves. It should be mentioned that the association between high *Iris yellow spot virus* titers and thrips location might be coincidental and must be experimentally evaluated.

On symptomatic leaves, samples from regions surrounding the symptom would probably provide a reliable indication of *Iris yellow spot virus* in plants. In symptomless plants, however, the studies provided information on which leaves and what sections of leaves (Tables 1-4) are most likely to enhance detection of *Iris yellow spot virus*. This information is useful in situations where large samples of symptomless plants (e.g., Diaz-Montano et al. 2010) need to be tested and using all samples would be expensive and time consuming. For field sampling for detection of *Iris yellow spot virus*, Leaves 3-5 of pre-bulb plants probably would provide a good indication of virus because besides having the greatest titer, 62-90% of the plants sampled, would be positive for the virus if the plants are infected (Table 1). In post-bulb plants, samples can be taken from younger leaves. It should be noted, however, that although titer is greatest in younger leaves, frequency of detection is least (Table 2). Thus, sampling size should be adjusted to enhance the frequency of detection.

Information on virus distribution within infected plants is a vital resource in studies of *Iris yellow spot virus*, especially in detecting infected but symptomless plants. The information also is important in quarantine control of *Iris yellow spot virus* because symptomless plant materials are frequently moved across state and international borders. Although there is lack of a distinct trend in virus distribution among leaves of infected plants, the detailed information provided by this study will aid in faster, more efficient, and more reliable evaluation of plant materials to ascertain *Iris yellow spot virus* in evaluations of germplasm, epidemiological studies, and quarantine controls.

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