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The Quest to Identify a New Virus Disease of Sunflower from Nebraska

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Abstract

Between 2010 and 2018, sunflower plants exhibiting virus-like symptoms, including stunting, mottling, and chlorotic ringspots on leaves, were observed from commercial fields and research plots from four sites within three distinct counties of western Nebraska (Box Butte, Kimball, and Scotts Bluff). Near identical symptoms from field samples were reproduced on seedlings mechanically in the greenhouse on multiple occasions, confirming the presence of a sap-transmissible virus from each site. Symptomatic greenhouse-inoculated plants from the 2010 and 2011 Box Butte samples tested negative for sunflower mosaic virus (SuMV), sunflower chlorotic mottle virus (SuCMoV), and all potyviruses in general by ELISA and RT-PCR. Similar virallike symptoms were later observed on plants in a commercial sunflower field in Kimball County in 2014, and again from volunteers in research plots in Scotts Bluff County in 2018. Samples from both of these years were again successfully reproduced on seedlings in the greenhouse as before following mechanical transmissions.

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Symptom expression for all years began 12 to 14 days after inoculation as mild yellow spots followed by the formation of chlorotic ringspots from the mottled pattern. The culture from 2014 tested negatively for three groups of nepoviruses via RT-PCR, ruling this group out. However, transmission electron microscopy assays of greenhouse-infected plants from both 2014 and 2018 revealed the presence of distinct, polyhedral virus particles. With the use of high throughput sequencing and RT-PCR, it was confirmed that the infections from both years were caused by a new virus in the tombusvirus genus and was proposed to be called *Sunflower ring spot mottle virus* (SuRSMV). Although the major objective of this project was to identify the causal agent of the disease, it became evident that the diagnostic journey itself, with all the barriers encountered on the 10-year trek, was actually more important and impactful than identification.

Keywords: oilseeds and legumes, pathogen detection, sunflower, sunflower virus, Tombusvirus, viruses and viroids

Identifying the causal agents of newly discovered plant diseases can be among the most formidable—but stimulating—challenges in a plant pathologist's career. Laboratory skills, communication skills, and stubborn persistence may all be called upon. Below, we relate the story of one such adventure.

The common sunflower, *Helianthus annuus*, is an important field crop in the U.S., grown on approximately 1.5 million acres (600,000 ha) in 2020. Sunflower has multiple uses including oil, confection, and ornamentals. Cropping of sunflowers is highly important for the economy of production farming systems throughout the Great Plains, stretching from Texas in the south to the Dakotas in the north. Compared with other major crops such as soybeans and corn, roughly 90 million acres (36 million ha) each, and wheat (47 million acres, 19 million ha) in 2021, sunflower acreage is minuscule, yet its production was valued at almost $600 million in 2020. The vast majority of sunflower production (95%) occurs in North Dakota, South Dakota, Minnesota, Kansas, Nebraska, Colorado, and Texas (Gulya et al. 2019) (**Fig. 1**).

Historically, Nebraska's cultivated acres have fluctuated greatly, but the sunflower has still served as an effective alternative crop in dryland wheat/fallow rotations for many systems. A decade ago, in the panhandle of western Nebraska, sunflowers were also being increasingly used to lengthen the traditional irrigated rotations of dry beans, corn, and sugar beets. Acreage in 2010 was up to 65,000 (26,000 ha), a 25% increase over 2009. It was assumed that as production in the

Fig. 1. Flowering sunflower field in western Nebraska. a new, unknown obligate

state continued to increase, the potential for disease problems would also increase. It was also thought that the two types of production—irrigated and rain-fed (dry-land)—could experience different disease problems due to the different environments for each. Consequently, the first extensive disease surveys of Nebraska production sunflower fields were conducted over the 2009 through 2011 seasons with the purpose of identifying the most prevalent diseases and establishing their relationships with crop growth stage (early season, late system, etc.) and disease distribution in both irrigated and dry-land fields (Harveson, *unpublished*).

As a result of these disease surveys, a suspected virus disease was uncovered in two of the three years of the study. During both 2010 and 2011, plants possessing almost identical symptoms characteristic of viral infection (stunting, yellow ring spots, and chlorotic mottle-type leaf patterns) were noted in mid-July from two distinct commercial fields in Box Butte County of western Nebraska (one each year), and fields were separated by approximately 25 to 30 miles (**Fig. 2**).

Historical Background of Sunflower Viruses

More than three dozen diseases are known to affect sunflowers. However, reports of the natural occurrence of virus diseases on

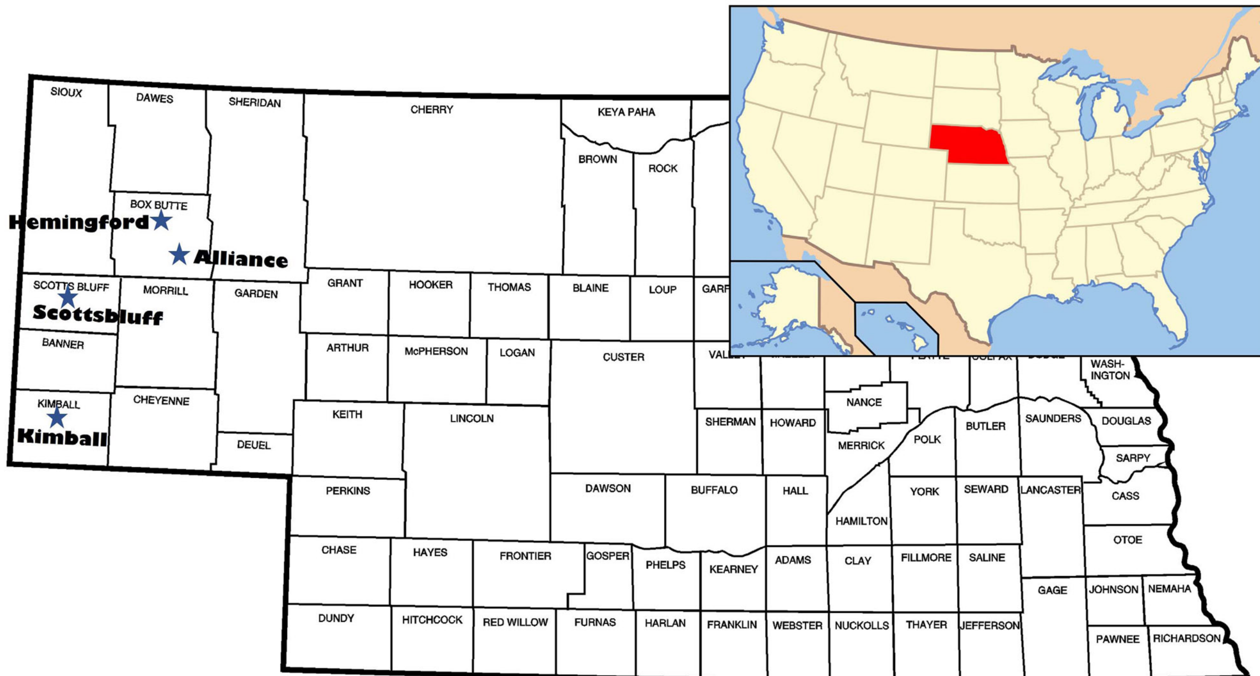


Fig. 2. Map exhibiting the four locations in the western panhandle of Nebraska where the disease was recognized (stars). Hemingford (2010), Alliance (2011), Kimball (2014), and Scottsbluff (2018).

sunflowers worldwide have been rare (Gulya et al. 1997). The ilarvirus *Tobacco streak virus* has caused significant damage and yield loss in Queensland, Australia, and regions of India and Iran (Sharman et al. 2009, 2015). Other similar diseases attributed to two luteoviruses (*Sunflower yellow blotch virus* and *Leaf crinkle virus*) have also been associated with sunflower production problems in east Africa (Gulya et al. 1997; Lenardon and Harveson 2016).

Conversely, virus diseases have frequently occurred in Argentina sunflower production, becoming potentially problematic issues. The most prevalent and widespread virus reported from Argentina is the potyvirus *Sunflower chlorotic mottle virus*, which has two different strains (common and chlorotic ringspot) (Dujovny et al. 1998; Giolitti et al. 2010). Other viruses identified from natural infections in Argentina include another potyvirus, *Sunflower mild mosaic virus*, and the bromovirus *Pelargonium zonate spot virus*, as well as the infrequently observed *Sunflower ringspot*, presumed to be an ilarvirus (Gulya et al.

2016; Lenardon and Harveson 2016; Markell et al. 2015). A number of additional disorders with virus-like symptoms have been reported from various countries in Asia and Africa but were not formally confirmed with contemporary diagnostic tools.

Curiously, reports of sunflower diseases have been even less common in North America, the apparent site of origin of the cultivated sunflower and the genus *Helianthus*. Until the late 1990s, reports of the natural occurrence of any sunflower virus disease in the U.S. or Canada were limited to single event descriptions of *Cucumber mosaic virus* in USDA research plots in Beltsville, MD, *Sunflower mosaic virus* (SuMV) occurring in wild sunflowers near Austin in central Texas, and *Tobacco ringspot virus* infections from wild sunflowers from the Rio Grande Valley of far south Texas (Arnott and Smith 1967; McLean 1962; Orellana and Quacquarelli 1968).

SuMV is the first and only virus to date that has been exhaustively characterized and found occurring naturally on sunflowers in North America (Gulya et al. 2002). Furthermore, it appears to be restricted to south Texas. In 1997, wild sunflowers with virus-like symptoms were found in an abandoned sorghum field in south Texas. In subsequent years, more symptomatic wild plants were observed from additional fields and road ditches from multiple locations in that region.

Since no virus diseases had previously been documented from sunflower production fields of the central or northern Great Plains, the presence of a rare virus disease sporadically being observed in sunflowers in the southern portion of the U.S. was concerning. The pathogen was identified as SuMV and thoroughly evaluated with studies aimed at the pathogen's host range, virus transmission and purification, and phylogenic assays with other known viruses (Gulya et al. 2002).

New Virus Infections in Nebraska

Between 2010 and 2018, both cultivated and wild/volunteer sunflower plants possessing symptoms characteristic of a virus infection were observed from multiple sites from three different counties in western Nebraska (Harveson, *unpublished*; Lenardon and Harveson 2016) (Fig. 2). Although this disease was not feared as an economically damaging

Box 1 Virus discovery timeline

Survey begins	2009
Field discovery	2010 (Hemingford)
	2011 (Alliance)
	2014 (Kimball)
	2018 (Scottsbluff)
Arrival of collaborators	2010 (Gulya and Karasev)
	2011 (Lenardon and Bradshaw)
	2015 (Tian)
	2016 (Al Rwahnih)
Ruled out SuMV	2010
Ruled out SuCMV	2011
Ruled out all Potyviruses	2011
Characterization studies, yield reductions, seed and insect transmission	2011
Found polyhedral particles	2015 (Kimball field)
	2015 (Scottsbluff alley)
	2016 (Scottsbluff alley)
	2018 (Scottsbluff field)
Ruled out all Nepoviruses	2016
Identified samples as an unknown Tombusvirus (Scottsbluff field culture)	2016 (Kimball field culture) 2018
Pathogen survival studies	2018, 2019, and 2020
Loss of virus cultures in greenhouse	2011, 2013, 2017, and 2020

threat to the industry, this report could still serve as a case study for investigating organism with no previous references for which to relate. The primary goal of this project was to identify the pathogen and record its continual, but infrequent presences within western Nebraska. However, the story of the long arduous odyssey, successfully overcoming obstacles and discouraging failures, is in reality more important and significant than diagnostic classification of the causal agent of the disease (see **Box 1**). We have additionally learned several valuable lessons as outcomes of this scientifically investigational journey, including the positive results of cooperation, melding of modern and classical diagnostic tools, and an avid curiosity.

First Discovery—2010

In mid-July 2010, a group of 10 to 15 plants with symptoms characteristics of a virus disease (**Fig. 3**) was observed within an irrigated,

Fig. 3. Virus-like symptoms on commercial sunflower from 2010 field infection near Hemingford.

confectionary sunflower production field near Hemingford, Box Butte Co., Nebraska (Fig. 2). They were not widely distributed throughout the field, but clustered in a single spot. This location was flagged and then monitored at three additional times over that season.

Affected plants were stunted and never produced normal seed heads, remaining very small (**Fig. 4**). This field had severe weed pressure and in subsequent visits, the infected plants were difficult to

Fig. 4. Virus-infected sunflower plant from first disease discovery from Hemingford in 2010. Note the stunting and delayed head development (arrows) compared with adjacent maturing, uninfected plants in the background.

locate again. In fact, if the pathogen's presence had not previously been known or if this area had not been marked earlier, it is likely that these diseased plants would never have been noticed. Furthermore, the virus-like symptoms faded substantially as the season progressed, and the stunted, symptomatic sunflowers were hidden within chest-high weeds and scattered among the unaffected crop plants (Fig. 4), now reaching six to eight feet in height.

Virus Transmission and Culture Maintenance

Mechanical transmission was successfully performed (based on symptom development) multiple times on sunflower seedlings in the greenhouse from both initial and new field samples obtained in subsequent trips. This proved that the symptoms were caused by some pathogenic agent and not an environmental or abiotic problem. Plastic pots were seeded with three to four seeds and placed in plastic trays (12 total pots) for a total of 45 to 50 plants per tray (**Fig. 5**). Inoculations consisted of grinding symptomatic leaves in a buffer and rubbing the solution on cotyledons of the seedlings after dusting with carborundum. The buffer was a 0.01 M phosphate solution with sodium sulfite (1% wt/vol) and polyvinylpyrrolidone (0.25% wt/vol) added as antioxidants,

Fig. 5. Infected sunflower seedlings in greenhouse after inoculations from field samples.

Fig. 6. First symptoms (small chlorotic spots) observed on seedlings in the greenhouse 12 to 14 days after inoculation (left). Symptoms observed after 4 to 6 weeks on the same plant consisting of mottling, ring spots, and line patterns (right).

as previously described for transmitting SuMV by Gulya et al. (2002). After inoculation, plants were additionally sprayed periodically with a growth regulator (B-Nine, daminozide) to avoid the spindly, leggy growth of sunflowers in the greenhouse (Gulya et al. 2002).

It was soon noted that better results were obtained with younger seedlings (emergence of the first true leaves) and after this process was optimized, we consistently achieved up to 80 to 85% of inoculated plants exhibiting symptoms after 12 to 14 days (Fig. 5). Symptoms began on newest leaves as small chlorotic spots (**Fig. 6**, left), followed after 4 to 6 weeks by the formation of mottled patterns becoming ring spots and occasionally, line patterns (Fig. 6, right).

In a similar manner to field infections, symptoms in the greenhouse after artificial inoculation also tended to fade over time, particularly when ambient air temperatures increased (35 to 40°C). Nevertheless, the causal agent was apparently still viable and infectious (**Fig. 7**). Transmission was still possible, but with more difficulty and lower percentages of inoculated plants becoming infected and developing symptoms.

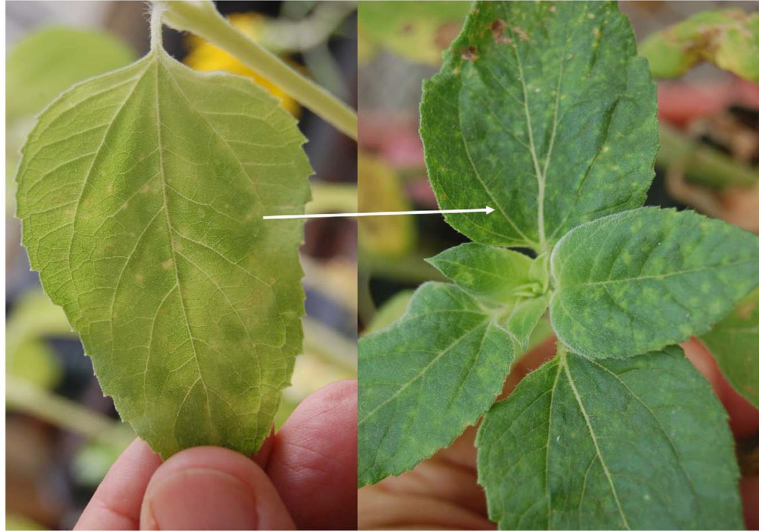


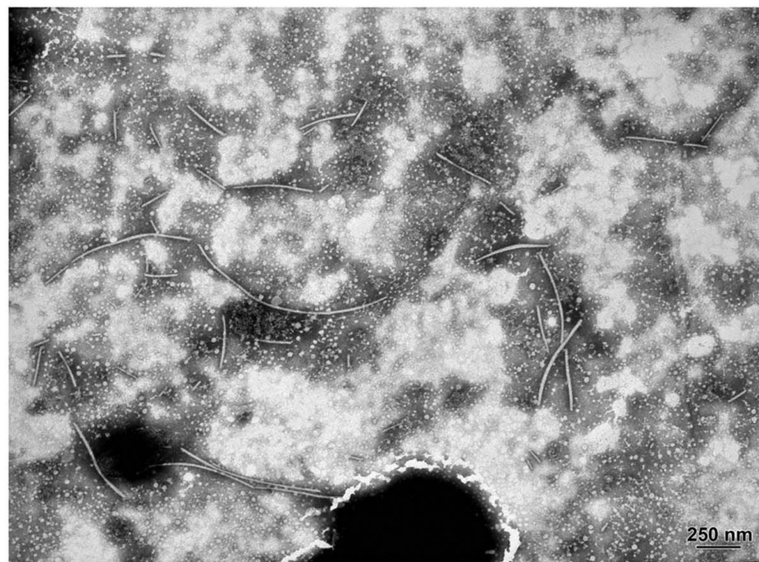
Fig. 7. Fading of ringspot symptoms on leaf of greenhouse-inoculated sunflower plant (left). Symptoms on new sunflower seedling (right) after being inoculated using tissue from the still-infectious leaf on the left.

Furthermore, symptoms on inoculated plants appeared to be more pronounced in the greenhouse during the fall (October to December). Until this pathogen could be identified, we maintained the culture in the greenhouse by continuous transmission events conducted approximately every 2 to 3 weeks (**Fig. 8**) until they were regrettably lost in late spring 2011 due to pest issues, particularly two-spotted spider mites (*Tetranychus urticae*).

Fig. 8. Maintenance of the pathogen in the greenhouse with continuous inoculations at 2- to 3-week intervals.

Diagnostic Efforts and Ruling Out SuMV

Due to the discovery of a biotic cause for the symptoms in sunflowers, we sought interested collaborators and began testing all available measures for the identification of unknown viral infections. Infected plants were sent to Dr. Tom Gulya at the USDA-ARS Northern Crop Science Lab in Fargo, ND. With the assistance of North Dakota State University's electron microscopist, structures were seen via leaf dips from a 2010 field sample that were presumed to be virus rod particles resembling potyviruses (**Fig. 9**). Leaf dips are crude or partially purified samples, utilized for viewing tiny structures with transmission electron microscopy (TEM). These particles appeared to be broken fragments of flexuous virus rods with highly variable lengths. Similar structures were never seen from samples derived from greenhouse transmission in 2010. Nevertheless, this observation provided a starting point for our efforts to identify the causal agent for the sunflower disease. We anticipated finding SuMV eventually as the cause of infection based on similar symptoms with SuMV, flexuous rod fragments observed from field samples, mechanical transmission, and previous reports of the disease in the U.S. (south Texas). All are characteristic of potyviruses such as SuMV.



18134 T. GULYA 58,700x
102832 NEB INFECTED SUNFLOWER 8/24/2010

Fig. 9. Leaf dip sample from initial Hemingford field infection in 2010 viewed with transmission electron microscopy (TEM). Note the slender fragments of varying lengths.

Mechanical transmission of the virus was achieved in the greenhouse three to four times that season with sunflower seedlings, based on symptoms. Based on results from the previous report of SuMV infections from south Texas (Gulya et al. 2002), we also inoculated zinnias and incubated them in the greenhouse as described for the sunflower seedlings. Gulya et al. found that zinnias were highly susceptible to SuMV, resulting in the production of strong symptoms as a host (Gulya et al. 2002). Virus titers in zinnias were determined to be much higher than those with infected sunflowers, so they theoretically should have served as excellent sentinel plants for detection of the virus. After treating the zinnias with the same inoculation procedures as the sunflowers, no definitive symptoms were ever noted, suggesting that zinnias were not a good host for our unknown pathogen.

As another result of the Gulya et al. (2002) characterization study of SuMV, antiserum for SuMV had been created and archived in freezers at the University of Idaho in Moscow, ID. Four samples of inoculated zinnias and symptomatic sunflowers were sent to Dr. Alexander Karasev in Idaho in November 2010 for further SuMV testing. The use of Western blots with this antiserum did not detect any expected bands for either the zinnias or sunflowers. Furthermore, with primers designed for SuMV, tests with reverse transcription (RT)-PCR were also conducted with no reactions toward SuMV from any of the zinnia or sunflower samples submitted. Thus, SuMV had convincingly been ruled out as the causal agent of this disease.

Another Discovery—2011

In mid-July 2011, within another irrigated, confectionary sunflower production field in Box Butte Co. near Alliance (Fig. 2), plants exhibiting near-identical symptoms to those from 2010 were noted with yellow blotches on leaves forming into ringspots (**Fig. 10**). As in 2010, incidence again was very low with symptomatic plants distributed randomly in several clusters of four to five plants each. This spot in the field, as well as individual symptomatic plants, were tagged and the field was monitored four more times that season before harvest. Mechanical transmission of the pathogen was successfully achieved once

Fig. 10. Virus-like symptoms on commercial sunflower from the Alliance 2011 field infection.

again on sunflower seedlings from tissue of symptomatic plants from this field and maintained in the greenhouse by continuous transmission as previously described. Late in the season (mid-September), the tagged, affected plants displayed conspicuous yellow rings on upper leaves (**Fig. 11**), and as before, symptoms faded over time.

Fig. 11. Symptomatic plants late in the season with faded yellow ringspots from the 2011 field infection near Alliance.

Diagnostic Efforts and Ruling Out SuCMV

Tests were again attempted with leaf dips from symptomatic leaves and viewed with TEM in the same manner as that from the 2010 infections. However, no evidence of potyvirus particles or fragments was detected this time, only a few circular structures that could possibly have been polyhedral-like viral particles.

Based on reports from South America, we learned of a new sunflower potyvirus, *Sunflower chlorotic mottle virus* (SuCMV), that was widespread in the production areas of Argentina and produced symptoms very similar to the ones from the unknown Nebraska infections (**Fig. 12**) (Dujovny et al. 1998). Contacts were made with a virologist (Dr. Sergio Lenardon) from the CIAP-INTA in Cordoba, Argentina, who was willing to cooperate with us in the identification of this assumed virus pathogen. NCM-ELISA was utilized with antiserum for SuCMV and nitrocellulose membranes (NCM). Symptomatic leaf tissues from plants inoculated directly from field infections, and plant tissues from one and two sequential transmissions from the initial transmission were rolled into cylindrical bundles, cut at one end, and squeezed, and the exuded sap was blotted onto membranes and allowed to dry. Healthy, nonsystematic sunflower plants were additionally treated in the same manner for controls. Blotted membranes were

Fig. 12. Chlorotic ringspot symptoms of Sunflower chlorotic mottle virus (SuCMV) from Argentina on sunflower leaves. (Courtesy of S. Giolitti and S. L. Lenardon.)

sent to Lenardon's lab and assayed by NCM-ELISA for SuCMV. These tests were negative for SuCMV from all samples sent to Argentina from Nebraska.

The samples additionally tested negative after assaying in Argentina with ELISA, utilizing monoclonal antibodies targeting all potyviruses in general. This same test was also repeated in Nebraska with the use of potyvirus ELISA kits (Agdia, Elkhart, IN), resulting in identical results and no positive responses or answers for our project goal.

Biological Characterizations

Estimates for potential yield reduction. Severe yield reduction potential was readily seen based on infected plants that survived until harvest (**Fig. 13**) in 2011 from the Alliance field, but we also attempted to roughly validate this observation by obtaining yield reduction estimates from the 2011 field epidemic. The heads of approximately 10 infected (previously tagged) and 10 adjacent noninfected (not stunted, with no foliar symptoms) plants were collected, dried, and shelled. Weights were taken of total seeds per head with averages of 60.4 g (2 oz)/head from infected plants, and 650 g (1.4 lbs)/head from healthy plants.

Fig. 13. Sunflower heads from the Alliance field in September 2011. Note the size of the head taken from the symptomatic plants (left) compared with the head from an adjacent unaffected plant.

Seed transmission? It was difficult to obtain enough viable seeds from infected plants for proper estimates, but after combining matured seeds from the infections of both 2010 and 2011 we were able to retrieve about 1,500 to 2,000 seeds from infected plants. In the effort to establish possible seed transmission of the pathogen, one half of these seeds were then planted in the greenhouse in the same manner as with the virus-inoculated seedlings and observed with healthy plants for comparison. The study was repeated once with the remainder of the seeds from the infected plants. No symptoms or signs of disease were ever observed from any emerged seedlings derived from infected plants after approximately 2 months, suggesting no seed transmission.

Insect transmission? An effort to evaluate possible insect transmission in a nonpersistent manner, characteristic of potyviruses, was also attempted. A colony of yellow sugarcane aphids (*Sipha flava*) was being raised and kept in Scottsbluff by entomologist Dr. Jeff Bradshaw. We chose this species of aphids for testing because they were available and had previously been noted to survive and reproduce on sunflower (Kindler and Dalrymple 1999). Within a cage, an aphid-infested barley leaf containing eight to 10 aphids from the colony was placed in a pot containing symptomatic, greenhouse-inoculated plants. Another pot with healthy plants was also placed next to the one containing symptomatic plants within the cage. Pots were watered as required and incubated within cages in the greenhouse for approximately 7 days. Pots were then removed from the cage and monitored for the appearance of any virus-like symptoms on the healthy plants. This effort was repeated once with no symptom development for any plants from either test. Furthermore, no evidence for virus transmission by sugarcane aphids was procured after assaying plants exposed to aphids with the potyvirus ELISA method once again.

At this point, we still did not know what we had, but we did know what we did not have. We now were certain that the disease was not caused by SuMV, SuCMV, nor any other potyvirus. We continued to maintain the culture from the 2011 infections in the greenhouse by continuous transmission every 3 to 4 weeks for about 18 months. Sadly, this culture was also lost sometime in February of 2013. The process of keeping an obligate organism alive on plant hosts in the greenhouse for extended periods of time is an onerous task. The loss

of cultures after continuously transferring the pathogen for months and sometimes years was a discouraging setback. However, our luck began to change the next year.

New Discovery—2014

In September of 2014, in a low area of a large dry-land, oil-type sunflower field north of Kimball, NE (Fig. 2), a small group (five to six) of stunted sunflower plants was discovered with faint yellow ringspots on leaves (**Fig. 14**). The plants were clustered in the field in a similar manner as the distribution of symptomatic plants in previous seasons.

Fig. 14. Symptomatic plant with faded yellow ringspots late in the season from the Kimball 2014 infection.

The virus-like symptoms of the pathogen from this site were reproduced again by mechanical transmission in the greenhouse on sunflower seedlings, resulting in near-identical symptoms to those from the previous 2010 Hemingford and 2011 Alliance infections. This culture continued to be maintained in the greenhouse with periodic transfers as described previously.

New Collaborators and Tests

In 2015, through our sunflower contacts, an interested virologist (Dr. Tongyan Tian) with the California Department of Food and Agriculture (CDFA) and electron microscopy expertise joined our group to pursue the identity of the mysterious sunflower pathogen. New results with his TEM leaf dip assays revealed polyhedral (icosahedral- shaped) particles, varying in dimensions of 25 to 28 nm, from both the greenhouse-transmitted infections from 2014 and symptomatic sunflower volunteers from a residential home alleyway in Scottsbluff in 2015 (Fig. 2 & **Fig. 15**). The same result was obtained the next summer in 2016 after utilizing leaf dip TEM tests on samples collected from plants exhibiting ringspot symptoms from the same residential alley in Scottsbluff (**Fig. 16**), now suggesting a potential soilborne nature.

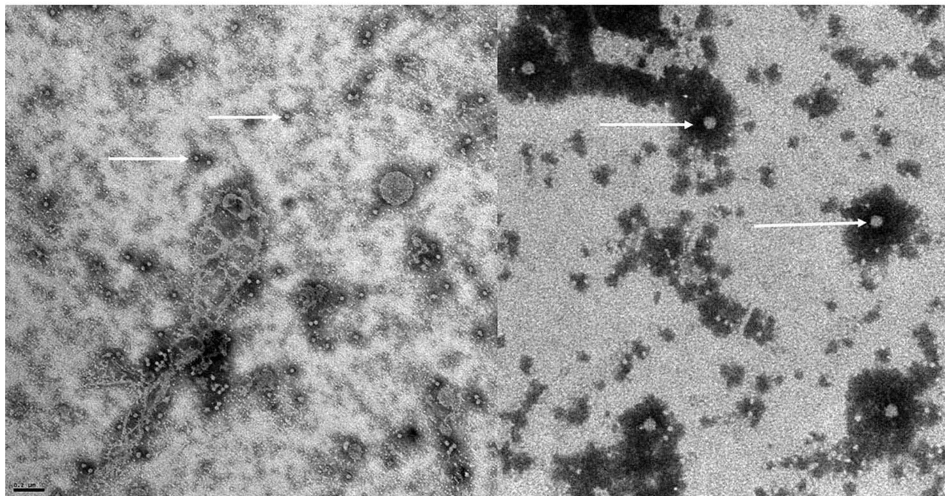


Fig. 15. Polyhedral virus-like particles (note white arrows) viewed from TEM leaf dips in 2015. Samples were derived from a symptomatic volunteer plant leaf from a residential alley in Scottsbluff.

Fig. 16. Sunflower volunteer in 2016 with faded ringspots on upper leaves from the same alley in Scottsbluff as shown in Fig. 15.

At this point, with another culture continuing in the greenhouse for a third time and the discovery of polyhedral-shaped structures within symptomatic plants from both the 2014 Kimball field sample and the alley in Scottsbluff, it was decided to discontinue the flexuous rod hypothesis. We then changed directions and concentrated on investigating the polyhedral particles and search for potential viruses possessing this characteristic. Due to continued observations of the distribution of symptomatic plants randomly in small patches within commercial fields in 2010, 2011, and 2014 and the same Scottsbluff alley in 2015 and 2016, we now considered addressing the possibility of a soilborne pathogen such as a nematode-transmitted nepovirus.

In March of 2016, additional polyhedral particles of similar size were again observed from greenhouse-inoculated cultures from the 2014 Kimball field with partial purification (**Fig. 17**). Furthermore, the 2014 culture samples were assayed by RT-PCR using degenerate primers against three distinct groups of nepoviruses (Digiaro et al. 2007; Wei and Clover 2008). However, no amplification was ever obtained from either the alley or Kimball 2014 infections, which then ruled out the possibility of a nepovirus as the cause of this disease. Frustratingly, we still did not know the identity of the pathogen.

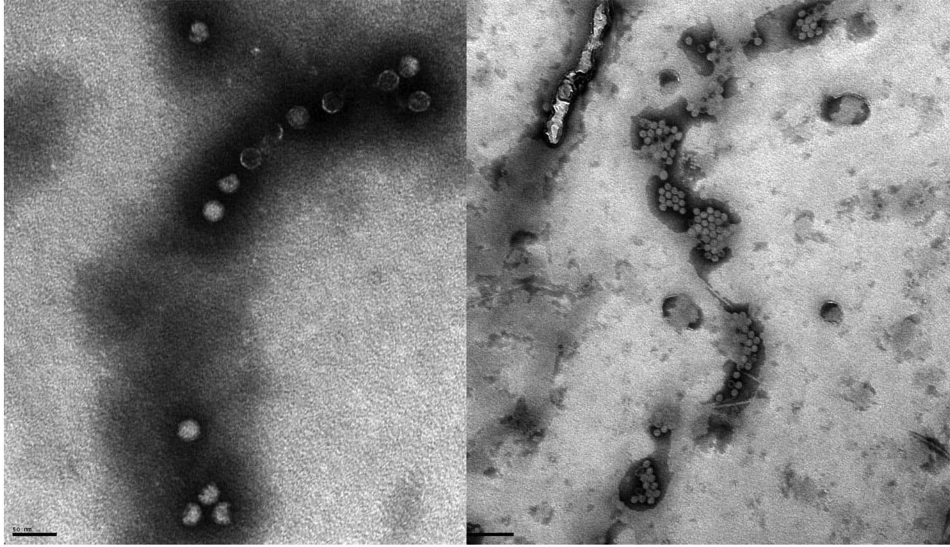


Fig. 17. Micrographs of partially purified samples from greenhouse-infected plants originating from the Kimball 2014 infections. Note again the rounded, polyhedral viral particles.

In September 2016, another virologist with the University of California, Davis, was recruited to join in this effort. New symptomatic samples from fresh greenhouse transmissions and nucleic acidextracted samples made from the 2014 Kimball infections by CDFA were then sent to the lab of Dr. Maher Al Rwahnih at Foundation Plant Services in Davis for further analysis. High throughput sequencing (HTS) produced several contigs from these samples with low identities to known members of the Tombusviridae, suggesting a new undescribed member of this family. This finding was confirmed by extracting total RNA from the greenhouse-inoculated plants and screening by RT-PCR using primers targeted for coat protein (S1-CP) and replicase genes (S1-RDRP), generating amplicons of the expected sizes of 423 and 512 bp, respectively, for the two sets of primers. A culture of this newly discovered virus from 2014 was kept alive with continuous mechanical transfers at the Panhandle Research and Extension Center (PHREC) in Scottsbluff monthly, until December 2017, when it was also lost.

Fig. 18. Symptomatic sunflower volunteers emerging within chickpea research plots at the Panhandle Research and Extension Center in Scottsbluff in 2018.

Another New Discovery—2018

In early June of 2018, four young (four to five leaf) volunteer sunflower plants were found exhibiting symptoms consistent with virus-like symptoms identical to those found in 2010, 2011, and 2014. These volunteers were located within chickpea research plots at the PHREC in Scottsbluff (Fig. 2) that had been planted to sunflowers the previous year (**Fig. 18**). They were clustered together in a small area of the field similar to previous findings, further suggesting a potential soil-borne origin for the disease (**Fig. 19**). Inoculations were performed as previously mentioned. The unknown pathogen was successfully transmitted mechanically to new plants for each of the four symptomatic volunteer plants from the field, producing four new distinct cultures.

2018–2020 Studies

Utilizing the same RT-PCR technique, both field and greenhouseinfected samples from 2018 in Scottsbluff were confirmed to be the same undescribed tombusvirus as the infection from Kimball in 2014. Each of the four distinct cultures obtained from the research plots in

Fig. 19. Distribution of the four symptomatic sunflower volunteers from the research field in Scottsbluff in 2018.

2018 was recognized as the same virus based on the same primers for coat protein and replicase (**Fig. 20**) utilized for the 2014 culture. Additionally, these samples all exhibited identical icosahedral virus particles of the expected size viewed from both leaf dip (**Fig. 21**) and purified samples (Fig. 21, inset) with TEM. Measurement of 100 particles showed that the average diameter was 28 nm, ranging from 25 to 31 nm.

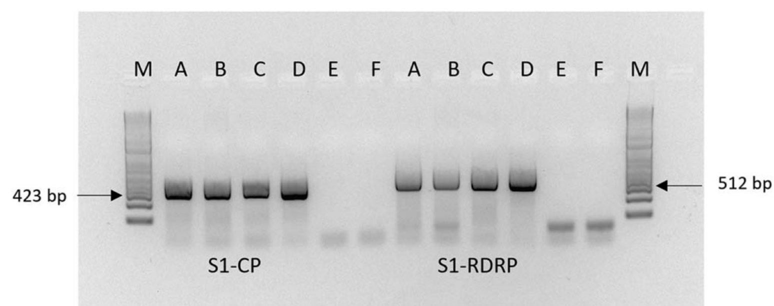


Fig. 20. Agarose gel electrophoresis of the four samples (A-D) from Scottsbluff research plots in 2018. Results show identical amplicons for both sets of primers, coat protein (S1-CP) and replicase genes (S1-RDRP), which were utilized in the 2014 Kimball infections. (E): Extraction buffer; (F): water control; (M) 1 Kb Plus DNA Ladder marker.

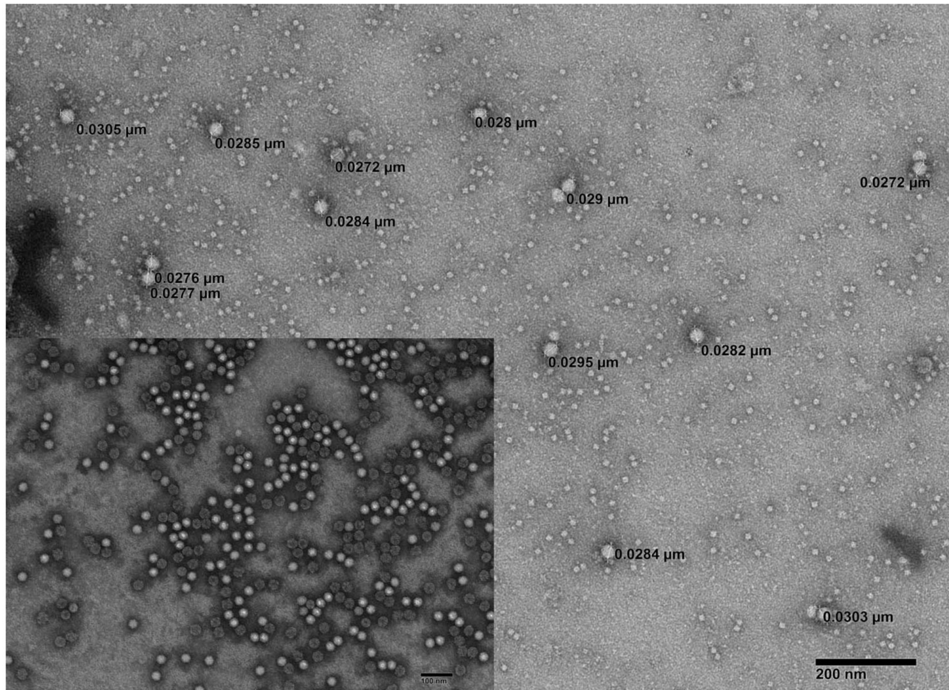


Fig. 21. Icosahedral particles of the expected size (25 to 28 nm) from Scottsbluff research plot infections in 2018 through both leaf dips and purified samples (inset).

Pathogen Survival

The four symptomatic volunteer plants were allowed to grow to maturity (**Fig. 22**), and in September of 2018, all leaves were removed, wrapped in paper towels within plastic bags, and quickly placed in a –40°C freezer. In December 2018, a sample of each of the four initial plants was taken from the freezer and inoculated to new seedlings. After approximately 14 days, similar symptoms began appearing, but the disease incidence for each culture dropped to about 25%. Additional inoculations were implemented, and successful transmission was achieved on greenhouse seedlings after 6, 12, and 18 months of storage in the freezer, but the percentages of infected plants were reduced to 25%, 12 to 15%, and 8 to 10%, respectively. Unfortunately, all cultures are now gone after loss of power for the greenhouse and freezer in November 2020.

Fig. 22. The four symptomatic sunflower volunteers at maturity from 2018 Scottsbluff research plot infections, shortly before leaves were removed for freezer storage and pathogen survival testing.

Since the disease was suspected as being soilborne, we additionally set up assays in the greenhouse attempting to bait the pathogen out of a soil sample and potentially identify a vector for the disease from the four 2018 Scottsbluff field samples. In late September, the same four symptomatic plants from the 2018 infections were dug, and soil from the rhizosphere clinging to the roots was removed, stirred with additional soil near roots, and placed into plastic pots. Sunflower seeds were planted (four per pot) into these soils and emerging plants were treated in the same manner as in those from the virus inoculations with standard practices of watering, fertilizing, and spraying with growth regulators. After 3 months, plants were removed, roots were cleaned of soil, stained, and observed as wet mounts with compound microscopy. The goal was to find resting spores of any obligate, soilborne parasites known to vector viruses, such as *Olpidium* and *Polymyxa*. This assay was repeated twice, but no foliar symptoms characteristic of prior infections from the virus pathogen ever developed on the seedlings. Furthermore, no resting spores or any other evidence suggesting the presence of these vectoring parasites was ever detected from roots.

Concluding Remarks

The now decade-long pursuit for studying and identifying this unknown viral pathogen has been a lengthy and extended process (Box 1). The persistent determination to put a name to the pathogen has been an often frustrating but eventually satisfying activity, fueled greatly by its novelty and multiple recurrences (Harveson et al. 2012, 2017). Thanks to perseverance, patience, and academic curiosity, we have finally established this pathogen as a virus new to science and have positioned it into a group with other related viruses. We also propose that this is a new member of the genus tombusvirus, yet to be fully characterized, and should be termed *Sunflower ring spot mottle virus* (SuRSMV).

Diseases caused by members of the tombusvirus genus have been reported occurring on varied hosts ranging from annual crops to woody perennials and ornamental plants. More than a dozen members of this genus have been recognized and in nature, most have relatively narrow host ranges with modest distributions.

The identification of this new pathogen as a tombusvirus makes sense biologically, based on a number of known traits associated with this genus that were repeatedly noted with each encounter. The pathogen is presumed to be soilborne due to its distribution within fields, namely occurring in small patches arranged randomly with no discernable pattern. Furthermore, icosahedral particles ranging from 25 to 28 nm were consistently found in symptomatic tissues, and the pathogen was easily transmitted mechanically. No known or detectable vector, with strong senses of stability are additional features characteristic of other known viruses in the tombusviridae (Morris 2001). This stability is illustrated by the pathogen's capability to survive in frozen tissues at –40°C and remain viable for at least 18 months.

Due to the loss of the early cultures in the greenhouse from the 2010 and 2011 infections, we will never know for certain whether they were caused by the same pathogen found in the succeeding years. Furthermore, we cannot fully explain the presence of potyvirus-like flexuous rod fragments found in the first infection from the Hemingford field in 2010, but never again from any other sample (field or greenhouse). It's possible that this was the result of multiple virus infections from that one site. In California, another potyvirus, *Turnip*

mosaic virus, was previously identified from a single sunflower exhibiting symptoms similar to the new sunflower tombusvirus (T. Tian, *unpublished data*). However, through trial and error and the process of elimination, blended with new technology and collaboration, we were able to confirm that the infections in 2014 and 2018 were both caused by the same unreported, uncharacterized member of the tombusviridae family.

We first began our search for identification using personal contacts with investigators experienced with sunflower production and problems rather than through the national plant diagnostic network. Working from the ground floor with an uncharted, obligate pathogen, causing no economic losses within a low acreage crop, is a difficult sell for recruiting collaborators to take part in this type of study. Furthermore, this was always a back-burner project with no specific funding, and all participants made their contributions generously by gratis.

One of the important lessons attained from this exercise is the efficiency of group projects employing individuals with different skills and backgrounds. Each of the authors contributed and played a critical role in this hunt, and collectively, we achieved our goal of producing an identity for the causal agent of this disease. Without the partnership created among these pathologists possessing diverse talents and abilities, the effort would have faltered.

Another powerful lesson was the effectiveness of modern diagnostic tools for assisting in the search. High throughput sequencing and RT-PCR were invaluable additions, integrated with the more traditional methods of diagnostics such as ELISA, electron microscopy, mechanical transmission, and biological characteristic studies. However, it is also necessary to recognize the synergistic benefits of jointly employing both methods of identification.

Lastly, we have learned the importance of inquisitive observation and the dogged desire to explore, learn, and report new scientific findings and add them to the literature as references. The significance of this idea is clearly illustrated by the seminal sunflower virus publication of Gulya et al. in 2002, and their foresight to presciently save antiserum to SuMV. This action allowed us to rule out SuMV two decades later with our discovery. It is hoped that our report may serve as a template or trailblazing guide for future investigations into new or unnamed virus diseases.

Fortunately, this new disease does not appear to pose a severe threat to the sunflower industry in Nebraska, or anywhere else this crop is produced. The affected plants are certainly damaged, stunted with small heads, and reduced seed yields. However, disease incidence and distribution within and among fields is low and not overly concerning. It remains a novel disease of more academic interest than economic importance. Based on the multiple observations of sunflower plants exhibiting similar symptoms since 2010 from production fields, research plots, and residential alleys from three western Nebraska counties (Box Butte, Scotts Bluff, and Kimball), our results have also remarkably revealed the presence of a hermit-like disease apparently more common than we ever realized, but just not often noticed due to minor economic damage to sunflower crops.

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