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Triticale as a Source of Resistance to Wheat Streak Mosaic Virus and Triticum Mosaic Virus

Marcos Winicius Goncalves de Souza
University of Nebraska-Lincoln

TRITICALE AS A SOURCE OF RESISTANCE TO WHEAT STREAK MOSAIC VIRUS AND TRITICUM MOSAIC VIRUS

by

Marcos Winicius Goncalves de Souza

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Marcos Winicius Goncalves de Souza, M. S.

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Advisor: Katherine Frels

Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV) present a great threat to wheat production in the Great Plains area of the U.S. due to the loss of photosynthetic area causing reductions in crop yields. Effective control of these viruses is limited to a few available strategies including controlling volunteer wheat and using currently identified resistant genes to both the vector and viruses. While effective, these genes show temperature sensitivity and yield drag, and evidence that the vectors and viruses can overcome them has been found. Thus, new methods of genetic resistance are urgently needed. Field observations have indicated that triticale (x Triticosecale Wittmack) exhibits strong tolerance to both viruses, suggesting it is a potential source of resistance genes to be introgressed in wheat. Our project aimed to characterize resistance to both viruses in a controlled environment with manual inoculation of WSMV and TriMV and under field conditions with natural inoculation. We tested 92 triticale genotypes from the University of Nebraska-Lincoln's Small Grains breeding program and analyzed their phenotypic response to WSMV and TriMV. Under an initial screening in the greenhouse, eight genotypes were selected based on their response to the viruses and a validation study was conducted on them where they were inoculated with WSMV,

TriMV, or co-infected with both. Our results show that four genotypes (NT23244, NT23245, NT23246, and NT21436) were not affected by WSMV and exhibited low symptoms of TriMV. These four tolerant genotypes also exhibited the lowest disease severity when co-infected with both viruses. Additionally, under field conditions, the four genotypes also exhibited lower symptoms, confirming their tolerance under different conditions. These findings highlight the potential of triticale as an alternative source for WSMV and TriMV resistance genes, thus helping manage these viral diseases to preserve wheat yield

DEDICATION

I dedicate this work to my friends and family for their unwavering support throughout my master's degree. Special thanks to my wife, Amanda Snyder, for everything she has done for me during this period.

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CHAPTER 1

LITERATURE REVIEW OF WHEAT STREAK MOSAIC VIRUS (WSMV) AND TRITICUM MOSAIC VIRUS (TRIMV)

1. Introduction

Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV) are two viruses that affect wheat production in the Great Plains region of the U.S. Wheat curl mite (WCM, *Aceria tosichella* Keifer) is the vector that transmits these two viruses (Singh et al. 2018). The origin of the WCM remains unclear, and its global spread is uncertain. Due to unreliable records of its initial occurrence in various regions, tracing its colonization routes is challenging (Navia et al. 2006).

Viral diseases can affect wheat crops in the Central Plains of the US and lead to a high annual reduction in yield. The average of loss is usually moderated ranging from 5% to 10%, but in severe cases, it can lead to a total crop loss, affecting 100% (Burrows et al. 2009; Hadi et al. 2011).

The objectives of this literature review are to provide a comprehensive overview of the current knowledge on WSMV and TriMV, which are now part of the wheat streak mosaic disease (WSMD) complex that also includes the High Plains Wheat Mosaic Virus (HPWMoV). However, it should be noted that HPWMoV is not commonly present which is why it is not the focus of this review. This review will cover the genomic features of WSMV and TriMV, their vectors, the life cycle of these diseases, and their management.

2. Wheat Streak mosaic virus

Compared to other viral diseases within the wheat streak disease mosaic complex, WSMV is the most extensively researched. WSMV was first observed in 1922 in Nebraska (McKinney, 1937). The virus consists of filamentous virions measuring 690-700 nm in length and 11-15 nm in diameter, encapsulating a single positive-strand RNA genome composed of 9,384 nucleotides (Stenger et al., 1998). WSMV belongs to the *Tritimovirus* genus within the *Potyviridae* family (Stenger et al., 1998). Its genome, approximately 9.3 kb in size, encodes a polyprotein that undergoes enzymatic cleavage to produce 10 mature proteins (Choi et al., 2002; Chung et al., 2008; Tatineni et al., 2011; Tatineni and French, 2014, 2016; Singh et al., 2018).

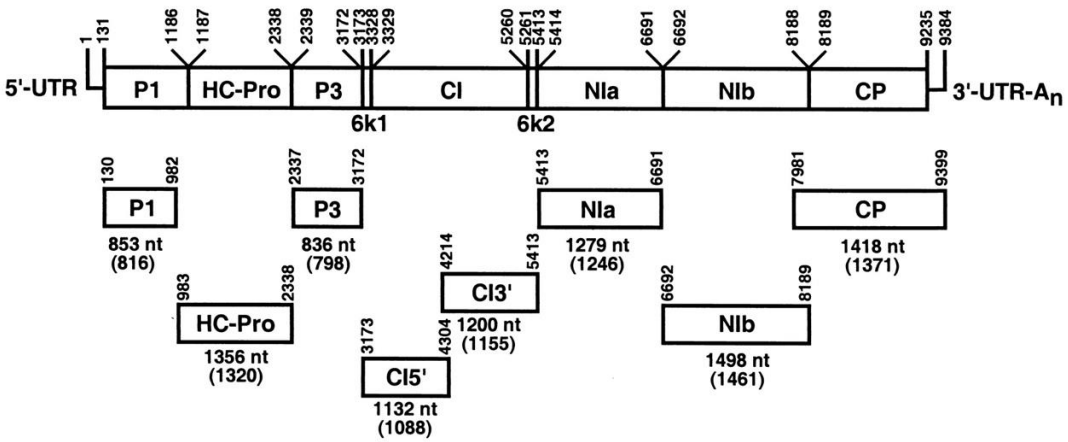

Figure 1. Genome map of wheat streak mosaic virus (Hall et al. 2001)

3. Triticum Mosaic Virus

In 2006, a new virus was identified in the western part of Kansas and named Triticum mosaic virus. TriMV belongs to the same family as WSMV, *Potyviridae*, but it is

classified under a different genus, *Poacevirus* (Seifers et al. 2008; Fellers et al., 2009; Tatineni et al., 2009). The TriMV genome measures around 10.2 kb and contains an mRNA strand with 10,266 nucleotides, encoding a polyprotein with 3,112 amino acids, which is processed into 10 mature proteins, similar to WSMV (Fellers et al., 2009). A notable difference between the two viruses is the untranslated region at the beginning of TriMV's mRNA strand, spanning 739 nucleotides, which is only 130 nucleotides in WSMV (Fellers et al., 2009; Tatineni et al., 2009). The TriMV coat protein shares a 45.9% similarity with Sugarcane streak virus strain AP, but only a 23.2% similarity with WSMV.

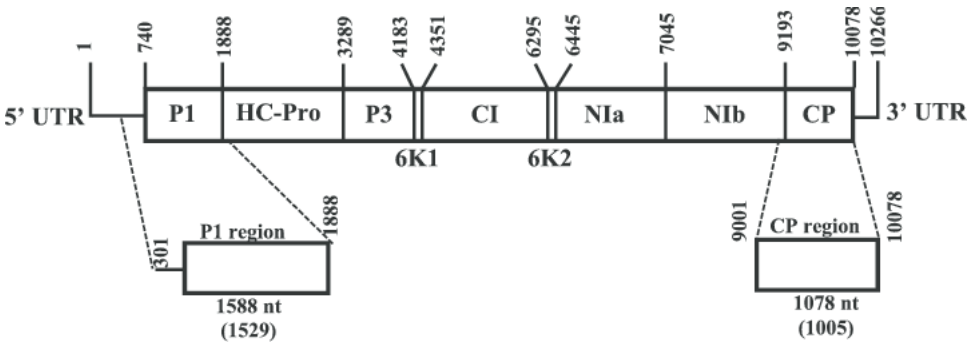

Figure 2. Genome map of Triticum mosaic virus (Bartels et al. 2016)

4. Disease life cycle

4.1.Vector transmission

The wheat curl mite (WCM) is the only identified vector of WSMV and TriMV, specifically biotypes 1 and 2 (Slykhuis, 1955). WCM uses around 90 grasses and various cereal crops, such as barley, oats, maize, and rye, as hosts (Navia et al., 2013). Since WCM cannot fly, it relies on wind dispersal for transmission. WCM acquires the virus during feeding, which can occur in as little as 15 minutes. The virus remains infective for up to 9

days at 20–25 °C after being removed from an infected plant, even after molting (Orlob, 1966; Siriwetwiwat, 2006; Slykhuis, 1955). All stages of WCM, except for eggs, can transmit viruses, but adult mites can only transmit if they acquired the virus during an immature stage (del Rosario and Sill, 1965; Orlob, 1966; Siriwetwiwat, 2006; Slykhuis, 1955). Although adults can acquire the virus, they likely cannot inoculate the plant (Orlob, 1966).

WCM transmits WSMV by feeding on the thin-walled epidermal cells of wheat leaves, specifically targeting bulliform cells within the whorl of a developing leaf. Their very short chelicerae (about 0.02 mm) limit their feeding to these superficial tissues. This feeding causes the leaves to curl, creating a humid environment that the mites prefer. Additionally, the feeding activity of WCM reduces the plant's photosynthetic capacity, thereby aiding in the transmission of the virus as the mites continue to infest new plants (Royalty and Perring, 1996).

4.2 Infection Process

The infection in the winter wheat can start in the fall, when the WCM will be blown by wind from the infected volunteer wheat or spring wheat to the fields with recently emerged winter wheat seedlings, where the vector will transmit the disease (Singh et al., 2018). In years when fall temperatures remain warmer for an extended period, mites are more active, leading to a higher infection rate during a time when winter wheat seedlings are more susceptible compared to later infections, which will likely cause a higher disease severity (Hunger et al., 1992; Slykhuis et al., 1957). The mites will survive through the

winter in the form of eggs, larvae, and nymphs in wheat plants, and the viruses will overwinter in the live tissue of alternative hosts and volunteer wheat. When the temperature rises in the spring, the mites will acquire the virus from infected plants and migrate to infect healthy plants. When winter wheat starts to mature, mites will look for a new host with live tissue to keep increasing its population and survive in the summer until the following fall, when the new season of winter wheat will start.

Similar infection patterns occur in spring wheat. The difference is that the infection begins in the spring after the germination, and the full disease cycle will be completed in the following spring (Singh et al. 2018).

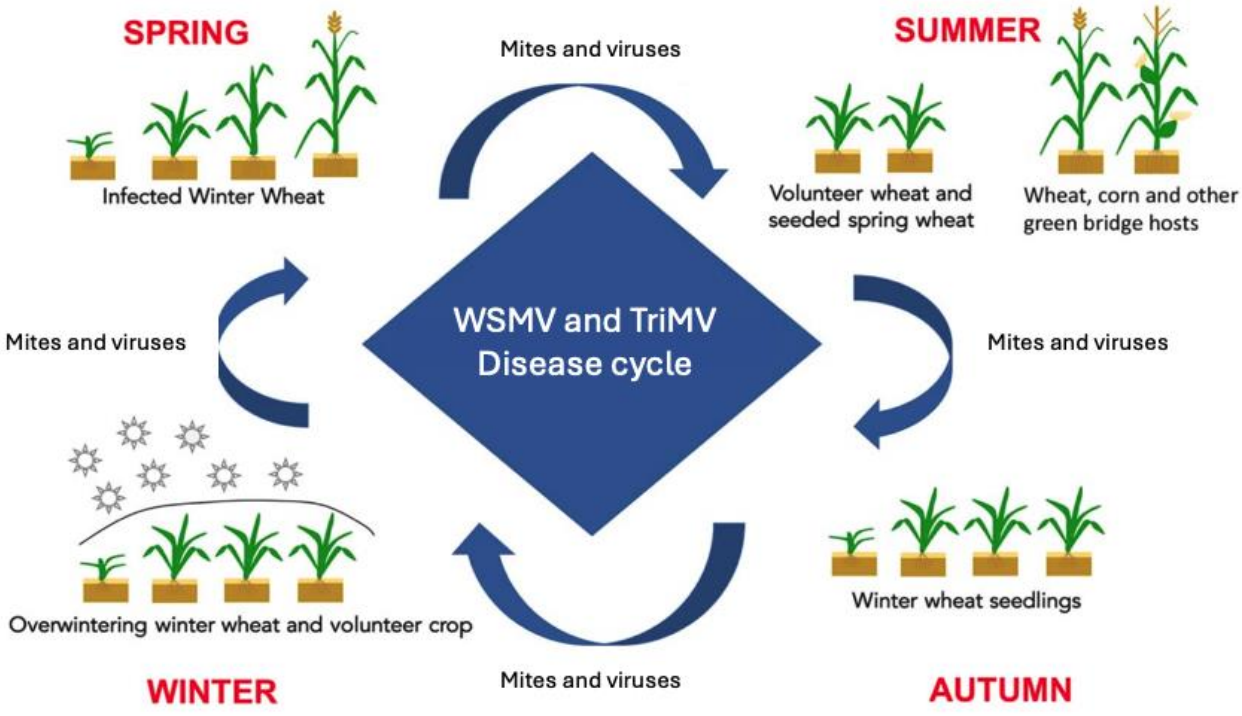

Figure 3. Life cycle of Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV) (Adapted from Singh et al. 2018).

5. Symptoms of WSMV and TriMV

The initial symptoms of the infection manifest as chlorotic streaks on the leaves, forming a mosaic pattern (Byamukama et al. 2011; Hadi et al. 2011*)*. When plants are infected at an early stage, such as during the fall in winter wheat, it can lead to stunted growth, floret sterility, reduced yield, and lower test weight (Singh. et al. 2018).

6. Co-infection and synergistic effect of WSMV and TriMV

Co-infection of the same plant by two or more viruses may generate an interaction, either positive or negative (Garcia-Cano et al., 2006; Renteria-Canett et al., 2011; Untiveros et al., 2007). A positive synergistic interaction occurs when multiple viruses infect the same host, amplifying the extent of damage, where one or both viruses benefit from the interaction. In contrast, a negative interaction occurs when only one virus benefits from the interaction, and its presence might lower the fitness of the second virus (Alcaide et al., 2020; Moreno and Lopez-Moya, 2020). The problem of synergistic infections in plants has attracted the interest of producers and researchers due to their potential to cause more severe damage to plants, such as in growth and yield, when compared with a single infection (Fodong et al. 2000: Gutierrez et al. 2003: Mahuku et al. 2015, Redinbaugh and Stuart, 2018).

The co-infection of WSMV and TriMV is asymmetrical, meaning that the success of the infection depends on the order in which the viruses first infect wheat. Prior studies show that when wheat is infected first with TriMV, the long-distance movement of WSMV

is facilitated. Conversely, when wheat is infected first with WSMV, TriMV experiences delayed infection in the early stages. However, in the late stages, the concentration of TriMV in wheat increases rapidly, and long-distance movement occurs more quickly (Tatineni et al., 2010, 2019). The molecular mechanism of the co-infection between WSMV and TriMV is currently unknown. Notably, the impact of the co-infection on damage and yield reduction is more pronounced in susceptible cultivars (Tatineni et al., 2010).

7. Disease management

Effective disease management for WSMV and TriMV involves an integrated approach combining cultural practices and resistant varieties. Controlling the WCM, the vector for both viruses, is essential. This includes eliminating volunteer wheat and other grassy weeds that serve as green bridges for WCM survival during the off-season, delaying planting of winter wheat until after the WCM population has decreased, and maintaining proper crop rotation and field hygiene to minimize virus transmission (Singh et al., 2018).

Controlled farming practices and cultural methods can effectively manage viral diseases in wheat by eliminating the ‘green bridge’ for overwintering mites and viruses. First, controlling volunteer wheat that emerges between harvest and planting is crucial through herbicide applications or manual removal. Second, shifting the planting date to later in the season after summer crops like maize have dried down can reduce potential virus reservoirs. However, this strategy must be balanced with other factors such as weather conditions and crop variety considerations.

7.1 WSMV and TriMV resistance

Limited genetic resistance to WSMV and TriMV is available. There are four genes conferring resistance to WSMV and TriMV: ***Wsm1, Wsm2, Wsm3***, and ***C2652***. ***Wsm1*** is an alien introgression from *Thinopyrum intermedium* ((Host) Barkworth & DR Dewey), located on chromosome translocation 4DL.4AgS (Friebe et al., 1996). *Wsm1* confers resistance to both WSMV and TriMV and was first released in the cultivar Mace (Friebe et al., 2011; Graybosch et al., 2009; Divis et al., 2006). Molecular markers for ***Wsm1*** have been developed to facilitate marker-assisted selection (MAS) in breeding programs. However, ***Wsm1*** is temperature-sensitive, being effective up to 27°C but losing its efficiency at temperatures above 30°C (Kumssa, 2016). A new translocation for ***Wsm1*** was discovered: the T4DL·4DS-4JsS rec213 translocation, which shows no effect on heading date and has a positive outcome on grain yield, though it reduces plant height (Guttieri et al., 2023).

A Colorado breeding line originating from TAM107 was the source for the discovery of ***Wsm2***, located on chromosome arm 3BS (Lu et al., 2011) and molecular markers, have been developed to enhance the efficiency of (Tan et al., 2017). ***Wsm2*** provides resistance to WSMV but not to TriMV (Haley et al., 2002). ***Wsm2*** is also temperature-sensitive, being effective up to 18°C (Seifers et al., 2013). Recently, WSMV isolates were identified as virulent to ***Wsm2***, indicating that additional resistance sources are urgently needed (Kumssa et al., 2019).

Wsm3 was found in *Thinopyrum intermedium*, conferring resistance to WSMV up to 27°C but losing effectiveness at higher temperatures. It also provides resistance to TriMV up to 24°C (Kumssa, 2016). ***Wsm3*** is located on the chromosome T7BS·7S#3L (Liu et al., 2011) and molecular markers have been developed and are used to facilitate its incorporation into wheat breeding programs through MAS (Danilova et al., 2017).

The ***C2652*** gene, identified in a Canadian hard red spring wheat population, confers resistance to WSMV but not to TriMV and is effective at temperatures up to 28°C (Haber et al., 2006, Fahim et al., 2012). Currently, no specific markers associated with **C2652** have been reported in the literature.

7.2 Wheat curl mite resistance

Genetic resistance to the wheat curl mite (WCM) is also limited. Currently, there are four known genes that limit mice colonization: *Cmc1, Cmc2, Cmc3, and Cmc4/*Cmc_{TAM112}*. Cmc1 and Cmc4/CmcTAM112* were introgressed from *Aegilops tauschii* to chromosome 6DS in wheat; although both are on the same chromosome, they segregate differently *(*Thomas and Conner, 1986; Whelan and Thomas 1989; Malik et al., 2003), molecular markers are available for both genes (Zhao et al., 2021). *Cmc2* was introgressed from *Thinopyrum elongatum (syn. Agropyron elongatum or Lophopyrum elongatum) (Host) Beauv. to* chromosome 6DL of wheat *(*Whelan and Hart, 1988). *Cmc3* was transferred from rye to wheat via triticale and is located on chromosome 1AL (Martin et al., 1984; Whelan and Hart, 1988). Additionally, several other unnamed genes have been identified. One gene is located on the short arm of chromosome group 6 from a

Wheat-*Haynaldia villosa* (L.) translocation line (Chen et al., 1996). Another gene from a wheat-*Thinopyrum ponticum* partial amphiploid line called ‘Agrotana’, provides immunity to WCM (Chen et al., 1998). There is also a gene in a wheat-*Thinopyrum ponticum* 6Ae/6DL Robertsonian translocation line (Thomas et al., 1998) and one from a wheat-*Thinopyrum intermedium* partial amphiploid (Chen et al., 2003).

Certain WCM populations have developed virulence against the *Cmc3* resistance gene, which has been documented in various studies (Dhakal et al., 2017; Harvey et al., 1997). This adaptation allows the mites to overcome the genetic resistance provided by Cmc3, leading to infestation and the spread of the viruses.

8 Triticale as an Alternate Source for WSMV and TriMV Resistance

Triticale (× Triticosecale Wittmack) is a hybrid crop derived from a cross of wheat (*Triticum* spp.) and rye (*Secale cereale* L.) (Mergoum et al., 2009). This hybrid demonstrates different levels of tolerance and resistance to WSMV and TriMV compared to wheat (Li et al., 2007; Seifers et al., 2010). While some lines of rye and triticale may be vulnerable to WSMV and TriMV, the identification of resistant and/or tolerant genotypes could provide a novel source of viral disease resistance for wheat.

Hexaploid triticale (AABBRR, 2n=6x=42) can efficiently transfer rye genes to hexaploid bread wheat (AABBDD, 2n=6x=42) (Li et al., 2018; Saulescu et al., 2010). A notable example is the WCM resistance gene Cmc3, which was transferred to wheat from

the rye variety ‘Insave F.A.’ via the triticale variety ‘Gaucho’ (Wood et al., 1995) via a Robertsonian translocation.

Figure 4. Wheat streak mosaic virus screening nursery in Dighton, KS. Rows from left to right: Triticale, LCS Chrome, experimental BDV line, and TAM114. The triticale line does not exhibit any chlorotic streaks or mosaic symptoms (Photo Guttieri, M. J 2021)

9 Robertsonian Translocation

Robertsonian translocation is a type of chromosomal rearrangement that involves the fusion of two acrocentric chromosomes, forming a single metacentric chromosome. This process can transfer entire arms of chromosomes between species, which is particularly beneficial for transferring desirable traits, such as disease resistance, from one species to another.

In the context of triticale and wheat, crosses between triticale and wheat result in F1 hybrids (AABBDR) (Figure 5a), which can produce whole-arm Robertsonian translocations transferring stress or disease resistance genes to wheat (Lukaszewski et al., 1983) (Figure 5b). The hexaploidy of both triticale and wheat is particularly beneficial in generating whole-arm Robertsonian translocations. This process allows chromosomes from different species to fuse, facilitating the transfer of desirable genes, such as those conferring disease resistance or stress tolerance, from triticale to wheat. Sharing the same genome as common bread wheat (genomes A and B) facilitates the crossbreeding process between triticale and wheat. Their expression increases the ability to differentiate rye genes in the presence of two wheat genomes (Saulescu et al., 2011).

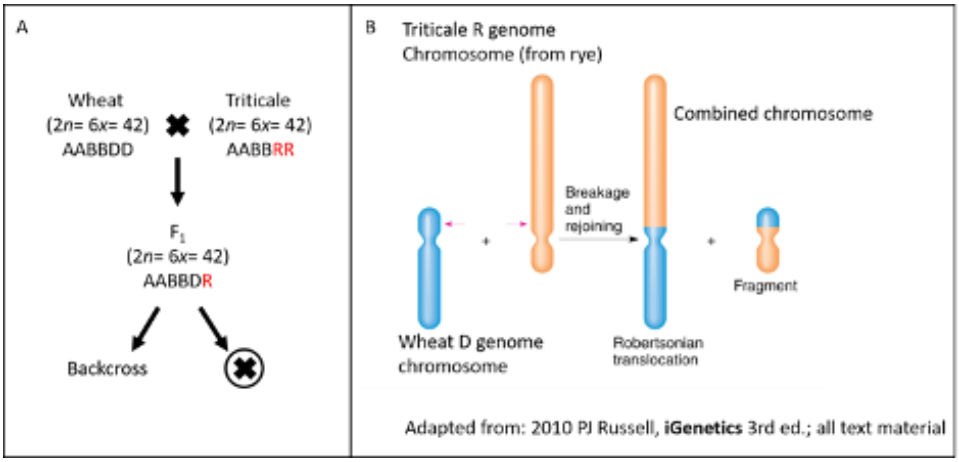

Figure 5. Utilizing triticale for wheat improvement. A) Hybridization method for introgressing rye genome segments into wheat via triticale. B) Robertsonian translocation between D and R genomes.

10 Conclusion

Wheat breeders and stakeholders are concerned with the limited number of strategies to fight against diseases transmitted by WCM, such as the WSM complex. Only four resistance genes have been identified for WSMV and TriMV, and only two have been deployed into commercial varieties (*Wsm1* and *Wsm2*). *Wsm2* only provides resistance against WSMV, and its temperature sensitivity compromises its effectiveness. Triticale and Rye present interesting and unexplored resources for discovering novel genetic resistance genes to these viral diseases. While the rye genome has been used for various disease-resistance and stress-resistant genes in wheat, transferring these genes to wheat can be quite a challenge. Identifying new WSMV and TriMV resistance within germplasm environmentally adapted to the region holds the potential to accelerate the development of commercial varieties with the new resistance genes. Introducing additional resistance genes in future varieties will mitigate the yield losses caused by the WSM complex.

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CHAPTER 2

PHENOTYPIC EVALUATION OF TRITICALE FOR WHEAT STREAK MOSAIC VIRUS AND TRITICUM MOSAIC VIRUS IN CONTROLLED ENVIRONMENT

1. Introduction

Wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV) are key viral pathogens that severely impact winter wheat production in the Great Plains of the US (Singh et al., 2023). Both viruses are transmitted by wheat curl mite (WCM, *Aceria tosichella* Keifer). When infection takes place early in the plant's growth stages, such as a fall infection in winter wheat, it can lead to stunted growth, floret sterility, reduced yield, and decreased test weight (Singh et al., 2018). The loss typically ranges from 5 to 10%, but in severe cases, it can lead to total crop loss, affecting 100% of the crop (Burrows et al., 2009). The impact of these viruses on wheat productivity in Kansas in 2021 was estimated at 11.6 million bushels, or approximately $84.1 million, underscoring the critical nature of these viral diseases (Hollandbeck et al., 2021).

Limited genetic resistance to WSMV (*Wsm1, Wsm2, Wsm3,* and *C2652*) and TriMV (*Wsm1* and *Wsm3*) is available. *Wsm1*, an alien introgression from *Thinopyrum intermedium* [(Host) Barkworth and DR Dewey] on chromosome 4AS (Friebe et al., 1996), offers resistance to both WSMV and TriMV and was later deployed in the cultivar Mace (Friebe et al., 2011, Graybosch et al., 2009; Divis et al., 2006). W*sm1* is temperature sensitive, presenting resistance at 20 °C but not in temperatures over 24 °C (Seifers et al., 1995). *Wsm2* was identified in a Colorado breeding line derived from TAM107 and is located on chromosome arm 3BS (Lu et al., 2011). *Wsm2* confers

resistance to WSMV but not to TriMV. This resistance is temperature-sensitive, being effective at 18°C but not at 24°C (Haley et al., 2002). Recently, studies discovered that some strains of WSMV can overcome *Wsm2* resistance, indicating an urgent need for additional sources of resistance (Kumssa et al., 2019). Additionally, two newer genes, *Wsm3* and *C2652*, were identified but are still being investigated, with little information available on their agronomic viability or utility. (Haber et al., 2006). *Wsm3*, identified in *Thinopyrum intermedium*, confers resistance to WSMV at both 18°C and 24°C and to TriMV at 18°C but not at 24°C. It is located on the chromosome T7BS·7S#3L (Liu et al., 2011). The *C2652*, identified in a Canadian hard red spring wheat population, confers resistance to WSMV but not to TriMV, and is effective at temperatures up to 28°C (Fahim et al., 2012).

New germplasm resources for WSMV and TriMV should be explored including evaluating close relatives that appear to be resistant or tolerant to the viruses such as triticale. Triticale (*x Triticosecale Wittmack*) is a hybrid crop derived from a cross of wheat (*Triticum* spp.) and rye (*Secale cereale*) (Mergoum et al., 2009) and its genome consists of chromosomes from both parent species, typically organized as AABBRR in hexaploid triticale (2n=6x=42). Triticale has shown better resistance to fungal diseases than its parent crops (Kiecana et al., 1987; Arseniuk et al., 1993, 1999; Miedaner et al., 2001, 2004; Langevin et al., 2004; Góral and Ochodzki, 2007). However, no studies have been made regarding viral diseases. As a hexaploid species, triticale may serve as a genetically compatible donor for genes resistant to wheat, facilitating the transfer of such genes. A successful example of triticale being used as a bridge to gene introgression is

the resistance gene to wheat curl mite *Cmc3*, which was introgressed from rye via triticale (Schlegel and Kynast, 1987). Triticale could provide practical solutions to viral disease resistance in wheat.

Recent observations indicate that triticale lines from the University of Nebraska-Lincoln Small Grains Breeding Program mostly exhibit strong viral tolerance, showing no symptoms when exposed to WSMV and TriMV under field conditions (Dr. Mary Guttieri, personal communication). This observation suggests potential unexplored resistance gene in triticale. The absence of comprehensive research into triticale's phenotypic characteristic for viral tolerance represents a gap in our current understanding. To address this gap, characterization of triticale response to viral infection is needed.

This research explores the phenotypic response of 94 triticale genotypes from the UNL Small Grains Breeding Program to both viruses; we aim to identify the varieties that exhibit the highest tolerance and susceptibility. A subset of the most susceptible and tolerant genotypes was used to validate the phenotypic response to single and dual virus infections. These findings will lay the groundwork for further research into the genetic control of triticale response to WSMV and TriMV by creating a bi-parental mapping population.

2. Methods

2.1 Initial screening

The study was conducted at the UNL Plant Pathology greenhouse complex, and plants were evaluated for response to mechanical inoculation of WSMV and TriMV on two different planting dates. The temperature in both greenhouses was set to 23 °C, but

the average was 30 °C due to warmer weather. Wheat cultivar Tomahawk and the triticale genotype NT441 were selected as the control genotypes. Tomahawk is notably susceptible to WSMV and TriMV, while NT441 has previously been observed to exhibit tolerance to both viruses (Dr. Mary Guittieri, personal communication). This experiment used 92 triticale genotypes from the UNL Small Grains breeding program. These genotypes were composed of 40 genotypes from the 2023 preliminary yield trial (PYT), an F3:6 generation, and 52 from advanced yield trial entries (AYT), an F3:7 generation. These genotypes were developed for forage and grain production in the central and southern Great Plains of the US. The 92 genotypes were planted randomly and assigned to places on the greenhouse bench in an augmented design, where the check genotypes (10 Tomahawk and 10 NT441) were spaced diagonally through the bench to ensure no spatial effects.

Each genotype, including the controls, was grown in 6-inch clay pots, with 10-15 plants per pot, on a standard greenhouse mix substrate. Lights were set to 14-hour days. During the experiments, the greenhouse temperature was maintained between 20°C and 25°C.

To assess the phenotypic response to WSMV and TriMV, all 92 triticale genotypes plus the checks were subjected to two separate planting and inoculation schedules. For WSMV phenotypic response, planting occurred on 06/30/23, with inoculation following 7-10 days post-emergence (DPE). Similarly, for TriMV phenotypic response, planting was on 08/11/23, with inoculation also occurring at 7-10 DPE. In both cases, inoculation involved all seedlings per pot.

Validation study.

Following the initial screening with both viruses, a subset of 8 triticale genotypes was selected based on their performance. The selection included four susceptible and four tolerant genotypes. After selection, the eight triticale genotypes and the control Tomahawk were evaluated in a replicated trial with three replications of each genotype. The trial was repeated twice, with planting dates of 12/21/2023 and 02/04/2024, in separate greenhouses distinct from those used for initial screenings. The same planting methods and greenhouse settings as the initial screens were used.

For each planting, we recorded the phenotypic response of WSMV, TriMV, and their co-infection based on three replications for each genotype. The results for each virus were aggregated from both planting dates to provide a comprehensive average score. Each of the eight genotypes, along with the control Tomahawk, was represented by three potted replications, resulting in a total of 27 pots per treatment. These replications were inoculated with WSMV, TriMV, and a combination of both viruses (WSMV+TriMV) 7-10 DPE. To minimize location biases, the pots within each greenhouse were arranged randomly.

2.2 Virus Preparation and inoculation

Virus inocula were prepared by following the procedures described by (Tatineni et al., 2010). Briefly, WSMV isolate Sidney 81 was derived from an infectious cDNA clone, with RNA transcripts generated in vitro and inoculated into wheat seedlings at the single-leaf stage. A Nebraska isolate of TriMV was obtained from wheat infected with in vitro transcripts from pTriMV (Tatineni et al. 2015). To ensure a fresh source of

inoculum, wheat seedlings at the single-leaf stage were inoculated with WSMV Sidney 81 or TriMV 10 days before inoculating the experimental plants. Leaves from young wheat plants infected with WSMV or TriMV at 10 to 12 days post-inoculation (dpi) were ground in an inoculation buffer (1 gram of leaves per 9 milliliters of buffer) using a mortar and pestle. The resulting inocula were combined in equal parts to create a 1:20 dilution of WSMV and TriMV mixture. Similarly, the WSMV and TriMV inocula were each diluted 1:1 with the inoculation buffer to achieve a final 1:20 dilution.

Inoculations were conducted manually on each seedling per pot. First, carborundum (silicon carbide) powder was applied to the surface of each seedling as an abrasive to facilitate the entry of the viruses caused by the superficial damage to the leaf tissue. Following this step, a pestle was dipped in the virus inoculum solution and used to scrub the surface of each seedling. After scrubbing each seedling, the pestle was re-dipped into the virus inoculum to ensure a fresh inoculation for a consistent virus application across all genotypes. This process was repeated for every genotype in the study, covering all the inoculation, including WSMV, TriMV, and the co-infection of WSMV+TriMV, to guarantee uniform exposure across the genotypes.

2.3 Phenotypic evaluation

The phenotypic data for all groups for the initial screening and validation study was collected following the same protocol. Assessments were performed three times post-inoculation: at 7 dpi, 14 dpi, and 21 dpi. During each assessment, the infection levels were scored on a scale of 0-5, with a score of 0 indicating no visible disease symptoms and a score of 5 representing the highest visible severity of disease symptoms (Figure 1).

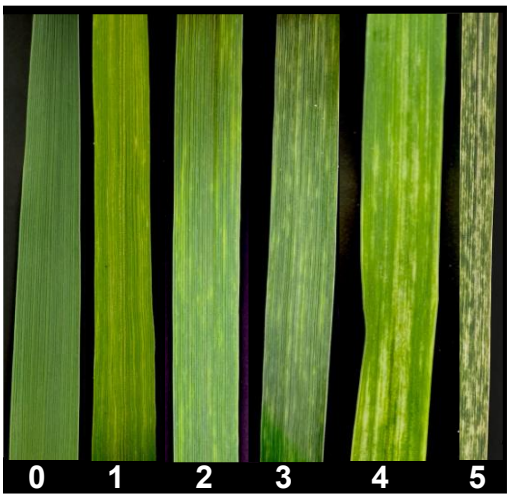

Figure 1: Disease severity scoring for Wheat streak mosaic virus (WSMV), Triticum mosaic virus (TriMV), and their co-infections in Triticale, assessed during phenotyping. Severity scores range from 0 (no visible symptoms) to 5 (extreme disease severity). Visible symptoms start with yellow chlorosis in a mosaic pattern and progress to more severe symptoms. At the highest severity score of 5, the chlorosis evolves into necrosis, characterized by extensive yellowing and tissue death, observable in infections by both viruses.

2.4 Chlorophyll content measurement

The chlorophyll content was measured at 7 dpi, 14 dpi, and 21 dpi using a chlorophyll meter (model SPAD 502 plus, Konica Minolta Sensing, Inc., Osaka, Japan). One reading was taken from 5 newest fully developed leaves per pot, and the average score per pot was recorded. Each measurement targeted approximately the same area on each leaf to ensure consistency across measurements.

2.5 Enzyme-linked immunosorbent Assay (ELISA)

Tissue samples for the initial study were collected for each of the 92 genotypes inoculated with WSMV and TriMV. Three random plants were selected from each pot, and 6-8 cm of leaf tissue was placed in a sample mesh bag (Agdia) at 21 dpi to ensure maximum viral expression for further analysis via ELISA. The tissue samples were stored at -80°C. We employed double antibody sandwich (DAS)-ELISA (Clark and Adams, 1977) to detect WSMV and TriMV in 92 triticale genotypes, including controls NT441 and Tomahawk. Following the protocol of Clark and Adams (1977), 2 ml of general extraction buffer (Agdia) was added to mesh bags (Agdia) containing triticale leaf samples. These samples were then homogenized using a motorized grinder. The resulting sap was loaded into WSMV or TriMV IgGs coated 96-well plates (100 µL per well, two wells per genotype), which had been washed three times with PBS-T (Agdia). The plates were incubated at 37°C for one hour in a moist chamber, then washed and loaded with 100 µL of ALP-conjugated virus-specific IgG. For WSMV, IgG was obtained from Agdia (1:400 dilution, SRA 47001/0096), and for TriMV, IgG was prepared according to the method described by Tatineni et al. (2013) (1:500 dilution) per well. After another hour of incubation at 37°C, the plates were washed and loaded with 100 µL of diluted PNP substrate buffer (Agdia) per well and incubated at room temperature in the dark until the color reaction developed. The intensity of the color reaction was quantified using a spectrophotometer (Molecular Devices - SpectraMax) at an optical density of 405 nm, 15 and 30 minutes after the addition of the PNP solution

(Agdia). A positive result was defined as an optical density value at least three-fold higher than that observed in healthy control samples.

2.6 Data analysis

The genotypes were scored on a per-pot basis, and the average infection level across all seedlings within a single pot was recorded. Scores were averaged across the six pots per genotype—comprising three pots per planting date across two dates to generate a single consolidated score for disease severity.

To assess the linear relationship between disease severity and chlorophyll content at 21 dpi and understand the impact of WSMV, TriMV, and the co-infection of WSMV+TriMV on the physiological health of the plants, the Pearson correlation coefficient and the significance level of the observed correlation was calculated for each of the diseases using the cor function in R.

3. Results

3.1 Phenotypic Responses of Triticale genotypes to WSMV infection

We assessed the response of 92 Triticale genotypes to WSMV at 7, 14, and 21 dpi. By 21 dpi, four genotypes had no disease symptoms that were scored 0 (NT23244, NT23245, NT23246, and NT21436), and NT23226 showed severe infection, receiving a score of 5 (Table 1). Disease symptoms gradually escalated from 7 dpi to 21 dpi in all genotypes and controls (Figure 2A-C). Most of the genotypes exhibited minimal or mild symptoms at 21 dpi, with 63% scored 1, 28% scored 2, and 5% scored 3 (Figure 2C). The resistant control NT441 averaged a score of 2, while the susceptible wheat control Tomahawk, scored an average of 5.

To characterize the effect of the disease on the photosynthetic area, we evaluated the correlation between leaf chlorophyll content and disease severity. As the disease severity increased, the chlorophyll content decreased. We saw a negative correlation of -0.55, with a p-value <0.0001, supporting the phenotypic evaluation made for WSMV inoculation, showing that the genotypes became unhealthy and more symptomatic over time (Figure 3A).

ELISA was conducted to detect the presence of the virus. Among the 94 genotypes sampled, including two checks, three genotypes (NT23212, NT23245, and NT22711) did not test positive for the virus, with scores of 0, 2, and 1, respectively. These results indicate that the majority of the genotypes were susceptible to the virus, with only a few exceptions showing tolerance.

3.2 Phenotypic Responses of Triticale genotypes to TriMV infection

We assessed the response of 92 Triticale genotypes to TriMV at 7, 14 and 21 dpi. The disease symptoms gradually escalated from 7 dpi to 21 dpi in all genotypes and controls (Figure 2 D-F). At 21 dpi, most of the genotypes exhibited mild symptoms, with 70% scoring 1 and 28% scoring 2, while only 3 triticale genotypes scored 3 (NT23207, NT23213, and NT23225) (Figure 2F). The resistant control NT441 averaged a score of 1, while the suzsceptible wheat, Tomahawk, scored an average of 5. Compared to the WSMV screen, the triticale genotypes demonstrated less resistance to TriMV since our phenotypic scores ranged from 1 to 3.

To assess the impact of the disease on the photosynthetic area, we evaluated the correlation between these parameters, and we noticed that as the disease increased, the

chlorophyll content decreased. Analyzing the relationship between disease severity and chlorophyll content levels at 21 dpi, there was a negative correlation of -0.69 with a p-value <0.0001, supporting our phenotypic evaluation for TriMV, and showing that the genotypes had an increase in the symptoms over time (Figure 3 B). These findings showed that all the evaluated triticale genotypes show symptoms of TriMV and are less resistant to this virus than to WSMV.

ELISA was conducted to detect the presence of the virus. All 92 genotypes, along with the two control checks, tested positive for the virus. These results indicate that triticale is susceptible to the virus.

3.3 Validation study: Phenotypic Responses to WSMV, TriMV, and co-infection using a subset of genotypes.

We assessed the viral response of the subset of eight triticale genotypes (NT23244, NT23245, NT23246, NT21436, NT23226, NT23207, NT23213, and NT23225) in a replicated study to validate the findings of the initial, unreplicated screen.

3.3.1 Phenotypic response to WSMV

For WSMV infection, the four immune genotypes (NT23244, NT23245, NT23246, and NT21436) were confirmed as exhibiting no symptoms of infection, thus scoring 0 at 21 dpi. The genotypes NT23207, NT23225, and NT23226 had reduced symptoms in the replicated trial compared to the initial screen, with average scores of 0.5, 1.17, and 3.5 respectively. However, the genotype NT23213 slightly increased from 2 to 2.33. The susceptible control Tomahawk was scored at 5 in the validation study (Table 1).

3.3.2 Phenotypic response to WSMV

For TriMV infection, all Triticale genotypes showed symptoms consistent with the findings from the initial trial. The genotype NT23244 was scored 1, the same score was obtained in the winter compared to the summer. Most of the genotypes showed less severity in the winter compared with the summer planting, with NT23245 and NT23246 scoring 1.17, NT23225 scoring 1.67, NT23226 and NT23207 scoring 1.83, and NT23213 scoring 2.33. At the same time, NT21436 was the only genotype that increased the score to 1.5 compared with the summer planting. The susceptible Tomahawk scored 4 in the validation study (Table 1).

ELISA was conducted to detect the presence of the virus. All 92 genotypes, along with the two control checks, tested positive for the virus. These results indicate that triticale is susceptible to the virus.

3.3.3 Phenotypic response to WSMV and TriMV co-infection

Finally, for the co-infection, some genotypes showed increased severity compared to a single inoculation. Specifically, NT21436 scored 1.83, NT23226 scored 4.5, NT23213 scored 3.17, and NT23225 scored 1.83. Meanwhile, genotypes NT23244 and NT23245 maintained the same scores of 1 and 1.17, respectively. Genotypes NT23246 and NT23207 showed a slight decrease in symptoms, with scores of 1 and 1.83, respectively. The susceptible control Tomahawk scored 5, having the highest severity (Table 1). These findings indicate that when there is co-infection of the viruses, the symptoms tend to increase, causing more damage to the plants and eventually will affect the performance of the genotype in the field.

The chlorophyll content was measured for plants infected with WSMV, TriMV, and the co-infection of WSMV+TriMV. When co-infected, the genotypes exhibited lower chlorophyll content compared to single infections (Figure 4). We evaluated the correlation between chlorophyll content and these infections at 21 dpi, and found correlation coefficients of -0.92, -0.91, and -0.86 for WSMV, TriMV, and the co-infection, respectively, with p-values < 0.001 for each analysis (Figure 4 A-C).

Table 1: Severity scores for Wheat Streak Mosaic Virus (WSMV), Triticum Mosaic Virus (TriMV), and their co-infection (WSMV+TriMV) in selected triticale genotypes[1] at 21 Days Post-Inoculation (dpi). Scores reflect disease severity in both summer and winter assessment dates.

		Summer		**Winter**		
		7/30/23	**9/11/23**	**Average of replicated trial**		
Genotypes	**Pedigree**	**WSMV**	**TriMV**	**WSMV**	**TriMV**	**WSMV+TriMV**
NT23244	**NT12440/NT05429**	0.00	1.00	0.00	1.00	1.00
NT23245	**NT12424/NT11428**	0.00	2.00	0.00	1.17	1.17
NT23246	**98T376-1-2-3-1/unknown//CT3/3/NT12440**	0.00	2.00	0.00	1.17	1.00
NT21436	**NT10429/NE03T416**	0.00	1.00	0.00	1.50	1.83
NT23226	**NT05421/NT15440**	5.00	2.00	3.50	1.83	4.50
NT23207	**LIRON_2/5/DIS B5/3/SPHD/PVN//YOGUI_6/4/KER_3/6/BULL_10/MANATI_1/7/DAHBI_6/3/ARDI_1/TOPO 1419//ERIZO_9/8/NT09423/9/NT16402**	2.00	3.00	0.50	2.17	1.83
NT23213	**CAAL/3/T1494_WG//ERIZO_10/2*BULL_1-1/3/NT09423/4/NT16402**	2.00	3.00	2.33	2.33	3.17
NT23225	**NT15440/NT14433**	3.00	3.00	1.17	1.67	1.83
Tomahawk		5.00	4.00	5.00	4.00	5.00

1. The 8 genotypes represent the extremes in susceptibility and tolerance observed within a group of 92 genotypes evaluated.

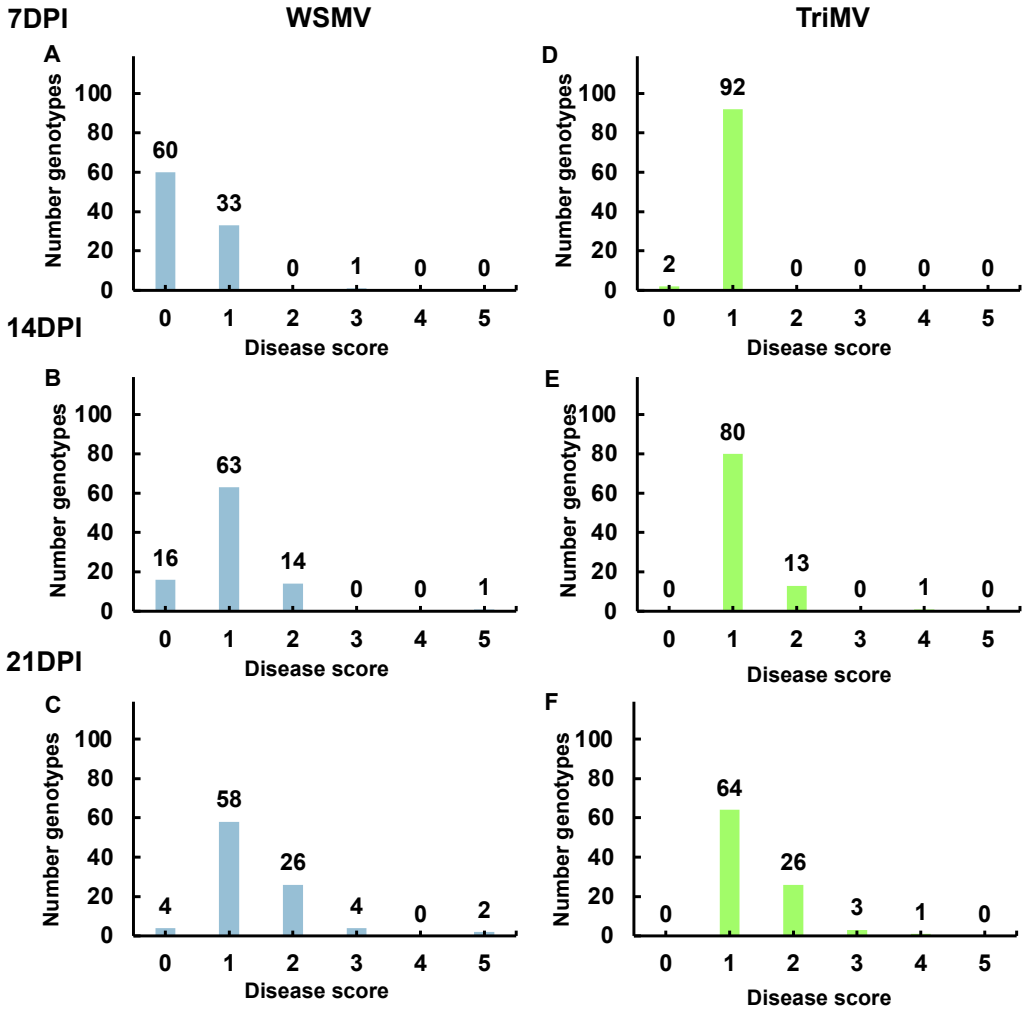

Figure 2: Frequency distribution of disease scores among triticale genotypes (n=92) and controls (NT441, Tomahawk; n=2) at 7, 14, and 21 Days Post-Inoculation (dpi) for Wheat streak mosaic virus (WSMV) panels A, B, C and Triticum mosaic virus (TriMV) panels D, E, F. Severity scores range from 0 (no visible symptoms) to 5 (maximum disease severity). Abbreviations: WSMV = Wheat streak mosaic virus, TriMV = Triticum mosaic virus, dpi = Days post inoculation.

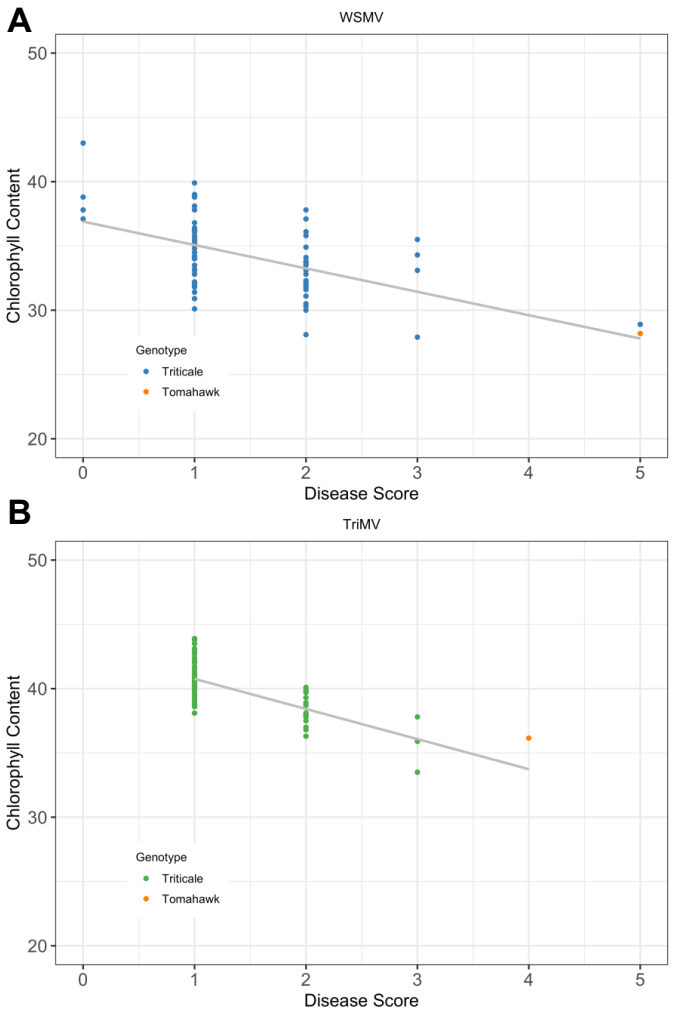

Figure 3: Correlation between disease severity and chlorophyll content in Triticale genotypes including 'Tomahawk' at 21 Days Post-Inoculation (DPI) with Wheat streak mosaic virus (WSMV, panel A) and Triticum mosaic virus (TriMV, panel B). Data include 93 samples: 92 Triticale genotypes and 1 'Tomahawk' sample. Abbreviations: WSMV = Wheat streak mosaic virus, TriMV = Triticum mosaic virus, DPI = Days post inoculation.

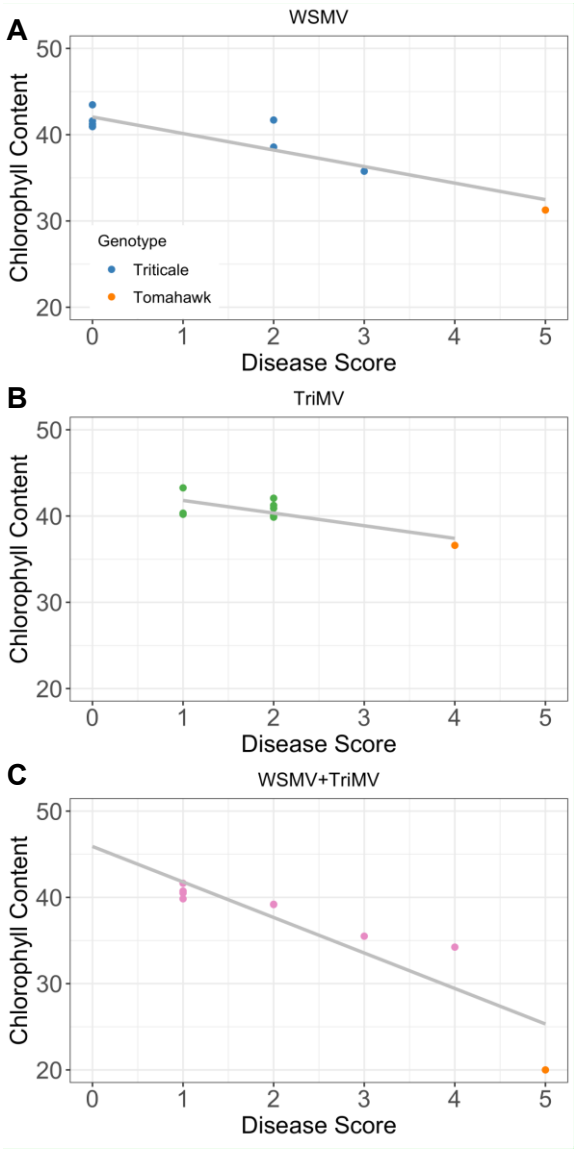

Figure 4: Correlation between severity of viral diseases—WSMV (panel A), TriMV (panel B), and co-infection of WSMV and TriMV (panel C)—and chlorophyll content in a replicated trial involving eight triticale genotypes and a wheat control. Pearson correlation coefficients are -0.92 (A), -0.91 (B), and -0.86 (C), all with p-values <0.001.

4. Discussion

Our research identified triticale as a promising source of resistance for WSMV but not for TriMV. The response to WSMV varied among the genotypes. Four out of 92 genotypes (NT23244, NT23245, NT23246, and NT21246) were immune, showing no signs of infection, while 58 out of 93 exhibited low infection scores of 1, thus being considered tolerant (Figure 2C). In contrast, one genotype, NT23226, showed strong susceptibility (Figure 2C). Under TriMV inoculation, all 92 triticale genotypes tested were susceptible and manifested low to mild symptoms. However, 64 genotypes showed low infection scores of 1, thus being considered tolerant (Figure 2F). In a replicated trial involving a subset of eight selected genotypes, we confirmed the consistency of the phenotypic responses to both viruses and all genotypes maintained the same level of infection as observed in the initial screening. However, co-infection with both viruses resulted in an increased level of disease, indicating synergistic interactions between WSMV and TriMV. This synergistic effect was evident through more severe symptoms and higher disease scores compared to plants inoculated with each virus individually. These findings highlight the importance of understanding virus-virus interactions in managing wheat diseases, as co-infections can lead to more significant yield losses than previously anticipated. (Table 1).

Other studies support our findings that triticale is a potential source for WSMV resistance, but not for TriMV. Gardner et al. (1969) reported that three out of 33 triticale lines inoculated with WSMV were immune, which aligns with our results. However, the lower disease severity in the triticale genotypes suggests a significant suppression of

TriMV, which is greater than the tolerance observed in wheat genotypes. These results also affirm the potential of triticale as a novel source of resistance to WSMV. The consistency of our findings with Gardner et al. (1969) suggests that the resistance mechanism in triticale is stable and robust. In contrast, for TriMV, our research aligns with the findings of Seifers et al. (2010), who reported susceptibility in all 15 triticale lines and cultivars inoculated with TriMV. However, the low infection levels observed in some triticale lines suggest that they could still be valuable sources of tolerance, potentially mitigating the impact of TriMV in infected plants. (Figure 2F).

When co-infected with both viruses, the genotypes that were immune to WSMV (NT23244, NT23245, NT23246, and NT21246) showed low symptoms, suggesting that the infection was due to only TriMV, without a synergism effect. In contrast, the genotypes that were susceptible to both viruses (NT23226, NT23225, and NT23213) showed increased infection when co-infected, suggesting a synergism effect when the genotype is susceptible (Table 1). This finding aligns with the research made by Tatineni et al. (2010), where it was observed that in wheat, when infected with WSMV and TriMV, the damage was enhanced, particularly in susceptible genotypes. Our research also found that susceptible genotypes exhibit an increased symptom severity compared to a single infection. Interestingly, the genotype NT23207 had decreased infection, suggesting that the synergism effect can be genotype-specific, where the infection will act differently for each genotype. Tatineni al. (2010) also reported that in wheat cultivars co-infected with WSMV and TriMV, the synergism effect might be cultivar dependent.

Tolerance and resistance are two mechanisms plants use to manage pathogens. Resistance involves the plant's ability to prevent pathogen multiplication through genetic traits, such as *Wsm1, Wsm2, Wsm3,* and *C2652*. In contrast, tolerance refers to the plant's capacity to minimize the impact of infection on its overall health and productivity, regardless of pathogen levels (Pagán and García-Arenal, 2018). Our study identified four genotypes (NT23244, NT23245, NT23246, and NT21246) that potentially hold resistance genes and 58 genotypes that are tolerant to WSMV. Additionally, 64 genotypes showed low infection levels with TriMV, indicating tolerance. These results highlight triticale's potential as a source of both WSMV resistance and TriMV tolerance, providing valuable insights for breeding disease-resilient varieties.

While this study was focused on phenotypic evaluation, confirmatory ELISA was conducted for TriMV and WSMV to check for the presence of a virus. For WSMV, the virus was not detected in three of the 92 genotypes (NT23245, NT22711, and NT23212) despite showing of symptoms. This discrepancy might be due to the virus load was below the detection level of the ELISA test. Additionally, the small amount of tissue collected (0.2 g) may have been too small for detection, suggesting that the actual infection severity in this study could be higher than observed. In contrast, for TriMV, all 92 genotypes that were susceptible tested positive by ELISA.

Moving forward, future studies should use the insight gained in this research to look deeper into the subset of the eight triticale genotypes. A genetic analysis of both resistant and susceptible genotypes could be conducted to identify promising candidates for resistance. This would involve creating a bi-parental mapping population to study the

genetic diversity underlying resistance traits. Our research identified four genotypes (NT23244, NT23245, NT23246, and NT21246) as potential resistance parents. These genotypes could be crossed with NT23226, a susceptible genotype, to facilitate the mapping of resistance genes and further understand the genetic basis of the resistance. Also, research about the molecular mechanism observed between both viruses with triticale and the physiological response of the triticale lines when infected with WSMV, TriMV, and co-infection of WSMV+TriMV would help us understand the viruses and triticale connection. These future steps will solidify a firm ground for using triticale as a new source for WSMV and TriMV resistance genes.

In summary, we have identified four genotypes (NT23244, NT23245, NT23246, and NT21246) as a potential for a novel source of resistance genes for WSMV. These genotypes showed low infection with TriMV and exhibited the lowest phenotypic scores during co-infection, showing their resilience. Given the challenges caused by WSMV and TriMV to crop yields, the limited sources of resistant genes, and low effective management strategies, identifying new resistance sources is needed. Our findings showed that triticale holds potential for novel resistance genes to WSMV, which will be a great help in sustaining high-yield wheat production in the US.

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APPENDIX 1. LIST OF GENOTYPES AND PEDIGREES

This appendix provides a comprehensive list of the genotypes and checks used in this study, including their pedigrees and associated phenotypic scores for disease resistance. Each genotype has been evaluated for its response to viral infections, and the phenotypic scores reflect the severity of disease symptoms observed. Additionally, chlorophyll content measurements were recorded to assess the overall health and vigor of the plants under study. These data offer valuable insights into the genetic variability and potential resistance mechanisms present in the genotypes analyzed.

CHAPTER 3

PHENOTYPIC EVALUATION OF TRITICALE FOR WHEAT STREAK MOSAIC VIRUS AND TRITICUM MOSAIC VIRUS UNDER UNCONTROLLED ENVIRONMENT

1. Introduction

The Great Plains region of the U.S. is North America's largest hard red winter wheat producer (U.S. Department of Agriculture, Economic Research Service, 2024), producing around 40 percent of the total U.S. wheat production. However, two viral diseases, wheat streak mosaic virus (WSMV) and Triticum mosaic virus (TriMV) present in this region, can negatively affect the yield, causing up to 100% loss in the crop (Burrows et al., 2009). Transmission of WSMV and TriMV is facilitated by wheat curl mite (WCM, *Aceria tosichella* Keifer) (Singh et al., 2018). Currently, the management practices to control these viruses and their vector is very limited. This can be done by late planting, controlling volunteer wheat, and using resistant wheat varieties (Conner et al., 1991; Harvey et al., 1994). However, delays in planting dates can significantly affect yield. Earlier planting dates are positively correlated with higher yields. Additionally, test weight declines by an average of 2.3 lb/bu for each month of delay (Witt 1996). The control of volunteer wheat needs to be done in advance, and growers in the same region need to be involved in preventing the wheat curl mite (WCM) from finding a green bridge to winter wheat (Texas A&M AgriLife Extension, 2015). These limitations show

the need for effective genetic resistance to maintain high-yield wheat crops and secure food production and supply.

Current genetic resistance to both viruses and mites is limited. There are four available genes for both viruses (*Wsm1, Wsm2, Wsm3, and C2656*). The genes *Wsm1 and Wsm3* were identified in intermediate wheatgrass (Thinopyrum intermedium [(Host) Barkworth & DR Dewey)] and confer resistance to WSMV and TriMV (Graybosch et al., 2009 and Liu et al., 2011). Both of these genes are temperature sensitive, where the resistance loses effectiveness over 24 °C (Seifers et al., 1995 and Divis et al., 2006). *Wsm2* was identified in a Colorado hard red winter breeding line, confers resistance to WSMV only, and is also temperature-sensitive (Haley et al., 2002; Seifers et al., 2013). However, recent isolates of WSMV have been found to be virulent to the *Wsm2* gene (Kumssa et al., 2019), and 13% of the plants infected by viruses are co-infected with WSMV and TriMV (Burrows et al., 2009). This indicates that *Wsm2* alone is not sufficient to control these viruses, underscoring the need for new resistance genes. C2652 was identified in a Canadian population of hard red spring wheat (Haber et al., 2006) and remains under investigation.

There are four genes that limit mite reproduction (*Cmc1, Cmc2, Cmc3* and *Cmc4*). *Cmc1* and *Cmc4* are located on chromosome 6DS but at a different locus (Whelan and Thomas 1989; Malik et al., 2003) and were introgressed from *Aegilops tauschii (*Thomas and Conner 1986). *Cmc2* was introgressed from *Agropyron elongatum and is located on* chromosome 6DL of wheat *(*Whelan and Hart 1988). *Cmc3* was

introgressed from rye via triticale and is located on chromosome 1AL (Schlegel and Kynast 1987; Sebesta et al., 1995). Furthermore, certain populations of WCM have developed virulence against the *Cmc3* resistance gene, which has been documented in various studies (Dhakal et al., 2017; Harvey et al., 1997). This adaptation allows the mites to overcome the genetic resistance provided by *Cmc3*, leading to infestations and the spread of the viruses.

Given the limited genetic resistance for either vector or viruses, a need to explore new alternative sources of genetic resistance is emerging. Triticale (*x Triticosecale Wittmack*) is a man-made hybrid crop between wheat (*Triticum* spp.) and rye (*Secale cereale*) (Mergoum et al., 2009) and can potentially be a great source of disease resistance. Studies have shown that triticale is a great source of viral resistance, such as for barley yellow dwarf virus (Collin et al., 1990). In addition, triticale has been bred for the same Great Plains environment as the hard red winter market class for over 50 years; thus, as an adapted crop for the environment, with great grain qualities (citation), it could speed up the development of virus resistant wheat with reduced yield drag compared to other low grain quality penalties. However, triticale resistance to WSMV and TriMV remains unexplored. Two mechanisms are used by plants to control pathogens: resistance and tolerance. Tolerance is the ability of the plant to minimize the adverse effects of infection on its performance, such as growth and yield, without significantly reducing the pathogen load. In contrast, resistance refers to the plant's ability to reduce pathogen

multiplication using genetic traits, such as *Wsm1*, *Cmc1*, and others (Baucom and De Roode, 2011).

The University of Nebraska-Lincoln (UNL) Small Grains Breeding Program has observed strong tolerance in triticale, showing no symptoms in their triticale germplasm when exposed to WSMV and TriMV under field conditions (Dr. Mary Guittieri, personal communication). However, the mechanisms behind this phenotype remain unclear, and no comprehensive study has yet focused on the viral resistance of triticale. To address this gap, our study aims to explore the phenotypic diversity of triticale under field conditions, examining its resistance properties through natural inoculation with both viruses, thereby contributing to the development of genetic resistance strategies for wheat.

This research explores the phenotypic response of UNL triticale genotypes to WSMV and TriMV under field conditions with mite inoculation. We conducted experiments across two different nurseries, planting 92 triticale genotypes for natural inoculation: one nursery in Dighton, KS, and a second in Mead, NE. We aim to compare these results with those from a controlled-environment study, enhancing our understanding of the triticale genotypes' responses in both settings. This analysis will help us select the most susceptible and resistant genotypes to develop a genetic mapping population.

2.Materials and Methods

2.1 Study Design

This study was conducted under field conditions at two locations during the 2023 to 2024 season. The first location was Mead, NE (41.15227° N, 96.49286° W), where the soil pH ranges from 5.1 to 7.3, the soil contains 3.50% organic matter, and the texture is 32.3% clay, 6% sand, and 61.7% silt. The second location was Dighton, KS (38°33'16.66"N, 100°31'46.84"W), where the soil pH is 7.3, the soil contains 1.63% organic matter, and the texture is 27.8% clay, 20.4% sand, and 51.8% silt.

Plants were evaluated for two viruses: WSMV and TriMV. At Mead, NE, a semi-natural inoculation was used. The field was planted on October 10, 2023, and TriMV viruliferous WCMs were inoculated onto wheat plots. Natural inoculation for WSMV was expected. Three checks were used: wheat cv. Mace, which carries the *Wsm1* resistance gene and is resistant to both WSMV and TriMV (Friebe et al., 2011 and Graybosch et al., 2009); the triticale genotype NT441, which shows tolerance to both viruses; and wheat cv. Tomahawk, which is susceptible to both viruses. At Dighton, KS, natural inoculation was used, with all mites coming from WCM hosts. The field was planted on September 22, 2023, next to uncontrolled volunteer wheat with mites present. The planter used was a Hege 1000 pulled by a John Deere 5055E tractor. The seeds was planted into moisture conditions at a depth of 1.25 to 1.5 inches. The field was sprayed with 3oz Zidua SC and 12oz MCPA per acre for weed control on 4/22/24. This location has had natural infection with both viruses for multiple years. Two checks were used: triticale genotype NT441 and wheat cv. Tomahawk.

The study included 92 triticale genotypes from the UNL Small Grains breeding program. These genotypes comprised 40 from the 2023 Preliminary Yield Trial (PYT), an F3:6 generation, and 52 from the Advanced Yield Trial (AYT) entries, an F3:7 generation. These genotypes were developed for forage and grain production in the central and southern Great Plains. At Mead, NE, the 92 genotypes, along with the three checks, were planted randomly in the field with two replications in single rows using a Wintersteiger Rowseed planter with UNL's four rows spaced 12" apart. At Dighton, the first replication was planted in sequence from 1-92 and the second was randomly planted.

The weather conditions in the season, October 2023 through May 2024, were particularly conducive to disease infection due to a warmer fall. The warmer conditions increased mite activity, leading to a greater spread of the disease (Table 1).

Table 1: Average temperatures (°C) in Mead-NE and Dighton-KS, from October 2023 to May 2024.

Month	Location	Average High (°C)	Average Low (°C)
October	**Mead, NE**	18.9	7.2
	Dighton, KS	19.2	8.5
November	**Mead, NE**	11.1	0.6
	Dighton, KS	12.9	2.1
December	**Mead, NE**	5	-5
	Dighton, KS	6.1	-3.2
January	**Mead, NE**	1.7	-7.2
	Dighton, KS	6.8	-3.2
February	**Mead, NE**	4.4	-5.6
	Dighton, KS	7.9	-2.7
March	**Mead, NE**	11.1	-0.6
	Dighton, KS	14.9	2.8
April	**Mead, NE**	17.2	5
	Dighton, KS	18.9	6.4
May	**Mead, NE**	22.8	11.1
	Dighton, KS	24.2	11.6

2.2 Virus inoculation

During the summer of 2023, winter wheat was planted to provide a trap crop for naturally-occurring wheat curl mites and virus for subsequent screening efforts. During the summer, wheat curl mites were also reared in the greenhouse on plants inoculated with Triticum mosaic virus, and these mites were released into the winter wheat trap crop to supplement virus inoculum. On September 25, 2023, the virus screen was planted with triticale entries planted in 1.2 m long rows in two randomized complete block replications. Plots of Mace (virus resistant) and Tomahawk (virus susceptible) winter wheat were interspersed throughout the screen area. The rapid symptom development for the susceptible Tomahawk through the fall indicated that mite and virus pressure in the

plots was very high. Subsequent assay for virus (via PCR) in the plots the following spring indicated extensive and consistent presence of both wheat streak mosaic virus and Triticum mosaic virus across the screen area.

2.3 Phenotypic Evaluation

The phenotypic evaluation for both fields followed the same protocol. The infection levels were visually scored on a scale of 0-5, with 0 indicating no visible disease symptoms and 5 representing the highest severity of disease symptoms (Figure 1). Each genotype was scored on a per-row basis, and the average infection level across a single row was recorded. At Mead, NE, the assessment was conducted at the flag leaf stage, while at Dighton, KS, the assessment was conducted at the flowering stage.

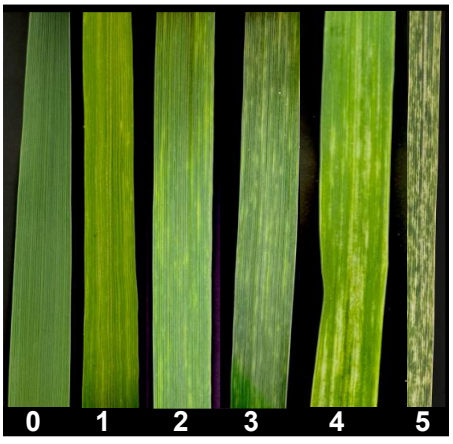

Figure 1: Disease severity scoring for Wheat streak mosaic virus (WSMV), Triticum mosaic virus (TriMV), and their co-infections in Triticale, assessed during phenotyping. Severity scores range from 0 (no visible symptoms) to 5 (extreme disease severity). Visible symptoms start with yellow chlorosis in a mosaic pattern and progress to more severe symptoms. At the highest severity score of 5, the chlorosis evolves into necrosis,

characterized by extensive yellowing and tissue death, observable in infections by both viruses.

Chlorophyll content was measured for each genotype at the same stage and time as the scoring using a chlorophyll meter (model SPAD 502 Plus, Konica Minolta Sensing, Inc., Osaka, Japan). One reading was taken from five plants, and the average score per single row was recorded. Each measurement was taken on the flag leaf, targeting approximately the same area on each leaf to ensure consistency.

Tissue samples were collected from each field for eight genotypes in both replications. These eight genotypes (NT23244, NT23245, NT23246, NT21436, NT23207, NT23226, NT23213, and NT23225) were previously selected in the first chapter of this thesis to represent a range of phenotypic responses to both viruses. Two random plants on each row were selected for each of these eight genotypes, and 4-6 cm of leaf tissue was collected in a sample mesh bag (Agdia) for further analysis via enzyme-linked immunosorbent assay (ELISA). These samples were stored at -80°C until the assay was performed.

2.4 Data Analysis

The genotypes were phenotypically scored based on observed disease symptoms on a scale from 0 to 5. To analyze the relationship between disease severity and chlorophyll content and assess the effects of WSMV and TriMV on plant health, we calculated the Pearson correlation coefficient and tested the significance of the correlation using the cor function in R.

Enzyme-linked Immunosorbent Assay (ELISA)

For both field studies, the ELISA test was performed for the detection of WSMV and TriMV in the eight triticale genotypes and the checks. The methodology followed was adapted from Clark and Adams (1977).

In a sample mesh bag (Agdia) containing the triticale samples, 2 ml of general extraction buffer was added, and the samples were ground using a motorized grinder until sap was produced. Viral IgG-coated 96-well plates were prepared and washed three times with PBS-T (Agdia) at 3-minute intervals. The wells were loaded with 100 µL of extracted sap per well, using two wells per genotype, and incubated in a moist chamber for 1 hour at 37°C. After incubation, the plates were emptied, rinsed with PBS-T, and washed three times with PBS-T at 3-minute intervals.

Next, the wells were loaded with 100 µL of the secondary antibody (Agdia - ALP-conjugated virus-specific IgG, 1:400 in 1X ECI buffer for WSMV, and 1:500 for TriMV) and incubated in a moist chamber for 1 hour at 37°C. The 5X PNP substrate buffer (Agdia) was diluted to 1X, and PNP pellets (1 tablet per 5 ml) were dissolved in the PNP substrate buffer and stored at 4°C in the dark. After incubation, the plates were retrieved, emptied, rinsed with PBS-T, and washed three times with PBS-T at 3-minute intervals. The wells were then loaded with 100 µL of the PNP solution and incubated at room temperature in the dark.

To read the plates, the intensity of the color reaction, which indicates antigen presence and quantity, was measured using a spectrophotometer (Molecular Devices -

SpectraMax) at an optical density of 405 nm. Measurements were taken at 15 and 30 minute intervals post-PNP solution addition. A positive detection was defined as an optical density value at least three-fold higher than that observed in healthy control samples.

3 Results

This study aimed to evaluate the resistance and susceptibility of triticale genotypes to WSMV and TriMV under field conditions in two different locations (Mead, NE and Dighton, KS) and at different plant stages (flag leaf and flowering).

3.1 Mead, NE

The phenotypic scores of the genotypes were assessed at the flag leaf stage. The distribution shows a broad range of disease severity, with all triticale genotypes exhibiting symptoms of the viruses (Figure 2A). Among the 92 triticale genotypes, nine had the highest scores of five and three received the lowest score of one (Table 2). The highest frequency of genotypes had a disease score of three with 22.3%, followed by 17% scoring 3.5 and 14% scoring 2.5 (Figure 2A).

The relationship between chlorophyll content and disease severity under field conditions was evaluated using a Pearson correlation. It was observed that as disease scores increased from 1 to 5, chlorophyll content consistently decreased. The correlation between disease severity and chlorophyll content levels showed a negative correlation of

-0.71, with a p-value <0.0001 (Figure 3A). These findings suggest that higher virus severity is associated with reduced chlorophyll levels.

ELISA was employed to verify the presence of both viruses. Only two of the nine genotypes sampled (NT23226 and Mace) tested positive for WSMV. In contrast, seven out of nine genotypes (Mace, NT23225, NT23246, NT21436, NT23226, NT23213, and NT23225) tested positive for TriMV. These results confirm that both viruses were present in the field.

3.2 Dighton, KS

The phenotypic scores of the triticale genotypes were assessed at the flowering stage. All genotypes showed some level of symptoms; however, the distribution was skewed towards lower disease scores, with 43.6% scoring one, 32.9% scoring 1.5, 18% scoring two, and 4% scoring three. The wheat cv. Tomahawk had the highest score of five (Figure 2B). These findings indicate that plants tend to recover under field conditions at later stages, such as flowering, and the symptoms decrease, which is unlikely to affect the yield.

To investigate the relationship between disease severity and chlorophyll content, their correlation was checked. Our findings showed that as the disease progressed, chlorophyll levels were reduced. A lower negative correlation of -0.48 was observed at the flowering stage, with a p-value <0.0001 (Figure 3B). This lower correlation indicates that the disease does not affect the photosynthetic levels of the plant at this stage.

The ELISA assay was used to detect the presence of both viruses. None of the eight genotypes tested positive for WSMV. In contrast, five of the eight genotypes (NT23245, NT23226, NT23207, NT23213, and NT23225) tested positive for TriMV. Kansas State University also conducted ELISA on 33 samples, where 14 out of 33 were positive for WSMV and all co-infected with TriMV. Additionally, 24 out of 33 samples were positive for TriMV, with 10 of these positive only for TriMV. Among these samples, five triticale genotypes were tested; all genotypes were negative for WSMV and 2 out of 5 were positive for TriMV (C. Day, personal communication). These results confirm that TriMV and WSMV were present in the field; however, only TriMV infected the triticale lines.

Table 2: Phenotypic response of the most resistant and susceptible triticale genotypes

Location: Mead, NE	
Genotype	**Score**
NT23204	5
NT23220	5
NT23226	5
NT23228	5
NT23229	5
NT23236	5
NT23240	5
NT22736	5
NT23201	5
NT23244	1
NT23245	1
NT232212	1

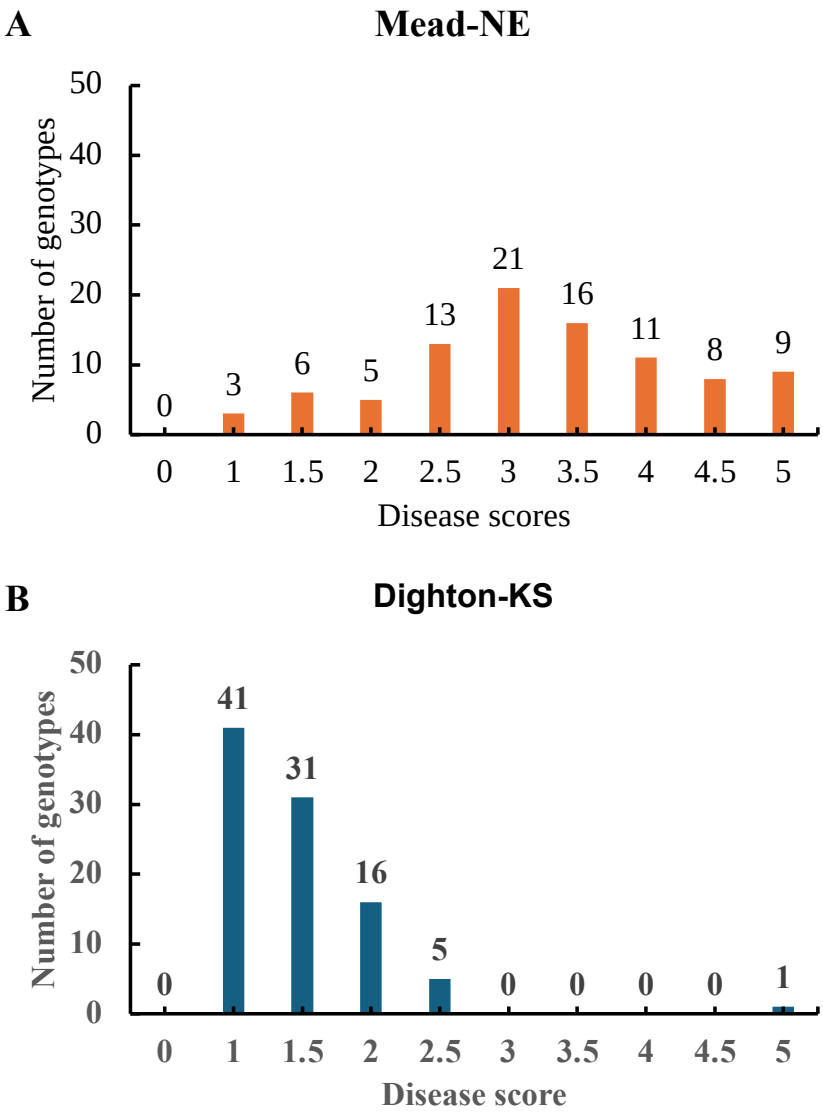

Figure 2: Frequency distribution of disease scores for 92 triticale genotypes and checks at two locations. In Mead - NE, the assessment was performed on the flag leaf (A), while in Dighton - KS, it occurred at flowering (B). A uniform 0-5 scoring system was applied, with a score of 0 indicating no visible symptoms and a score of 5 representing the highest severity of disease symptoms. Note: Two genotypes in Mead, NE were not scored.

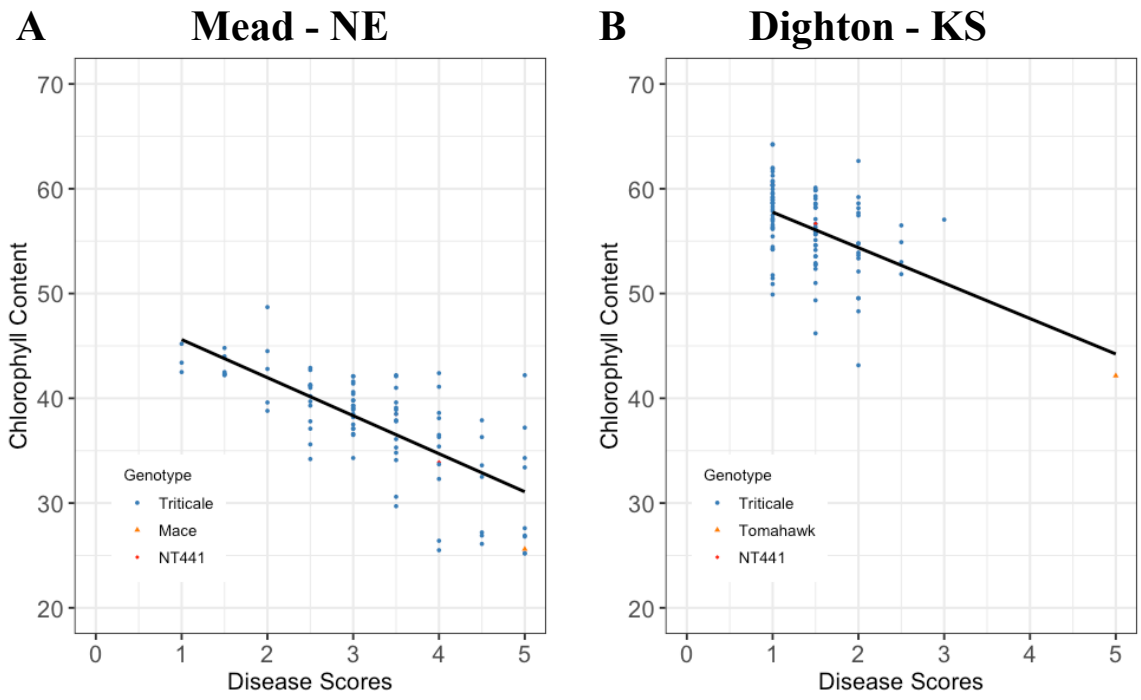

Figure 3: Correlation between disease severity and chlorophyll content in 92 triticale genotypes and checks. In Mead - NE, the assessment was performed on the flag leaf (A), while in Dighton - KS, it occurred at flowering (B). The Pearson correlation coefficients are -0.71 and -0.48, respectively, with p-values <0.001 for each analysis. Chlorophyll content represents a relative value indicating the greenness or chlorophyll concentration in the leaf.

4 Discussion

Our study demonstrates the phenotypic diversity of tolerance to WSMV and TriMV among triticale genotypes under field conditions. The genotypes at Mead, NE, exhibited higher symptom severity compared to those at Dighton, KS. Specifically, at

Mead, 70% of the genotypes were scored at 3 or higher, whereas all triticale genotypes at Dighton were scored below 3 (Figure 2). Notably, three triticale genotypes (NT23212, NT23244, and NT23245) that had the lowest score of 1 at Mead also received the same score at Dighton, indicating that these genotypes exhibit strong tolerance to both viruses. The genotypes NT23244 and NT23245 were also the most tolerant in a greenhouse experiment, showing no symptoms to WSMV and low symptoms to TriMV (Chapter 1). Additionally, three genotypes NT23204, NT23224, and NT23226 received the highest scores of 5 in Mead, NE, and 2.5 in Dighton, KS, showing consistent susceptibility in both environments. This consistent performance across different environments suggests that these genotypes could be promising candidates as a parent for a for a bi-parental mapping population.

At Dighton, KS, assessments conducted at the flowering stage showed that the triticale genotypes exhibited lower disease severity. Specifically, 43.6% of the genotypes scored 1, 32.9% scored 1.5, 18% scored 2, 4.2% scored 2.5, and the highest score of 5 was observed in the check Tomahawk (Figure 2B), indicating less severe infection at this location. Additionally, the lower negative correlation of -0.48 between disease severity and chlorophyll content observed at Dighton, KS, at the flowering stage, compared to the correlation of -0.71 at Mead, NE, at the flag leaf stage, suggests that the plant's photosynthetic capacity is less affected as they mature. This finding indicates that the yield will likely remain unaffected at Dighton, KS, due to plant recovery. Age-specific recovery has been demonstrated in various crops, such as potato with late blight, oat with stem rust, and wheat with root rot (Boardfoot, 1933; Peturson, 1944; Peterson and Mills,

1953). In these cases, age-specific recovery was associated with lower infection rates and reduced yield losses. Additionally, the age of the vector infecting the plants is associated with a lower impact on yield (Eigenbrode and Gomulkiewicz, 2022). These findings align with our results, where in Dighton, natural inoculation led to a slower spread of the vector and combined with later infection, resulted in a reduced impact on the triticale genotypes.

The broad variability of resistance to WSMV and TriMV observed under field conditions can be attributed to a range of environmental factors that are less controlled than in laboratory or greenhouse settings. In the field conditions of the Great Plains of the US, the presence of vectors such as the wheat curl mite, particularly Type 1 and Type 2 (Hein et al., 2012), which naturally disseminate the viruses, adds another layer of complexity. In contrast, greenhouse conditions require manual inoculation of the viruses, which can lead to more uniform infection rates and better repeatability and reliability (Langstroff et al., 2022). In greenhouse experiments (Chapter 1), 95% of the genotypes were scored below 3 for both viruses. In contrast, at Mead, NE, there was a broader range of susceptibility, with 70% of the genotypes scoring over 3. These findings suggest that environmental factors, such as the use of the vector WCM, affect disease severity compared to controlled environments where the diseases were manually inoculated.

In greenhouse experiments, four triticale genotypes (NT23244, NT23245, NT23246, and NT21436) consistently exhibited lower symptoms from the co-infection of both viruses (Chapter 1). Under both field conditions at Mead, NE and Dighton, KS, where ELISA tests detected primarily TriMV but also WSMV, these genotypes exhibited

low symptoms with scores of 1, 1, 1.5, and 3, respectively. This suggests that environmental factors had a minimal influence on the disease development in these tolerant lines.

While this study was based on phenotypic screening, ELISA was performed in both fields to check for the presence of both viruses. Only two out of nine genotypes tested positive for WSMV at Mead, NE (NT23226 and Mace). In contrast, seven out of nine genotypes were positive for TriMV, with Mace and NT23226 co-infected with both viruses. At Dighton, KS, no WSMV was detected, while five out of eight genotypes tested positive for TriMV. Kansas State University also conducted ELISA in Dighton, KS, where no WSMV was detected, while 2 out of 5 triticale samples were positive for TriMV. However, WSMV was found in 14 out of 33 wheat samples in the same field (C. Day, personal communication). These findings suggest that while WSMV was present in both fields, the triticale lines were not affected. In contrast, the triticale showed moderate susceptibility to TriMV, since the scores were very low. It is important to note that at Mead, NE, there was a manual inoculation of WCM infected with TriMV, which could have influenced the dissemination of this virus and increased the disease incidence. At Dighton, KS, where natural inoculation was the method, management of volunteer wheat in the fall could have likely contributed to the lower incidence of the viruses.

Despite the promising results from this study, some improvements can be made to further evaluate the triticale genotypes. The eight genotypes selected in Chapter 1, which showed consistency under field conditions, should be planted with more replications at each location and across multiple locations throughout the Great Plains of the US over at

least two years to account for annual variability in disease incidence. Additionally, more detailed quantification of the viruses should be implemented. Although ELISA tests were conducted in this experiment, they only confirmed the presence or absence of the viruses. Techniques such as quantitative qPCR could provide more precise measurements of viral load and help identify genotypes with stronger resistance. Expanding the range of environmental conditions and pathogen pressures in the trials will ensure that the selected triticale lines have durable resistance. Additionally, starting a bi-parental mapping population with the two genotypes that exhibit tolerance to the viruses under field and greenhouse conditions (NT23244 and NT23245) by crossing them with the susceptible genotype NT23226 and conducting QTL mapping will help identify specific genetic regions associated with resistance. This approach will facilitate the identification of new resistance genes to WSMV and TriMV.

In conclusion, this study demonstrates the potential of triticale as a valuable source of resistance to WSMV and TriMV under field conditions. The phenotypic diversity observed among the triticale genotypes highlights the possibility of identification of gene resistance that could be further introgressed into wheat varieties through targeted breeding programs. These findings align with the broader goal of improving disease management strategies to maintain high-yield wheat crops. By using the resistance properties of triticale, we can enhance the resilience of wheat, contributing to sustainable agricultural practices and ensuring a stable food supply.

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