Effect of Cultivars and Temperature on Synergistic Interaction Between Panicum Mosaic Virus and Satellite Panicum Mosaic Virus in Switchgrass

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Abstract

Panicum mosaic virus (PMV), the type species of the genus *Panicovirus* in the family *Tombusviridae*, naturally infects switchgrass (*Panicum virgatum* L.). PMV and its molecular partner, satellite panicum mosaic virus (SPMV), interact synergistically in co-infected millets with exacerbated disease phenotype and increased accumulation of PMV, compared to plants infected only by PMV. In this study, we examined the reaction of switchgrass cvs. Summer and Kanlow to PMV and PMV+SPMV infections at 24°C and 32°C. Switchgrass cv. Summer was susceptible to PMV at both temperatures. In contrast, cv. Kanlow was tolerant to PMV at 24°C but not at 32°C, suggesting that Kanlow harbors temperature-sensitive resistance against PMV. At 24°C, PMV was readily detected in inoculated leaves but not in upper non-inoculated leaves of Kanlow, suggesting that resistance to PMV was likely mediated by abrogation of long-distance virus transport. Co-infection by PMV and SPMV at 24°C and 32°C in cv. Summer but not in Kanlow caused increased symptomatic systemic infection and mild disease synergism with slightly increased PMV accumulation compared to plants infected only by PMV. These data suggest that the interaction between PMV and SPMV in switchgrass is cultivar dependent, manifested in Summer but not in Kanlow. However, co-inoculation of cv. Kanlow by PMV+SPMV caused an enhanced asymptomatic infection, suggesting a role for SPMV in enhancing symptomless infection in a tolerant cultivar. These data suggest that enhanced asymptomatic infections in virus-tolerant switchgrass cultivar could serve as a source for virus spread and play an important role in panicum mosaic disease epidemiology under field conditions. Our data revealed that cultivars, co-infection with SPMV, and temperature influenced the severity of symptoms elicited by PMV in switchgrass.

Introduction

Switchgrass (*Panicum virgatum* L.) is a hardy perennial warm-season C_4 grass native to the region east of the Rocky Mountains in the United States. Switchgrass is being used for soil conservation, forage, ornamental grass, fiber, biosequestration of atmospheric carbon dioxide, and biofuels [1, 2]. Switchgrass gained prominence since the beginning of the 21st century as research efforts were focused on lignocellulosic biomass crops for the generation of biofuels [1, 3]. Switchgrass can be grown on marginal lands with minimal agronomic inputs, resulting in net bioenergy production outputs of more than five times the total resource inputs [3-5]. With the advancement of switchgrass cultivation practices and the development of processing technology, the lignocellulosic-based biofuel yield from switchgrass could be similar to ethanol yield from maize [3, 4]. However, biomass production from switchgrass is dependent on stable yields over several years and requires good local adaptation of cultivars [6].

In Nebraska, tetraploid switchgrass upland cultivar (cv.) Summer and lowland cv. Kanlow are the backbone of the USDA ARS breeding programs [7, 8] and have been used to generate a dedicated bioenergy cv. ‘Liberty’ [2]. Switchgrass is susceptible to several fungal and viral pathogens, and these infections could cause significant losses to ethanol yields [9-14]. A multi-year survey of switchgrass breeding plots in Nebraska revealed a high prevalence of infections by panicum mosaic virus (PMV) and its satellite virus, satellite panicum mosaic virus (SPMV) [12]. In 2012, 72% of 139 symptomatic plants

were infected by PMV, with 19% of plants co-infected with PMV and SPMV. Infections by PMV or PMV+SPMV were more prevalent on Summer or its progeny with severe symptoms than on Kanlow [12], suggesting that cv. Kanlow harbors some level of resistance to PMV. However, the levels of cv. Kanlow resistance or susceptibility of Summer to PMV have not been comparatively demonstrated experimentally.

Though PMV and SPMV were first reported in 1953 and 1976, respectively, on switchgrass from Kansas in the USA [15, 16], most research on interactions between PMV and SPMV has been conducted using other experimental hosts such as proso millet (*Panicum miliaceum* L.) and pearl millet [*Cenchrus americanus* (L.) Morrone]. PMV is the type species of the genus *Panicovirus* in the family *Tombusviridae*. The genome of PMV contains a single-stranded plus-sense RNA genome of ~4300 nucleotides (nt) encapsidated in ~30 nm icosahedral virions [17]. PMV acts as a helper virus for its satellite virus, SPMV, and for two satellite RNAs, satS and satC [18, 19]. SPMV is a member of the genus *Papanivirus* and contains a single-stranded positive-sense RNA genome of ~820 nt encapsidated in 16 nm icosahedral virus particles [18, 20]. The SPMV genome contains a multifunctional ORF encoding for 17 kDa CP. In addition to virion assembly, the SPMV CP is also involved in synergistic interaction with its helper virus PMV [21, 22]. PMV elicits mild mottling or symptomless infection in millets. In contrast, co-infection of millets by PMV and SPMV caused exacerbated symptoms with drastically enhanced accumulation of PMV compared to infection only by PMV [21-23].

Experimental evidence is lacking in switchgrass on whether co-infection by PMV and SPMV elicits similar synergistic interaction that was previously observed in millets. A preliminary investigation on the effects of temperature, cultivar, and synergistic interaction between PMV and SPMV was conducted using anti-PMV sera [24]. These data suggested that Kanlow plants were more resistant than Summer plants, although accumulation of PMV and SPMV were not specifically quantitated. Additionally, it has been shown with other virus-plant interaction studies that an elevated temperature can inhibit plants' defense response and facilitate systemic infection at higher temperatures [25-27]. Therefore, this study was conducted to i) examine differences between Kanlow and Summer in their resistance to PMV, ii) determine whether the synergistic effect of co-infection with SPMV occurs in these two switchgrass cultivars, and iii) assess the effect of temperature on the reactions of the switchgrass cultivars to PMV and the PMV+SPMV co-infection. In this study, we found that Summer was susceptible to PMV and PMV+SPMV with a mild synergistic interaction. In contrast, Kanlow was tolerant to PMV at 24°C but not at 32°C, suggesting temperature-dependent resistance. However, co-infection of Kanlow by PMV+SPMV at 24°C resulted in enhanced asymptomatic infection.

Materials And Methods

Experimental design

A 2x2x3 factorial experiment was conducted comparing infection of switchgrass cultivars (Kanlow and Summer) at two temperatures (24°C and 32°C) following three viral inoculation treatments (mock, PMV,

and PMV+SPMV). The experiment was conducted twice, and the results from two independent experiments were combined for the analysis. In each experiment, ten switchgrass seedlings per cultivar were inoculated with each of the two virus treatments, and five seedlings were used for mock inoculation with buffer. Inoculated switchgrass seedlings were transferred into growth chambers (Conviron A2000) at 24°C or 32°C with 16 h light (300 mmole m^{-2} s^{-1}) and ~35% relative humidity. The inoculated plants were kept in growth chambers in a randomized array.

Propagation of switchgrass cultivars

Switchgrass cvs. Summer and Kanlow were grown in cone-tainers (Stuewe & Sons, Inc.; 3.8 cm diameter, 21 cm depth, and 164 ml volume) that were filled with a pasteurized greenhouse potting mix (1 part loam soil, 2 parts peat moss, 1 part sand, and 1 part vermiculite). Eight switchgrass seeds were initially planted per cone-tainer, and as seeds germinated, the seedlings were thinned to two per cone-tainer. Prior to inoculation, plants were grown in a greenhouse with an average temperature of 26°C for about three weeks, or until the second true leaf stage. Cone-tainers were arranged on racks (30 cm W x 61 cm L x 17 cm H) and watered uniformly by placing trays under the racks of cone-tainers and filling the trays with water to keep the bottom of the cone-tainers submerged. Once a week, trays were filled with a low dose of fertilizer (20-20-20 NPK) at 250 ppm.

Virus inoculation

A Nebraska strain of PMV (PMV-NE) and a Kansas strain of SPMV (SPMV-KS) were used in this study [23]. *In vitro* generated transcripts of PMV or PMV and SPMV (PMV+SPMV) were inoculated onto proso millet cv. Sunup seedlings at the two-leaf stage as described in Chowda-Reddy et al. [23]. PMV or PMV+SPMV infected proso millet leaves were harvested at 14 days postinoculation (dpi) and stored at -80°C until used. PMV or PMV+SPMV inoculum was prepared by grinding 1 g of virus-infected proso millet tissue in 19 ml of 20 mM sodium phosphate buffer, pH 7.0. Switchgrass leaves were rub-inoculated by dipping a pestle into the inoculum and rubbed on the adaxial side of the second leaf of each plant in an upward motion 4 times with medium pressure. Mock inoculations were performed with 20 mM sodium phosphate buffer, pH 7.0.

Sample collection and symptom severity rating

The treatment combinations were evaluated for their effects on local and systemic infection. For local infection, the inoculated leaf was collected from each plant at 7 dpi into a separate Ziploc® bag for examination of local infection of PMV. For systemic infection, the 4^{th} upper non-inoculated true leaf of each plant was rated at 21 dpi for symptom severity and collected into a separate Ziploc® bag. All samples were stored at -80°C until further processing. Symptom ratings were based on a 0-3 scale, 0 =no symptoms, 1=≤25% of leaf exhibiting chlorotic spots and mottling symptoms, 2=25-75% of leaf exhibiting chlorotic spots, mottling, and mosaic symptoms, 3=>75% leaf exhibiting chlorotic or bright chlorotic spots, mottling, mosaic symptoms.

Virus detection and quantification

Leaf tissue was ground in liquid nitrogen and processed for RNA extraction using Direct-Zol RNA Miniprep kit (Zymo Research, Irvin, CA), which required the use of TRIzol reagent (Life Technologies, Carlsbad, CA). PMV detection in inoculated leaf tissues was performed using reverse transcription, followed by polymerase chain reaction (RT-PCR). First-strand cDNA synthesis was conducted using SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA) with a PMV-specific reverse primer (PMV-R2, 5'-CACTGAACTACTCTGGAT TAGTAC-3', complementary to nts 4233 to 4210 of PMV) at 50°C for 60 min, followed by 70°C for 15 min. PCR was performed with a forward primer PMV-F2 (5'-AAGCCCATTTACT CGGGAAGTGC-3', corresponding to nts 3548 to 3570 of PMV) and a reverse primer (PMV-R2) with following amplification conditions: 2 minutes at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 59°C, and 1 minute at 72°C with final amplification of 5 minutes at 72°C. The RT-PCR products were examined through 1% agarose gel electrophoresis in 1XTAE (Tris-acetate-EDTA buffer) with a GeneRuler 1.0 Kbp ladder (Thermo Scientific) as a DNA size marker.

To determine relative amounts of PMV and SPMV associated with systemic infection, total RNA extracted from the 4th upper non-inoculated leaf was used for reverse transcription-quantitative PCR (RT-qPCR). The first-strand cDNA was synthesized by using iScript^{TM} Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA) by incubating 5 minutes at 25°C, followed by 20 minutes at 46°C, and 1 minute at 95°C. The RT-qPCR was performed with Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA) using forward and reverse primers specific to PMV and SPMV, as described in Chowda-Reddy et al. [23]. The RT-qPCR amplification started with a hot-start of 3 minutes at 95°C, followed by 44 cycles of 10 seconds at 95°C and 30 seconds at 60.3°C. Samples without RNA template and reverse transcription were included as negative controls. The switchgrass casein kinase 1-like protein 2 and 26S proteasome regulatory subunit S2 1A genes were used as internal controls for determining the relative expression of PMV and SPMV genomic RNA copies using the ΔCt method [28].

Data analysis

Data from the two repetitions of the experiment were pooled for statistical analysis. Quantitative data, i.e., disease severity ratings and virus titer measurements, were subjected to analysis of variance for a randomized design was conducted using treatment means that were computed with the Lsmeans package (version 2.27-62). The Least Significant Difference (LSD) was used for mean separation by using the LSD.test package and differences between treatment means at the 95% confidence level were considered to be significant. For proportional data, i.e., infection incidence, Chi-squared analysis of infection rates was performed using JMP (version 12.2.0), followed by Analysis of Means for Proportions (ANOMP) for significance grouping.

Results

Kanlow was tolerant to PMV infection at 24°C but not at 32°C

The reaction of switchgrass cvs. Summer and Kanlow to systemic infection by PMV at 24°C and 32°C was determined at 21 dpi on the basis of symptomatology. Switchgrass plants exhibiting chlorotic streaks and spots, mosaic, and mottling symptoms in upper noninoculated leaves were considered positive for infection. At 24°C, 60% of Summer plants inoculated with PMV alone exhibited symptoms, whereas only 15% of Kanlow plants developed systemic symptoms (Fig. 1). These data indicated that, at 24°C, the Kanlow population exhibited a higher frequency of genotypes with resistance to PMV compared to the Summer population. In contrast, the infection frequency at 32°C for Summer (75%) and Kanlow (70%) plants inoculated with PMV alone was higher than the respective plants held at 24°C, with the difference between Kanlow populations being significant (Fig. 1). None of the mock-inoculated plants of Summer or Kanlow exhibited symptoms. These data suggest that irrespective of temperature, the Summer population was susceptible to systemic infection by PMV. In contrast, the Kanlow population was tolerant to PMV at 24°C but not at 32°C indicating temperature-sensitive resistance against PMV.

PMV elicited efficient local infection on switchgrass cv. Kanlow

The temperature-dependent resistance of Kanlow to PMV was further examined to detect if this virus can cause a local infection that facilitates cell-to-cell movement on inoculated leaves before entering the vasculature required for systemic infection. Total RNA extracted from inoculated leaves of both cultivars at 7 dpi was used for RT-PCR. The RT-PCR assay revealed that PMV was detected in 95% of inoculated leaves of Kanlow at both temperature regimes (Fig. 2), suggesting that PMV initiated local infection and established cell-to-cell movement in inoculated leaves. The local infection of PMV in Kanlow was found to be identical to that of 95% of infections observed in inoculated leaves of Summer at 24°C and 32°C (Fig. 2). PMV was not detected in any mock-inoculated leaf (data not shown). These data suggested that PMV facilitated efficient cell-to-cell movement in inoculated leaves of Kanlow.

Interaction between PMV and SPMV on systemic infection of Summer and Kanlow

Interaction between PMV and SPMV on systemic infection was discerned by comparing the results of inoculations with PMV alone with those with PMV+SPMV. At 24°C and 32°C, co-inoculation of Summer plants with PMV and SPMV caused a statistically significant increase in the number of plants systemically infected, as indicated by the presence of symptoms, compared to those with PMV alone. At 24°C, inoculation of Summer seedlings with PMV+SPMV elicited symptoms in 90% of plants compared to 60% plants exhibiting symptoms by PMV alone (Fig. 1). At 32°C, 75% and 100% of Summer plants inoculated with PMV or PMV+SPMV, respectively, exhibited chlorotic streaks and mottling symptoms (Fig. 1). In contrast, there was no significant difference in infection of Kanlow plants by PMV with or without SPMV at either temperature as to the percentage of plants exhibiting systemic symptoms (Fig. 1). At 24°C, PMV+SPMV elicited symptoms in 20% of Kanlow plants compared to 15% of symptomatic infection by PMV. At 32°C, 70% and 75% of Kanlow plants produced symptomatic infection by PMV or PMV+SPMV, respectively (Fig. 1). These data suggest that the interaction between PMV and SPMV in switchgrass is cultivar dependent, manifested in Summer but not in Kanlow.

Co-infection of PMV and SPMV augmented asymptomatic systemic infection in Kanlow

To verify systemic infection determined by symptomology and to examine the possibility of asymptomatic infection by PMV in both switchgrass cultivars, total RNA isolated from the 4th upper fully expanded leaf was used as a template for RT-qPCR [23]. The RT-qPCR analysis revealed that all treatments have a higher number of infected plants than indicated by symptomatology (Fig. 1), except for PMV+SPMV-infected Summer at 32°C, in which 100% systemic infection was indicated by both methods. The additional plants shown to be infected by RT-qPCR were considered to be asymptomatically infected. For Summer plants incubated at 24°C, RT-qPCR revealed that 70% and 100% of plants were found to be positive for PMV and PMV+SPMV infections, respectively, compared to 60% and 90% of visually assessed symptomatic infections (Fig. 1); thus, 10% asymptomatic infections for both virus treatments. In Summer at 32°C, 100% of PMV inoculated plants were infected based on RT-qPCR assay compared to 75% of plants being symptomatic, leading to 25% additional asymptomatic infections (Fig. 1). All of the Summer plants inoculated with PMV+SPMV were infected at 32°C based on both assessment methods (Fig. 1).

RT-qPCR analysis indicated that, at 24°C, PMV or PMV+SPMV infected 35% and 70%, respectively, of Kanlow plants compared to 15% and 20%, respectively, being infected based on symptoms (Fig. 1). These data revealed that, at 24°C, PMV and PMV+SPMV caused asymptomatic systemic infection in 20% and 50% of Kanlow plants, respectively. At 32°C, RT-qPCR detected systemic infections in 95% and 100% of Kanlow plants inoculated with PMV or PMV+SPMV, respectively, compared to 70% and 75% symptomatic infections (Fig. 1). These data indicated 25% of asymptomatic infections resulted from both virus treatments in Kanlow at 32°C. Taken together, the data indicate a proportion of the Summer and Kanlow population to be susceptible to asymptomatic infection by PMV. That proportion was substantially increased in Kanlow at 24°C by inoculation with PMV+SPMV, suggesting that co-infection of PMV and SPMV can augment asymptomatic systemic infection.

Co-infection by PMV and SPMV elicited mild synergistic interaction in Summer but not in Kanlow

At 21 dpi, the upper noninoculated 4th leaves were rated for symptom severity rating on a 0-3 scale. No symptoms were observed in mock-inoculated plants. Symptoms were evaluated from symptomatic plants of two independent experiments of 10 plants each. To compare symptom severity, a mean rating was calculated for each treatment, with plants exhibiting no visible symptoms (rating of 0) being excluded from the mean calculation. In Summer plants, the temperature had no significant effect on symptom severity when the plants were inoculated with PMV alone, with mean symptom scores of 1.4 resulting from infection by PMV at 24°C and 32°C (Fig. 3). Infection of Summer plants with PMV+SPMV resulted in mean symptom severity scores of 2.0 and 2.2 at 24°C and 32°C, respectively, which were a modest increase over the corresponding scores from infection with PMV alone (Fig. 3). The difference between PMV and PMV+SPMV treatments was statistically significant at 32°C but not at 24°C.

In Kanlow, the severity of symptoms resulting from infection by PMV alone was temperature-dependent with a symptom score of 1.0 at 24°C compared to a statistically significant symptom score of 2.3 at 32°C (Fig. 3). However, co-infection of Kanlow plants by PMV+SPMV at 24°C and 32°C resulted in no increase

in symptom severity over infection with PMV alone (Fig. 3). Taken together, these data indicated that switchgrass cultivars, co-infection with SPMV, and temperature influenced the severity of symptoms elicited by PMV in switchgrass.

Synergistic interaction between PMV and SPMV caused slightly enhanced accumulation of PMV genomic RNA

The effect of interaction between PMV and SPMV in two switchgrass cultivars at 24°C and 32°C on the accumulation of genomic RNA copies of PMV was examined by RT-qPCR. Total RNA isolated from the true 4^{th} leaves of switchgrass cultivars symptomatically infected by PMV or PMV+SPMV was used for RT-qPCR. The $\log_2$ fold change in accumulation of genomic RNA copies of PMV and SPMV was presented in Figure 4.

At 24°C, the genomic RNA copies of PMV accumulated at a statistically insignificant level in PMV+SPMV-infected Summer plants at 1.29 $\log_2$ fold more copies than those in PMV-infected plants. However, at 32°C, the change was only 1.06 $\log_2$ fold in co-infected plants compared to those of PMV-infected Summer plants (Fig. 4). These data suggest that co-infection of Summer plants by PMV+SPMV caused only a marginal increase in PMV genomic RNA accumulation.

In contrast, in Kanlow plants, co-infection by PMV+SPMV caused a statistically insignificant increase in PMV genomic RNA copies (1.13 $\log_2$ fold) at 24°C compared to infection only by PMV (Fig. 4). At 32°C, the PMV genomic RNA copies accumulated at a slightly decreased level (0.89 $\log_2$ fold) in PMV+SPMV-infected plants compared to those infected by PMV (Fig. 4). These data suggest that the co-infection of Kanlow by PMV and SPMV did not cause a significant increase in accumulation of PMV genomic RNA copies at 24°C and 32°C. Notably, viral loads of PMV and SPMV were significantly lower in singly and doubly infected Kanlow plants compared to those in Summer plants, suggesting that virus propagation was potentially suppressed in Kanlow plants.

Discussion

Although PMV and SPMV were first reported as pathogens of switchgrass [15, 16], the majority of interaction studies between these two viruses were investigated in other experimental hosts, such as proso millet and pearl millet [21-23, 29-31]. Previously, Stewart et al. [12] surveyed switchgrass nurseries in Nebraska and found that fewer Kanlow plants were infected by PMV but not co-infected with SPMV. In contrast, a large proportion of Summer plants were co-infected by PMV and SPMV. The physiological reasons for these findings were not explored. In this study, we found that Kanlow was tolerant to PMV at 24°C with only 15% of inoculated plants developed visible symptoms of viral infection. However, the level of Kanlow resistance was drastically reduced at 32°C with 70% of plants showing visible symptoms of chlorotic streaks, mosaic, and mottling. These data suggest that Kanlow possesses temperature-sensitive resistance against PMV. As expected, cv. Summer was susceptible to PMV at both temperatures with 60 to 100% of plants infected. The nature of resistance of Kanlow to PMV is not known. However, extreme

resistance was not observed in Kanlow against PMV since it was detected in inoculated leaves at the site of infection, suggesting efficient cell-to-cell movement of PMV in Kanlow. It is unlikely that the virus would be detected by RT-PCR in inoculated leaves without cell-to-cell movement. Resistance has been observed for long-distance movement of viral infections, but not at the cell-to-cell level for other viruses [26, 38-41]. Our data suggest that resistance in Kanlow against PMV was temperature-dependent, similar to that reported for wheat cultivars with the *Wsm1* or *Wsm2* genes [26, 42, 43].

We observed a mild hypersensitive reaction to PMV infection on a few inoculated leaves of Kanlow, suggesting that Kanlow could harbor a dominant resistance (R) gene encoding nucleotide-binding site leucine-rich repeat proteins [32, 33]. The R gene-mediated resistance in a majority of pathosystems leads to a hypersensitive reaction with localized cell death or extreme resistance [34, 35]. The switchgrass genome encodes over 1000 R genes with variations in their expression across different switchgrass cultivars. Additionally, the expression of these genes was developmentally regulated in field-grown Summer plants [36]. More recently, a global transcriptomic study over a developmental time course of the 4th leaf of greenhouse-grown Kanlow and Summer plants was reported [37]. Under greenhouse conditions, both cultivar-dependent and -independent gene networks were detected. Cultivar-independent gene networks included those governing normal leaf developmental processes such as photosynthesis and senescence. The cultivar-dependent gene networks included several genes coding for nucleotide-binding leucine-rich repeat (NB-LRR; R) proteins and cell wall kinases orthologous to genes that typically respond to environmental cues and influence plant defense responses. Notably, genomic resequencing data indicated that many of these NB-LRRs were not expressed or potentially absent in the genomes of Summer plants [37], raising the intriguing possibility that these differences could underlie differential viral responses observed in the current study.

In this study, we found cultivars, co-infection with SPMV, and temperature played important roles in disease development in switchgrass by PMV. Co-infection by PMV+SPMV caused significantly enhanced symptomatic systemic infection in Summer, but not in Kanlow, compared to respective infections by PMV, suggesting that SPMV interaction with PMV is a cultivar specific for enhanced systemic infections. Incubation of switchgrass cultivars at 32°C produced enhanced symptom phenotype similarly by PMV or PMV+SPMV in Kanlow but not in Summer, suggesting that Kanlow but not Summer produced high temperature-specific interaction with host factors as reported in wheat [26]. Co-infection by PMV+SPMV interacted synergistically at 24°C and 32°C in Summer but not in Kanlow with enhanced symptom phenotype and a slight increase in PMV accumulation compared to infections only by PMV. These data suggest that PMV and SPMV interacted modestly, independent of temperature with enhanced symptom phenotype in Summer but not in Kanlow.

Synergistic interaction between PMV and SPMV in proso millet caused enhanced accumulation of PMV compared to infection only by PMV [22, 23]. A two amino acid difference in the CP of SPMV isolates was responsible for differential synergistic interaction with PMV [23]. In this study, we found that co-infection of Summer at 24°C and 32°C caused mild disease synergism with a slight increase in symptom phenotypes compared to severe disease synergism in co-infected millets. Although, some Summer plants

infected only by PMV also elicited symptoms similar to those by PMV+SPMV pointing to differences in underlying genetics with the population of switchgrass cultivars. PMV causes symptomless or mild mottling symptoms on proso millet, but coinfection with SPMV caused disease synergism with severe necrosis and occasional death of plants [22, 23]. In contrast, PMV elicits mosaic, chlorotic streaks, and mottling symptoms with little or no synergistic interaction with SPMV in coinfections in switchgrass cultivars. Why the same viruses interact differently in two different hosts is not known. Perhaps, these viruses interact differently with the host factors of these two hosts, thus causing a differential synergistic interaction in these hosts.

The temperature-sensitive resistance of Kanlow to PMV and SPMV has two implications as to the potential for disease development in the field. First, spring temperatures during the emergence of tillers (shoots) from virus-infected crowns could be a critical factor as to the proportion of tillers in a plant that would subsequently display panicum mosaic disease symptoms. Should high temperature occur during this early period, Kanlow would likely display the same degree of disease as cv. Summer. Second, higher summer temperatures during the vegetative growth phase would increase virus replication and symptom severity, further limiting yield in virus-infected plants regardless of the cultivar.

Our data indicated that there was a much greater number of co-infected Kanlow plants with no apparent visual disease symptoms. We observed drastically enhanced asymptomatic infections in Kanlow plants co-infected by PMV+SPMV compared to those only by PMV. These data suggest that the co-infection of Kanlow by PMV with SPMV caused an enhanced asymptomatic systemic infection, which could be a different kind of synergistic interaction between PMV and SPMV in a resistant cultivar. Also, asymptomatic infections by PMV+SPMV in switchgrass cultivars could play a significant role in disease epidemiology because these infections could act as a natural reservoir for PMV and SPMV for other susceptible switchgrass cultivars.

Declarations

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Author Contributions

ST, GS, and GY: formulated and designed the experiments; AM: performed the research; GS, NP, and GY: supervised the research; SE and GY: provided funding for the research; ST, AM, NP, and GS: analyzed the data; SE and RM: provided resources and analyzed the data; ST and AM: wrote the manuscript; ST, AM, NP, SE, GS, GY, and RM: edited and improved the manuscript and approved the submitted version.

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Conflict of interest: None

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Figures

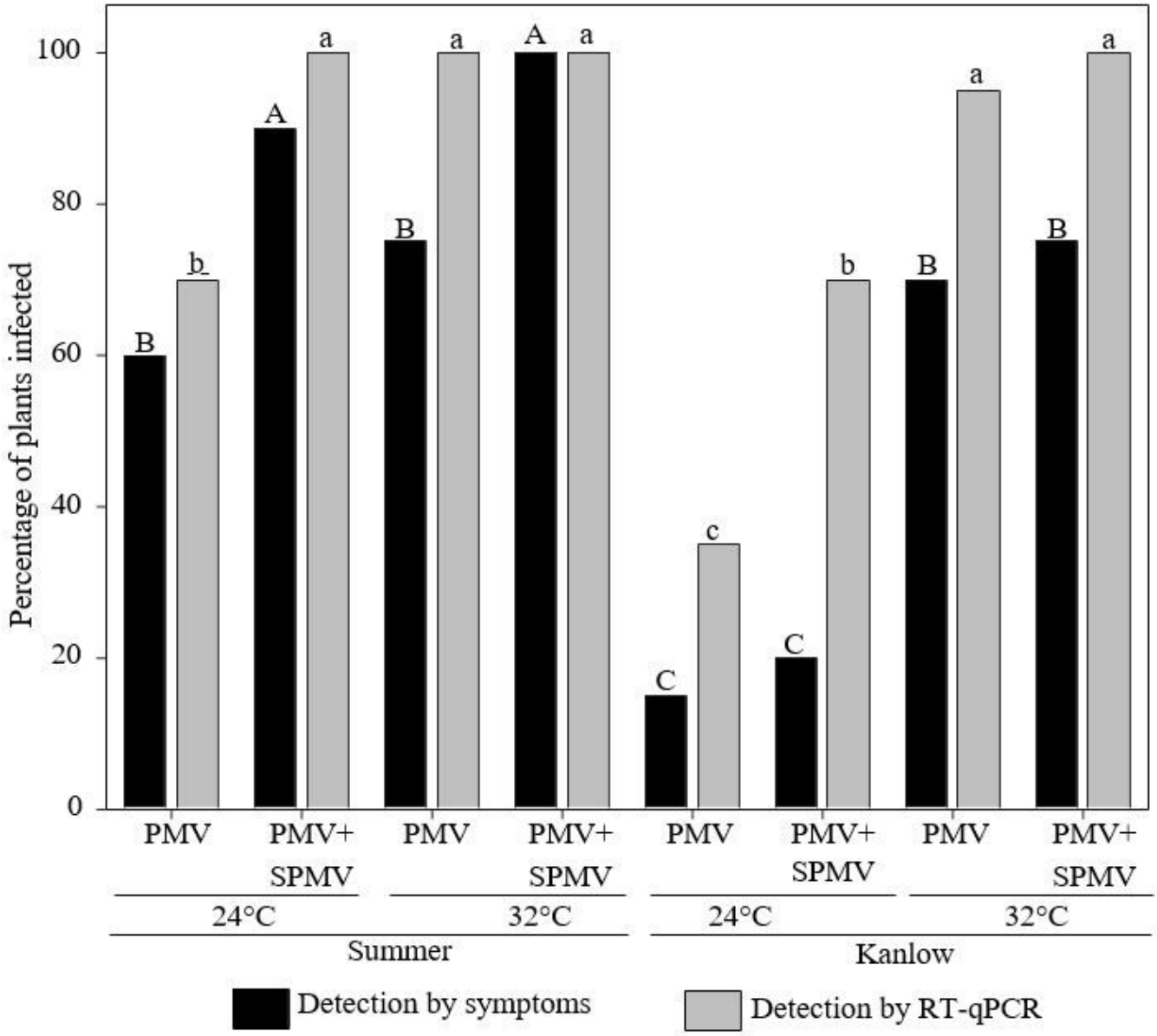

Figure 1

Evaluation of PMV and PMV+SPMV infections at 24°C and 32°C on switchgrass cvs. Summer and Kanlow by visible symptoms and reverse transcription-quantitative PCR (RT-qPCR). Note that co-infection of switchgrass cv. Kanlow by PMV+SPMV caused enhanced asymptomatic infection compared to those

by PMV. A Chi-squared test was performed on all of the ratios, followed by Analysis of Means for Proportions (ANOMP) to assign the significantly different groups. Treatments with different letters indicate significantly different, while treatments with the same letter are not significantly different.

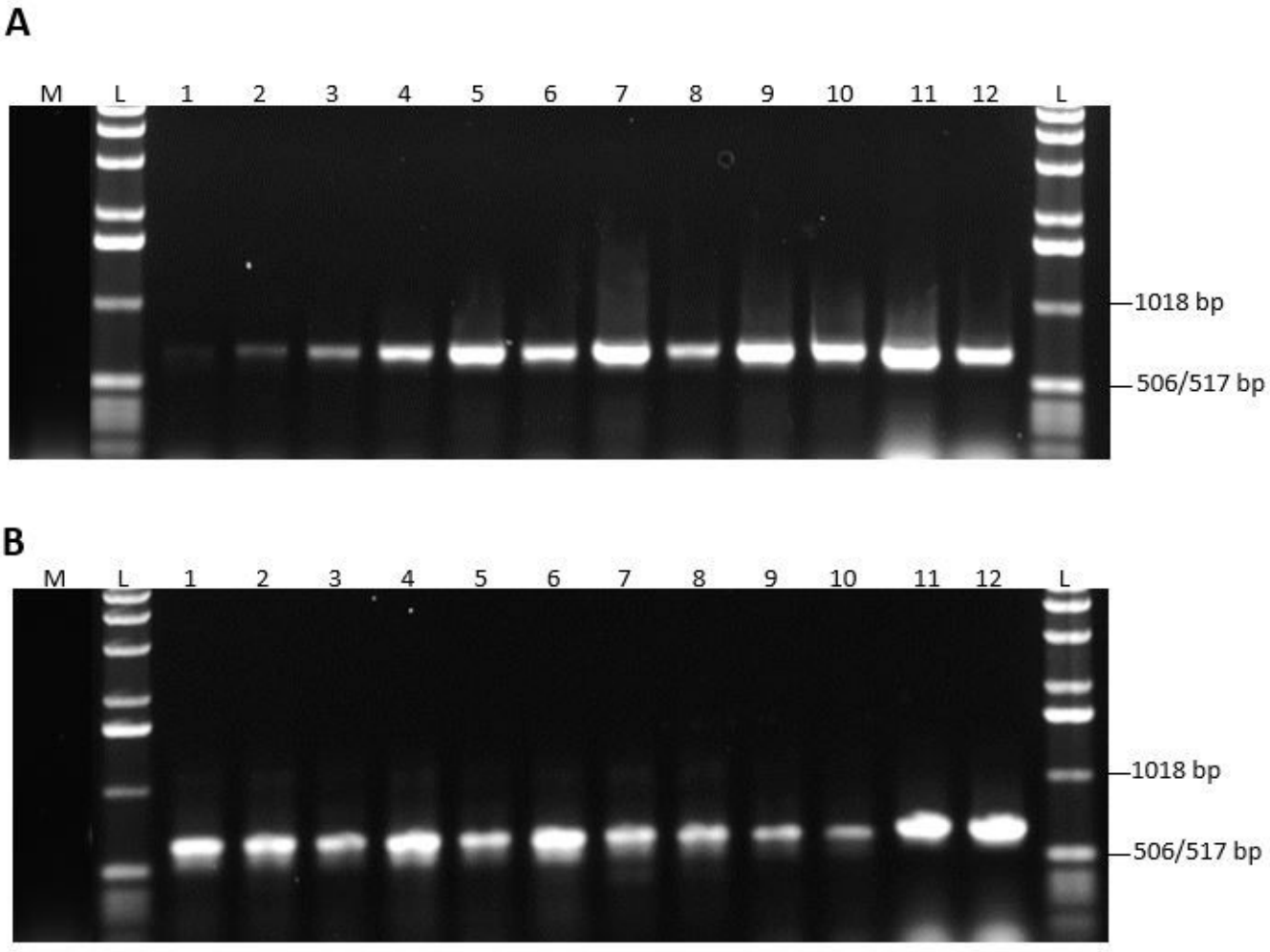

Figure 2

Detection of PMV from PMV-inoculated leaves of switchgrass cv. Kanlow at 24°C (A) and 32°C (B) by RT-PCR. The RT-PCR products of PMV from inoculated leaves of Kanlow (lanes 1-10) and Summer (lanes 11-12) were separated through 1.0 % agarose gels in TAE (Tris-acetate-EDTA) buffer. Five µl of RT-PCR product was loaded onto agarose gels. Out of twenty Kanlow plants analyzed, the results of only 10 plants were presented. M: mock-inoculated Kanlow; L: 1.0 kbp DNA ladder.

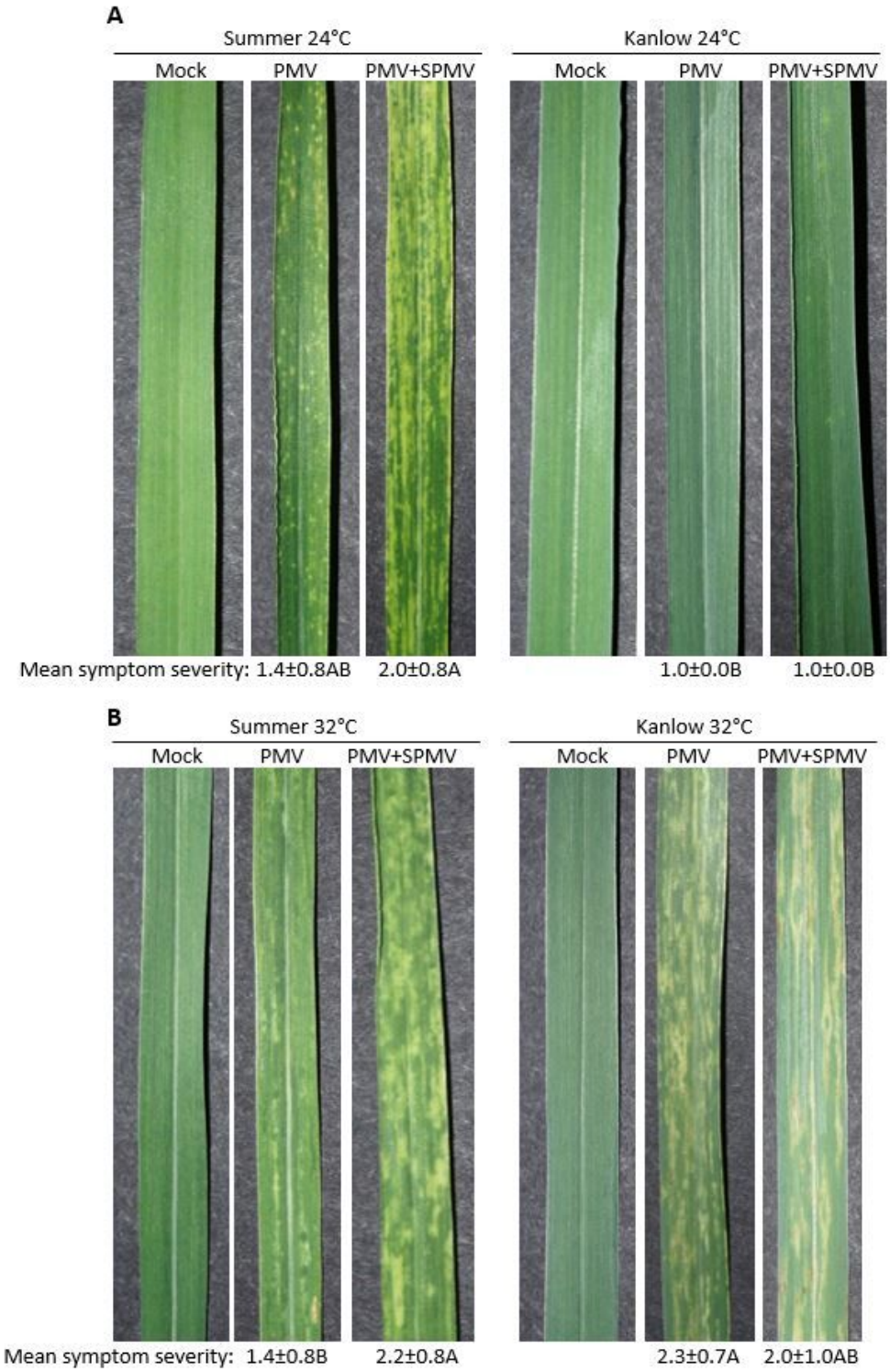

Figure 3

Symptoms elicited by PMV or PMV+SPMV in switchgrass cultivars at 24°C (A) and 32°C (B). The second true leaf of switchgrass seedlings was inoculated with crude sap of PMV or PMV+SPMV at 1:20 dilution in 20 mM sodium phosphate buffer, pH 7.0. Symptoms elicited by PMV or PMV+SPMV on upper uninoculated leaves of switchgrass plants at 21 days postinoculation (dpi) with a mean symptom severity score ± standard deviation. A representative leaf of switchgrass was presented in the picture.

Note that moderate synergistic interactions were observed on switchgrass cv. Summer at 24°C and 32°C inoculated with PMV+SPMV compared to PMV. PMV+SPMV elicited only a mild or symptomless infection in switchgrass cv. Kanlow at 24°C, but elicited symptoms at 32°C with no significant enhancement of symptom severity in plants inoculated with PMV+SPMV compared to only by PMV. Mean separations were calculated using Tukey's HSD test in JMP 12. A and B indicate the mean separations of relative symptom severity. Treatment with different letters indicates significantly different, while treatments with the same letter are not significantly different.

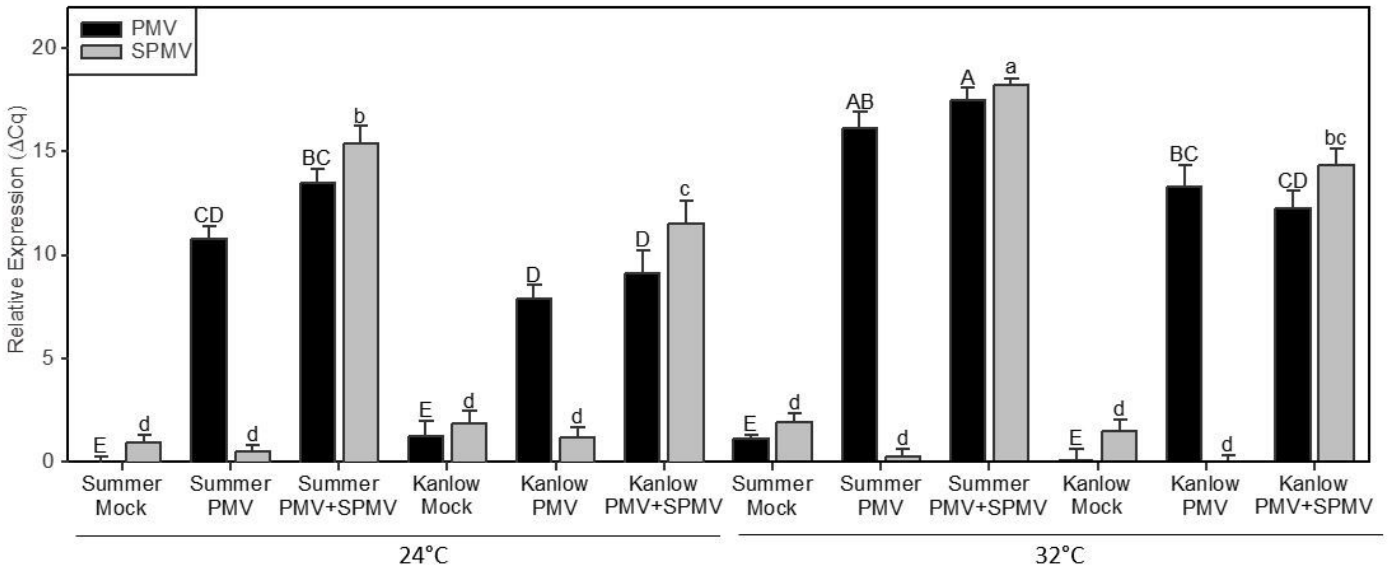

Figure 4

Quantification of relative expression of genomic RNA copies of PMV and SPMV by RT-qPCR from switchgrass plants infected with PMV or PMV+SPMV at 21 days postinoculation (dpi). Mean separations were calculated using Tukey's HSD test in JMP 12. A, B, C, and D, and a, b, c, and d indicate the mean separations of relative expression of PMV and SPMV genomic RNA copies, respectively, at p=0.05. Treatments with different letters indicate significantly different means, while treatments with the same letter are not significantly different.