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Panicum Mosaic Virus Complex and Biofuels Switchgrass

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PANICUM MOSAIC VIRUS COMPLEX AND BIOFUELS SWITCHGRASS

by

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New switchgrass (Panicum virgatum L.) cultivars are being developed for use as a biofuel pyrolysis feedstock. Viral pathogens have been reported in switchgrass, but their importance in biofuel cultivars is not well known. In 2012 surveys of five switchgrass breeding nurseries in Nebraska, plants with mottling and stunting— symptoms associated with virus infection—had an incidence of symptomatic plants within fields as high as 59%. Leaves from 120 symptomatic plants were analyzed by ELISA for Panicum mosaic virus (PMV) and four other viruses known to infect switchgrass. Most samples (87%) were positive for PMV, and fewer than 8% for the remaining viruses. Among PMV-positive samples, 36% tested positive for the presence of PMV's satellite virus (SPMV) by immunoblotting.

In 2013 fields were assessed for PMV- and PMV+SPMV-infection incidence and associated symptoms. PMV and SPMV were detected by ELISA and RT-PCR, respectively, in leaf samples from randomly selected plants. Symptom severity was assessed on these plants using a 1 to 5 scale (1 = no symptoms; 5 = plants stunted and

>50% foliage with mottling). PMV incidence varied among fields and switchgrass populations within fields. Common among sampled populations was dual infection by PMV and SPMV. Few plants were infected with PMV alone and these exhibited symptoms at the 1-3 rating. There also were many PMV+SPMV-infected plants and these exhibited symptoms at the 1 to 5 rating.

To assess potential resistant switchgrass, four strains of switchgrass were grown in a growth chamber and rub-inoculated with PMV and PMV+SPMV. These were observed for 30 dpi and then collected. During the 30 dpi there was little symptom expression. Samples were weighed and tested for the presence of PMV or SPMV via RT-PCR. This study is on-going; however presently there is no evidence of resistance to either PMV or PMV+SPMV infection. However, the four switchgrass strains had less biomass accumulation if infected with either PMV or PMV+SPMV. There was no significant difference in biomass accumulation between PMV and PMV+SPMV infection.

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LITERATURE REVIEW

LITERATURE REVIEW

To provide new and sustainable energy sources for the future, there are public and private efforts nationwide to develop new crops as feedstock for biofuel generation. This project is part of the USDA AFRI-funded project CenUSA Bioenergy (http://www.cenusa.iastate.edu/) that seeks to develop a new biofuel extracted by the pyrolysis method using perennial grasses, such as switchgrass (*Panicum virgatum*), as the feedstock. My efforts in this project focused on identifying viral pathogens that potentially can be problematic on new biofuel switchgrass crops.

Historically, pathogen problems in traditional agricultural crops were addressed in a reactive manner, after the grower has experienced some amount of loss. There is no history of intensive monoculture cropping with switchgrass developed for biofuel feedstock, so this is an opportunity for the biofuels development community to address potential pathogen problems in a proactive manner. This unique opportunity is especially critical given that the expected duration of yield from a biofuels switchgrass crop is ten years. Once a grower plants a switchgrass crop, it would not be feasible for the grower to take reactive measures such as replanting with a different cultivar or crop rotation. Proactive efforts can be directed towards ensuring that new cultivars will be resistant to diseases while ensuring high yields. This requires first that the main disease problems be identified; second, an assessment of the levels of resistance within existing populations; and lastly, whether needed sources of resistance could be identified for use in minimizing losses due to disease.

To better understand the framework for the project, it is important to understand the historical aspects of switchgrass: as a crop, the current knowledge base of pathogens known to be associated with switchgrass, and the importance of resistance as the primary disease management strategy. Key gaps in our knowledge will be pointed out in italics.

Switchgrass as a crop

Switchgrass has not always been seen as a biofuel feedstock and it is pertinent to understand the origins of its cultivation in understanding the context for the research questions to be addressed in the completion of this thesis. The story of switchgrass and its first transition from a wild grass to a cultivated crop begins with the First World War. There was a significant rise in the demand for wheat and, therefore, millions of acres of grasslands were converted to agricultural fields (Montgomery, 1953). The removal of the protective grass cover and the severe drought that followed resulted in the Dust Bowl of the 1930's, which caused significant damage to crop, pasture, and rangelands. There was a demand to re-vegetate these damaged lands with native grassland species. In 1935 L. C. Newell a U.S. Department of Agriculture scientist stationed at the University of Nebraska – Lincoln, became one of the first switchgrass breeders. Switchgrass then was used for erosion control as well as for livestock feed. In the 1980's research began to optimize switchgrass for another purpose - a biofuel feedstock. (Wright, 2007)

Switchgrass has two discrete ecotypes, lowland and upland (Vogel, 2004). These ecotypes have significant genetic differences. The lowland ecotype plants are tetraploids, whereas upland ecotype plants are either tetraploids or octaploids (Vogel, 2004). Lowland types are typically found in areas prone to flooding, while upland ecotypes are found in upland areas, which are less prone to flooding. Also lowland types are typically

taller and produce more rapid growth than upland types. Lowland types generally have more coarse leaves, potentially contributing to them being less susceptible to rust (*Puccinia* spp.). Because switchgrass is photoperiod sensitive, needing short days to induce flowering, switchgrass must be adapted for specific ecoregions. If southern ecotypes are moved north, they will remain in a vegetative state longer with the reverse being true if a northern ecotype is moved south. The induction of flowering seems to be tied to winter survival capabilities. If a southern ecotype is moved too far north, it will not survive the winter (Vogel, 2004). In order to distribute switchgrass across the country, it must undergo breeding and selection to ensure that strains used by growers are not only able to survive but able to produce enough biomass to produce a profit. Because switchgrass must cross-pollinate, there is genetic diversity within any given population. Switchgrass as a species is a native grass to N. America east of the Rocky Mountains. Its broad native range reflects its adaptability to different climates and soil types, as well as precipitation gradients, found across the US. There is the possibility to collect switchgrass germplasm from many diverse areas of the country with ecological characteristics, such as winter hardiness, that can be combined with disease or pest resistance and desired agronomic traits to develop the optimum cultivars for any given region.

Pathogens of Switchgrass with an Emphasis on Virus Species

The diversity of pathogens that can infect switchgrass has been reviewed in a number of publications (Gravert and Munkvold, 2002; Tiffany and Knaphus, 1995). Therefore, there will be no attempt to list all pathogens of switchgrass here except for viruses. What has been reported as pathogens of switchgrass include species of fungi,

oomycetes, and nematodes, as well as viruses, but no bacterial pathogen is known. Considering there are numerous wheat and corn bacterial pathogens (*Compendium of Wheat Diseases,* 3rd Ed.; *Compendium of Corn Diseases*, 3rd Ed.), it is highly likely that there are bacterial pathogens of switchgrass as well. Due to the historic uses of switchgrass for erosion control and forage, considered to be of relative economic importance, there is little information in the older literature pertaining to the potential impact of diseases in general on switchgrass. In some recent surveys of fungi and nematodes associated with switchgrass, there was no verification of pathogenicity (Cassida et al., 2005; Crouch et al., 2009; Krupinsky et al. 2004). On the other hand, several recent studies identified new switchgrass pathogens and provided precise descriptions of symptomatology (Carris et al., 2008; Etheridge et al., 2001; Vu et al., 2012). Furthermore, the incidence and impact of a few diseases caused by fungi also has been documented. One example, smut caused by the biotrophic fungus *Tilletia maclaganii*, was found to have incidences of up to 70% in Iowa biofuel switchgrass fields (Gravert and Munkvold, 2002), and disease incidence was shown to have a strong relationship to yield (Thomsen et al., 2008). However, the reality for many switchgrass pathogens, particularly viruses, is that our current knowledge of their biology and epidemiology is in its infancy.

This project focuses on viral pathogens, in part because viral symptoms were observed to be the most dominant symptoms in Nebraska field experiments during the initial stages of the CenUSA project (see Chapter 2). Another justification for focusing on viruses is the potential threat they could pose to switchgrass biofuel production. Plant viruses can be systemic; thus, entire virus particles, or virions, of a virus potentially may

be present in all the tissues of an infected plant and be stable in infected residue after the plant has died. Given the extensive nature of the foliage and the root system of switchgrass, this represents a tremendous pool of virus inoculum for infection of healthy switchgrass plants and, potentially, nearby cereal crops. In addition, viruses generally can infect or replicate throughout the life of the host plant. In biofuel switchgrasses, which have expected productive life spans of eight to ten years, there is an opportunity for infection of any given plant at any point in the life of the plant, and once it becomes infected the negative impact of infection in respect to biomass production could accumulate over many years. In addition, a virus-infected switchgrass crop also could serve as a continual reservoir of inoculum for infection of other agronomic crops if the viral pathogen was to have a broad host range.

Viruses found in or reported to infect switchgrass include:

Panicum mosaic virus (PMV) (Sill and Pickett, 1957)

Barley/Cereal yellow dwarf viruses (B/CYDVs) (Garret, et al., 2004; Schrotenboer, et al., 2011)

Sugarcane mosaic virus (SMV) (Agindotan et al., 2010)

Switchgrass mosaic virus (SwMV) (Agindotan et al., 2010)

In addition, eight new viruses were reported to infect switchgrass (Agindotan et al., 2013) but await further characterization.

These reported viruses are known generally to cause yellow mosaic symptoms on switchgrass. It has also been noted that sometimes virus infection does not cause noticeable symptom development (Schrotenboer et al., 2011); specifically in respect to B/CYDVs, symptoms on switchgrass are not consistent with symptoms observed on

cultivated cereal crops. Also, it has been reported that more cultivated types of switchgrass have a greater susceptibility to viral infection than native populations (Schrotenboer et al., 2011). In respect to yield loss, however, there are no reports relating switchgrass yields to viral infection.

It should be noted that virus pathogens also may play a beneficial roles to switchgrass, providing cross-protection for example. In cross-protection, a host plant is inoculated with a viral strain that is too weak to cause noticeable symptoms but able to replicate and spread throughout the plant. The plant infected with the weak strain is then resistant to infection by stronger, more damaging strains of the virus. Cross protection has proven effective against a variety of destructive viruses (*Citrus trizetza virus* and *Tomato mosaic virus*), but there are a variety of potential drawbacks. Potential negative impacts of cross protection include: the viral concentration encountered in field conditions not being able to overcome the weaker strain, the mild strain may spread to other unintended hosts, a weaker strain virus may cause the plant to be more susceptible to other pathogens, potential for the virus to mutate into something more harmful, as well as the difficulty and cost in inoculating multiple plants (Fulton, 1986). To implement cross-protection against a switchgrass virus, it must be determined first if there were multiple strains of the virus species occurring naturally in field conditions and then the option of using cross protection would have to be examined for its benefits as well as its potential negative impacts.

The Panicum Mosaic Virus complex

Because PMV was found to be the predominant virus species in Nebraska switchgrass breeding experiment fields (Chapter 2), the virus complex consisting of PMV

and its associated satellite virus, satellite panicum mosaic virus (SPMV), will be reviewed here in detail. The PMV complex also includes two distinct types of satellite RNAs (satRNAs). They have been found in nature in the southeastern US. In St. Augustinegrass, it is not possible to visually distinguish a plant infected with PMV and satRNAs from one infected with only PMV. The satRNAs may possibly play a role in the infection process, but very little is currently known about them and they were not included in this study. PMV is in the family Tombusviridae and the type species in the Panicovirus genus. Tombusviridae has not been assigned to an order. PMV is a single-stranded, positive sense RNA virus, which is especially common in plant viruses. It is an icosahedral virion that is 28-30 nm in diameter. The genome for PMV codes for two replicase proteins, a capsid protein that likely also functions to aid replication and movement. There are three proteins (p8, p6.6, and p15) that are not needed for infection of protoplasts, but are required for movement in millet plants. Thus these three proteins are believed to aid in viral movement. In respect to PMV's relation to other viruses, it is serologically related to *Molina streak virus* and *Maize mild mottle virus* (Batten and Scholthof, 2004). Currently, mechanical transmission is the only known method of transmission for PMV (Batten and Scholthof, 2004)

PMV has been found to infect the following in nature: St. Augustinegrass (*Stenotaphrum secundatum*), switchgrass, and centipede grass (*Eremochloa ophiuroides*). Millet species (*Setaria italic* L., *Panicum miliaceum* L., and *Pennisetum glaucum* L.) and crabgrass (*Digitaria sanguinalls* L.) can be used to propagate PMV. The virus has also been mechanically transmitted to maize (*Zea mays* L.) and some wheat cultivars (*Triticum aestivum* L.) (Batten and Scholthof, 2004).

PMV has a US distribution that extends north as far as WI, west to NE, and south to TX (see Fig. 1). This distribution is based on reports of PMV infection in both switchgrass and St. Augustinegrass. PMV has been found in switchgrass in Nebraska (L. Lane, http://lclane.net/text/pamv.html), but its occurrence was not been formally reported.

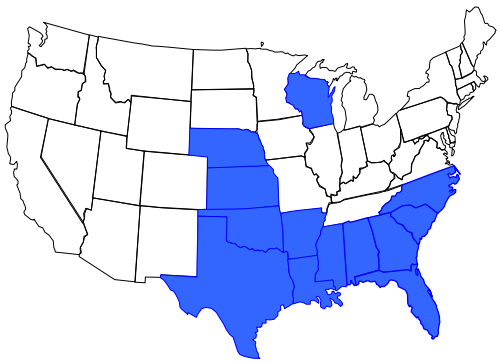

Fig. 1: Reported Distribution of PMV
Source: (http://www.panicovirusproject.org/Research/st-augustinegrass-decline)

Switchgrass, a primary host for PMV, had a native distribution that ranged the continental US, east of the Rocky Mts. (USDA Plant Fact Sheet) (see Fig. 2). This range is now expanded to the entire continental US due to the use of switchgrass in erosion control, forage, ornamental use, as well as more recently biofuel.

Fig. 2: Distribution of Switchgrass
Source: USDA Plants Database: *Panicum virgatum* L.

Therefore, it is possible that PMV eventually may have a wider distribution than what is currently reported, a range that corresponds to the current range for switchgrass. Because PMV is mechanically transmitted and not known to have a vector, the absence of PMV in areas where switchgrass is grown might be due to the switchgrass not being mowed or being planted solely for erosion control.

PMV was first discovered in 1953 in a switchgrass-breeding nursery in Manhattan, KS (Sill and Picket, 1957). Symptoms observed in this initial report of the pathogen on switchgrass included stunting, chlorosis, and necrosis at the tips of leaves. The chlorosis occurred as a mosaic pattern, a blotchy mottling, or a yellow streaking of the leaves (Sill and Picket, 1957). Some switchgrass plants inoculated with the virus were sterile or had limited seed development. It was also noted that symptoms typically developed in July, with some plants showing symptoms in early August. Symptoms were especially severe on some lines selected from cultivar Blackwell, an upland ecotype. In

addition to mosaic mottling, streaking of the leaves were common. Some plants presenting symptoms were severely stunted, while others were not. It is important to point out 'Blackwell' was developed for erosion control and foraging. This cultivar of switchgrass may not be representative of other cultivars or strains of switchgrass as to their reaction to infection. Thus, *there is little known about symptom expression within newly developed switchgrass strains when infected with PMV.*

After this paper there was little to no mention of PMV until 1987 when PMV was recognized as the causal agent of St. Augustinegrass decline (SAD) (Haygood and Barnett, 1988). SAD was identified previously in 1966 as a disease of St. Augustinegrass and, later, centipedegrass (McCoy, et al., 1969). Symptoms of SAD are similar to those reported for PMV infection in switchgrass. It is important to note that once St. Augustinegrass is infected with PMV, the virus will generally kill the grass within three years post-infection (McCoy, et al., 1969). *The long-term effects of infection of switchgrass by PMV have not been investigated.*

As mentioned above, PMV can be associated with SPMV to form a virus complex. The complex has been found in nature only in St. Augustinegrass (Niblett and Paulson, 1975), SPMV has been reported to occur only in association with PMV (citation). It has a synergistic relationship with PMV in pearl millet plants, St. Augustinegrass and centipedegrass, as well as Brachypodium. Plants suffering a mixed infection with PMV and SPMV exhibit symptoms similar to those caused by infection with PMV alone, but the symptoms caused by mixed infection progress at a more rapid

rate and generally are more severe than in plants infected with PMV alone (Scholthof, 1998). Whether or not this relationship occurs in switchgrass is unknown.

The reason for this synergistic effect is unknown. The genome of SPMV codes only for its capsid protein. Other proteins SPMV needs for replication and cell-to-cell movement are encoded in the PMV genome. SPMV is not serologically related to PMV and for this reason should be considered another species of virus (Batten and Scholthof, 2004). Transmission for SPMV beyond mechanical transmission and its origin are unknown. It was found that the satellite is lost after multiple passages through plant by inoculation (K.B. Schothof, personal communication).

The seminal PMV paper by Sill and Picket (1957), is important because it is the only report research involving switchgrass and PMV prior to this study. In that initial work, however, plants were inoculated with“viruliferous” plant-sap (ie. ground tissue from plants suspected of being virus infected) diluted in water, and no virus purification was performed.Subsequent characterization of isolates retained from that study revealed the satellite was present in some samples. (K.B. Schothof, personal communication)This suggests the satellite was present in the experiments conducted by Sill and Picket, and may explain some of the diverse symptoms reported. This association in switchgrass, however, has not been confirmed or formally reported. *SPMV had not been reported in switchgrass in the field. It also was not known if the synergistic relationship between PMV and SPMV observed in other grasses could occur in switchgrass.* Therefore, providing answers to these questions regarding PMV and SPMV in biofuel switchgrass is one focus of this thesis as these answers could benefit the biofuels energy community.

Resistance to virus diseases

It has been suggested that because switchgrass is a native grass that has coevolved with native pathogens, because it has a broad genetic background, and because screenings will be done throughout cultivar development, that these factors, "…will likely limit the negative impact of native pests." (Mitchell et al., 2008). The first two arguments are true, but only in respect to native switchgrass populations within a community of plants, and individual plants within the native population may be highly susceptible to certain pathogens. In switchgrass populations selected or bred for a particular use, genetic diversity will be narrower.

The third point references the fact that selections will be made throughout the development of new biofuels cultivars. Plant breeding work will be conducted to improve for multiple characteristics including increased yield, improved winter tolerance and for biomass composition including altered lignin concentration. As a result of the breeding work, there may be reduced genetic diversity in some strains or cultivars and in addition, a potential unintentional loss of other genetic traits such as pathogen resistance. The presence or absence of resistance to a pathogen will not always be apparent when new cultivars are evaluated in the field because a lack of pathogen inoculum or the occurrence of unfavorable environmental condition might prevent disease from occurring. Therefore, screenings performed throughout the cultivar development process cannot ensure that native pathogens will not impact new cultivars unless screenings are also performed under conditions in which pathogen and environmental conditions are controlled, i.e. greenhouse conditions.

Another concern in relation to potential pathogen problems in switchgrass grown as a biofuel crop is the limited number of other options available for disease control. Management practices such as tillage and crop rotation that can help to limit soil and residue – borne diseases cannot be done. Burning can be done with switchgrass, however this is not generally environmentally desirable. Therefore these pathogens could have the potential to serve a significant threat in switchgrass production (Cox, et al., 2004) if resistance is unavailable.

Resistance is critical for virus control in other grasses. Research on viruses of other grass hosts has shown that resistance to a virus can be manifested in three ways, the first being resistance to feeding by the virus vector and the second being resistance to virus replication or spread within the plant. In the third kind of resistance, referred to by some as tolerance, the virus can replicate and spread throughout the plant, but the plant exhibits minimal, if any, symptoms. In respect to PMV and SPMV, very little is known about resistance, especially resistance in switchgrass. Because no vector is known to transmit PMV and SPMV, resistance of the first type, i.e. resistance to feeding by an insect vector, would be unimportant for the management of disease caused by the PMV complex.

Resistance to virus replication, however, may be very important for controlling PMV and SPMV. There are strains of St. Augustinegrass that are resistant to PMV, 'Floratam' being one such cultivar. They use of resistant cultivars is the recommended management strategy to control SAD. The exact mechanism for how replication of PMV is prevented is not understood. (Reinert et al., 1980)

However in other systems it is known that some plants can suppress viral infection via RAN silencing mechanisms. When ssRNA viruses replicate within a host plant they can form dsRNA or stem-loop structures. In work with Arabidopsis, a dicer-like protein (DCL) cleaves the dsRNA into smaller dsRNA pieces (typically 19-25 bp in length). One of the two strands from the small dsRNAs are incorporated into a protein complex. This protein complex (AGO) can then target similar sequences for degradation. (Ruiz-Ferrer and Voinnet, 2009) *It is unknown whether resistance of this type is present in switchgrass against the PMV complex.*

The third type of resistance that allows replication of the virus but suppresses symptom development is available against PMV in St. Augustinegrass. For example accession FA-108 of St. Augustinegrass is a symptomless carrier of PMV and PMV+SPMV (Bruton and Toler, 1983). Tolerance to PMV has also been previously identified in switchgrass. Sill and Picket (1957) reported some strains of switchgrass being developed for forage to be tolerant to PMV, i.e. to exhibit mild symptom development following greenhouse inoculation. Those strains, however, were low in cellulose. Furthermore, strains investigated by Sill and Pickett likely had a different genetic background than new biofuel strains studied in this project. *The presence or absence of tolerance to PMV complex in new biofuel switchgrass strains has not been identified.*

Research objectives

Given some of the important knowledge gaps relating to the PMV complex in switchgrass, the objectives of my research reported in this thesis were 1) to identify the main virus pathogen problems in Nebraska switchgrass breeding nurseries, 2) determine

the incidence of PMV and SPMV infection in these field experiments, 3) correlate infection to symptom expression under field conditions, and 4) assess if there are strains of switchgrass that are more resistant or tolerant to PMV and PMV+SPMV infection than others.

Chapter 2:

FIELD DYNAMICS OF *PANICUM MOSAIC VIRUS* AND ITS SATELLITE VIRUS

INTRODUCTION

In early summer 2012 switchgrass breeding field experiments in Mead, NE were examined for presence of disease symptoms. It became clear in these initial observations of the experiments that the most prevalent symptoms were those associated with viral infection. From this point the objectives in 2012 were to determine, on the basis of symptoms, the extent to which these experiments were infected by virus and the severity of symptoms among plants; and to identify the agent causing of the viral symptoms. Because PMV subsequently was found to be the predominant viral pathogen, objectives in 2013 were to assess the frequency of infection by PMV and its satellite virus SPMV; and to assess the relationship of single or dual infection on severity of virus symptoms.

MATERIALS and METHODS

Description of field experiments

Switchgrass field experiments involved in this study were located at the University of Nebraska Agricultural Research and Extension Center located near Mead, NE (41.166103^{o} N, 96.482938^{o} W). Three experimental nurseries (designated PV0910, PV1103, PV1104) were inspected or sampled extensively in 2012 and 2013. Experiments PV1103 and PV1104 were adjacent to one another in an area located approximately 2 km from experiment PV0910. The three experimental nurseries contained switchgrass plants grown in rows, with rows and plants within rows being spaced set distances apart. The nurseries were established by transplanting greenhouse-grown seedlings. Seedlings previously were grown from seed in Cone-Tainers containing a standard soil mixture and raised in a greenhouse with a 16 hr light/8 hr dark photoperiod until the 2-3 leaf stage prior to machine transplanting in the respective experiment. In the nurseries, soil between

plants and rows were cultivated with 0.6 m-wide roto-tillers creating 0.4 m x 0.4 m mini-plots containing individual plants. Nurseries were fertilized annually with 112 kg N per ha, and herbicides and hand weeding were used for weed control. Nurseries were mowed or burned each spring to remove the accumulated biomass from the previous year.

Experiment PV0910 contained plants from five switchgrass populations: cv. Summer, cv. Kanlow, Kanlow-early maturing (a population selected for earlier flowering from the base 'Kanlow' population), Kanlow-high yield (derived from 'Kanlow' base population for high yields) and F3 seeds derived from a population of plants arising from a cross between select 'Kanlow' (male) and 'Summer' (female) plants and hereafter referred to K x S. Seedlings of each population were transplanted into the experiment in spring of 2009. From each population, 125 seedlings were planted in a single plot consisting of five rows of 25 plants each on 1.1 m centers. Plants were randomized within a plot and the position of each plot was selected at random. The five plots planted in 2009 thus constituted one block. In 2010, two rammets (sections of live crown tissue) were mechanically dug from each plant using a 4" soil core tube and each rammet was transplanted into a plot within blocks 2 or 3. Some of the original transplanted seedlings and some rammets, i.e. clones, did not establish after transplantation, but the end result was a nursery with three clonal replicates of approximately 100 plants per population. The experiment was not harvested in 2010 or 2011, and was first harvested in 2012 for biomass yield after a killing frost. It was burned in early spring in 2011 and 2012 to remove previous year's residue.

Experiment 1103 was the cycle 3 breeding and selection nursery for the KxS HP 1 NETO2 population, which is based on progeny from 'Kanlow' x 'Summer' crosses in

which ‘Summer’ was the female parent. The recently released switchgrass cultivar ‘Liberty’ is based on the plants selected from the cycle 1 breeding nursery of this population (Vogel et al., 2014). The nursery contained one hundred eleven (111) half-sib families produced from seed produced on plants in the cycle 2 polycross nursery. Each family was identified by a half-sib family ID number. In addition to the 111 families, ‘Kanlow’, ‘Summer’, ‘Shawnee’, ‘Liberty’ (KxS HP1 NETO2 C1), and the experimental strain KxS HP C0 were included as check strains. Field experiment plots were single rows of five plants from the same family or check strain with rows spaced on 1.1 m centers. Spacing of plants within rows was 0.5 m. End plants of plots were separated by a 2 m alley. A randomized complete block experimental design was used with three replicates. The nursery was established in 2011 using greenhouse grown seedlings. Nursery management was as described previously except roto-tilling was only done between rows. No other mechanical procedure was conducted in 2011. In the spring of 2012, the nursery was mowed to remove the previous year’s residue before the start of the growing season. The nursery was harvested on a family plot basis for biomass yield after a killing frost at the end of October in 2012.

Experiment PV1104 was the cycle 3 breeding and selection nursery for the Summer Late Maturity High Yield (Summer Late Mat HYLD) population. The nursery had ninety half-sib families produced by harvesting seed produced on plants in the cycle 2 polycross nursery. Cultivars Kanlow, Shawnee and Liberty (KxS HP1 NETO2 C1) and the experimental strain Summer Late Mat-HYLD C1 were used as check strains. Summer Late Mat-HYLD C1 was the strain produced by the first breeding cycle for this

population. Experimental procedures for this nursery were the same as for Experiment PV1103.

2012 survey

The inspection and sampling of experiments PV0910, PV1103 and PV1104 in 2012 was conducted to assess virus symptom incidence and severity and to diagnosis of the causal agent(s). Every plant in the 3 fields was inspected in June or July for symptoms associated with virus infection: chlorotic mottling and stunting. The severity of symptoms in a plant was scored on a 1 to 4 scale, with 1 = no symptoms, 2 = indistinct mottling, or distinct mottling in <10 of the foliage, 3 = distinct mottling in <50 of the canopy, and 4 = distinct mottling in >50 of the canopy. Where stunting accompanied virus symptoms, '1' was one to the symptom score, giving a final severity scale of 1 to 5.

Foliage from randomly selected plants with moderate to severe mottling were collected June through August. A small number of samples also were collected from asymptomatic plants or plants exhibiting necrotic leaf lesions or discoloration atypical of virus infection. Each sample was placed in a plastic bag and put on ice in the field and transported in a cooler to the laboratory in Lincoln where most were placed immediately at -75C. Some samples were kept at 6-8C for several weeks until they were processed.

Virus identification in 2012 samples

Samples collected in 2012 were analyzed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS ELISA) kits (AC Diagnostics, Fayetteville, AR) specific for *Panicum mosaic virus, Sugarcane mosaic virus, Wheat streak mosaic virus,*

Barley yellow dwarf virus serotypes MAV and PAV, and *Cereal yellow dwarf virus*. Leaf tissue was ground in a 1:10 ratio with sample buffer as provided by AC Diagnostics, and the extracts tested in duplicate wells following methods specified by the manufacturer. Readings were taken 60 min after adding the substrate at 405nm. Negative controls included the negative control supplied in the DAS ELISA kits and extracts from two growth-chamber grown switchgrass seedlings that exhibited no symptoms. Duplicate wells of these negative controls were included in each ELISA plate. A negative-positive threshold was calculated for each plate from the mean of all the negative control optical density (OD) values plus 2 standard deviations from the mean. Any sample in a plate in which reactions in both of its wells exceeded the negative-positive threshold was considered to be positive for that virus test. Any positive sample with an average OD value lower than or exceeding 2x the average of the OD readings of the negative controls was rated as low positive and high positive, respectively. Samples with reactions that varied considerably between duplicate wells or which exhibited reactions that just exceeded the negative-positive threshold were retested.

A number of samples that were positive for PMV in DAS ELISA were retested for PMV and SPMV by Western blot. Tissue samples previously homogenized in PBST (same sample grinding buffer used in ELISA) buffer were centrifuged at 13,000 rpm for 5 minutes. The supernatants containing soluble proteins were mixed in a 1:1 ratio with 5X Laemmli protein extraction buffer. The prepared samples were boiled for 5 minutes to enhance protein denaturation, and separated by electrophoresis using 12.5% acrylamide gels and standard SDS-PAGE equipment (BioRad). Following separation, the sample proteins were transferred to nitrocellulose membranes using electrophoresis. Membranes

were incubated in blocking solution (5% fat-free milk in 1X Tris-buffered saline solution with 0.05% Tween 20, milk-TBST) for 1 hour at room temperature (RT) on a shaker. After blocking, membranes were incubated with primary rabbit polyclonal antibody solutions for either PMV (1:5000 dilution, antibody:milk-TBST) or SPMV (1:2000 dilution, antibody:milk-TBST) overnight on a shaker at 4°C. Following incubation with primary antibody, the membranes were washed three times: one quick rinse with TBST, two 5 minute washes in TBST on shaker at RT. Next, the membranes were incubated for 1 hour on the shaker at room temperature in the HRP-conjugated goat anti-rabbit secondary antibody (Thermo Scientific) solution (1:10000 dilution, antibody:TBST). After secondary antibody incubation, the membranes were briefly rinsed with TBST and washed for 5 minutes in TBST on shaker at RT. Prior to chemiluminescent substrate addition, membranes were given a final wash in 1X-Tris-buffered saline to remove traces of Tween detergent. Membranes were developed according to manufacturer's protocol using ECL Prime (Amersham) chemiluminescence substrate reagents and exposed using X-ray film (Agfa).

Sampling and symptom severity ratings in 2013

Select plants in experiments PV0910, PV1103 and PV1104 were sampled in 2013 to determine the incidence of infection by PMV alone and PMV in combination with SPMV and the same plants were inspected for virus symptoms to determine the relationship between virus infection and symptom severity. Fifty plants were identified at random from each of the 5 populations in experiment PV0910. The identified plants were scored for symptom severity and the foliage sampled at three times (May, June, and

July). Similarly, 50 randomly identified plants from each of experiments PV1103 and PV1104 were rated for symptom severity in May, June and July, but foliage samples were collected from the same plants only in June. In the sample collection, there was no attempt to select for particular leaves as to symptoms. Instead, leaves were collected by grasping a handful of leaves at random from the top portion of the plant and then the leaves were removed by tearing the leaves at a distance away from the hand to avoid contaminating the hand with liquid released at the tear.

A separate set of plants in experiments PV1103 and PV1104 were sampled in 2013 for the purpose of investigating changes in virus presence from 2012 to 2013. The plants sampled were among those sampled in 2012 and the absence or presence of PMV and SPMV was determined.

Leaf samples were transported to the lab, stored, and then tested for PMV using DAS ELISA as in 2012. Inconclusive or low-positive samples, as well some negative samples, were re-tested via reverse transcriptase PCR (RT-PCR) for the presence of PMV. Positive samples (high positives in DAS ELISA and verified low positives), were then tested for the presence of SPMV using RT-PCR.

RNA Extraction and RT-PCR methods

RNA was isolated from leaf samples using the Direct-zol RNA MiniPrep kit (Zymo Research) with TRI Reagent (Ambion) phenol-based solution. The homogenized PBST switchgrass leaf tissue samples prepared for DAS ELISA were used for total RNA isolation. For the initial phenol extraction, 50 µL of each sample homogenate was combined with 400 µL of the provided phenol. From this point all steps following the

phenol extraction were followed according to the manufacturer's protocol. RNA quality was visually assessed by electrophoresis separation on a 1% non-denaturing agarose gel and ethidium bromide staining. cDNA was prepared using SuperScript III Reverse Transcriptase (Life Technologies) following manufacturer's instructions. All first-strand cDNA synthesis reactions were primed using reverse primers either specific for PMV or specific for SPMV (Table 1). The generated cDNA samples were used as templates for standard Taq polymerase PCR amplification, using primers specific for PMV capsid protein (PMV-CP) and SPMV CP (SPCP). Sequences for primers used in PMV/SPMV RT-PCRs are listed in Table 1.

Primers for PMV and SPMV RT-PCR	
PMV p26 Forward Primer	ATGAATCGCAATGGAGCTAC
PMV p26 Reverse Primer	TTATGCGCTAACCCCACTGA
SPMV 87 Forward Primer	ATGGCTCCTAAGCGTTCCA
SPMV 297 Reverse Primer	ATACAGGCGCGCGTTATACATC

Table 1: List of primers used for either PMV or SPMV RT-PCR (primers provided by Karen-Beth G. Scholthof at Texas A&M University)

RESULTS

Field Symptoms

Fig. 3: Stunting (left) and mottling (right) are characteristic of virus infection; these were the most prevalent observed disease problems.

Stunting and mottling were the most prevalent viral infection-associated symptoms observed in the experiments in 2012 (Fig. 3). These symptoms were evident throughout each experiment (Fig. 4). There was no obvious pattern in the spatial distribution of symptomatic plants to indicate spread from point sources. Severity of observed symptoms varied among different strains of switchgrass. In some plots with a half-sib family, all of the plants exhibited symptoms while none of the plants in plots with other half-sib families might be symptomatic. Symptom severity also varied among plants within plots containing a half-sib family (Fig. 4). Incidence of symptomatic plants within experiments was as high as 59%, recorded in PV1104.

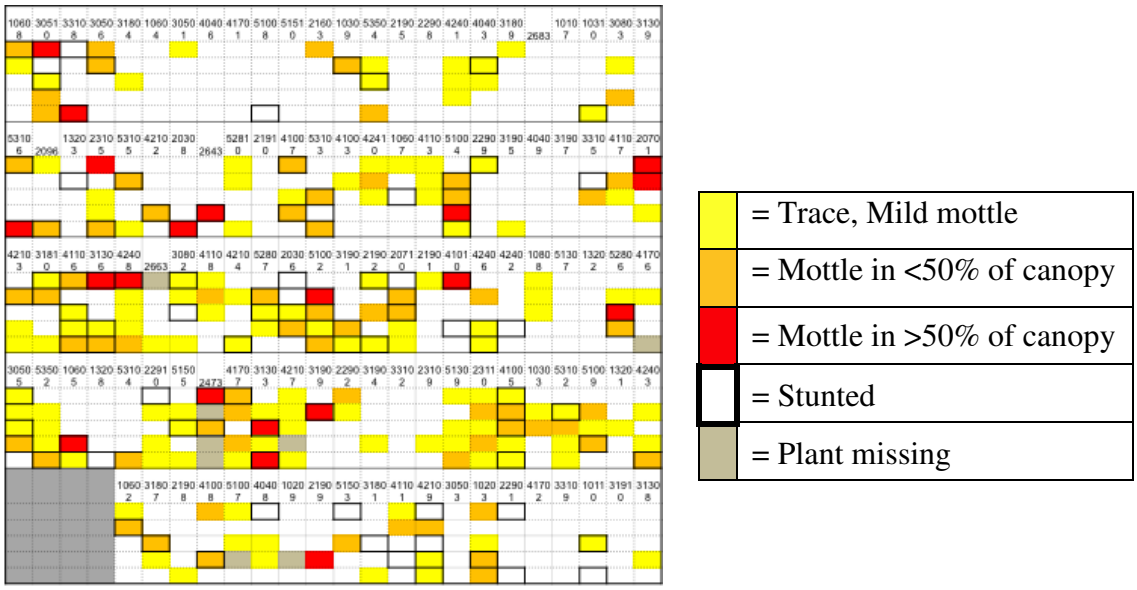

Fig. 4: A characteristic symptom distribution and severity in one section of one of the observed field experiments. Each box represents a single switchgrass plant, with (from top to bottom) every column of five plants being a half-sib family.

Virus Identification

PMV was found in 80% of 139 leaf samples from viral symptomatic plants collected in 2012. Fewer than 8% tested positive for any of the other four viruses tested (CYD, BYD-pav, BYD-mav, and SCM) via ELISA. Among the plants that tested positve for PMV, 28% were found also to be positive for SPMV via Western blot.

2013 Incidence of PMV and PMV+SPMV

Plants sampled at three different time points (May, June, and July) exhibited no marked changes in occurrence of PMV or PMV+SPMV between time points. Experiments PV1103 and PV1104, each having roughly 50 plants sampled, had different incidences of infection by PMV and PMV+SPMV (Table 2). Experiment PV1103 had 47% of plants testing positive for PMV+SPMV, while only 4% tested positive for PMV

only (no SPMV). Experiment PV1104 had 76% of plants infected with PMV+SPMV and no plants were found to be infected with PMV only.

Experiment	No PMV	PMV only	PMV+SPMV
PV1103	49%	4%	47%
PV1104	22%	0%	76%

Table 2: Incidence of detection of PMV alone and PMV+SPMV in experiments PV1103 and PV1104 in 2013.

Plants from experiment PV0910 were also sampled at three time points (May, June, and July). Similar to findings from experiments PV1103 and PV1104, there were no marked changes in virus detection between time points. Incidence of virus detection, however, varied among switchgrass strains, with ‘Kanlow’ and ‘Kanlow’-derived populations having similar low incidences of PMV alone and PMV+SPMV), ‘Summer’ showing the highest incidences, and the KxS strain having intermediate incidences.

Experiment PV0910 Switchgrass Strains	No PMV	PMV only	PMV+SPMV
Summer	23%	11%	66%
Kanlow Early Mat. High Yield	92%	2%	7%
Kanlow	91%	2%	9%
Kanlow Late Mat. High Vigor	86%	2%	14%
KxS	64%	18%	25%

Table 3: Incidence of dectection of PMV alone and PMV+SPMV among switchgrass strains in experiment PV0910 in 2013.

Relationship of symptom severity to single- or dual-virus infection 2013

Symptom severity ratings (1 to 5 scale) of individual plants in PV1103 and PV1104 were compared to the presence of PMV or SPMV within the same plants (Fig. 5). In both experiments, the vast majority of plants that were negative for PMV and SPMV exhibited no symptoms (rating of 1), while a small proportion (<20%) had trace amounts of mottling (rating of 2). In PV1103, equal proportions of plants with PMV only had ratings of 2 and 3 (moderate symptom severity); no plants in PV1104 were found to have PMV only. In contrast, plants with PMV+SPMV in both experiments had ratings ranging from 1 (no symptoms) to 4 (high severity) or higher, and the proportion of plants in each category were similar.

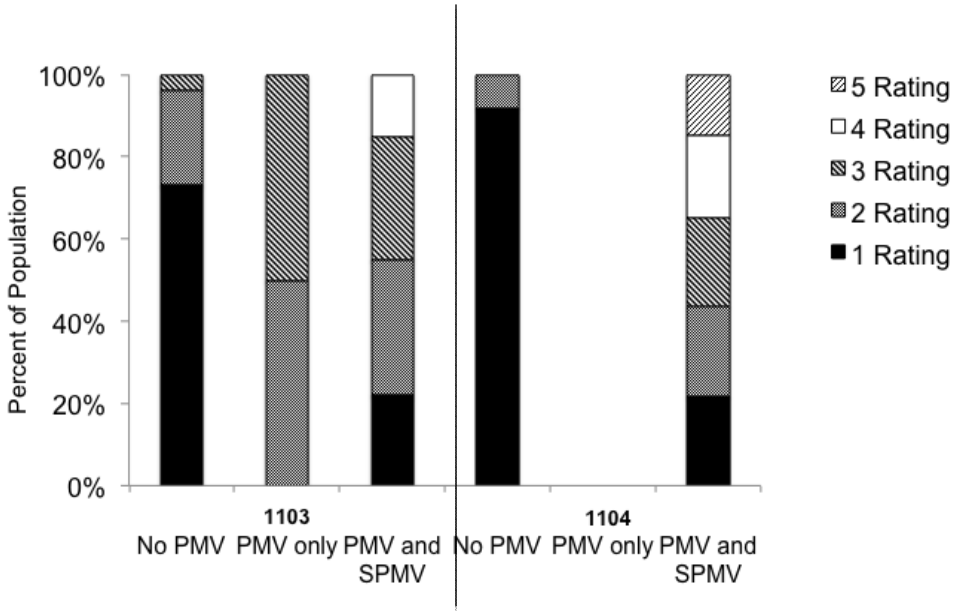

Fig. 5: Distribution of disease severity rating (1-5) among plants with no PMV, with PMV only, and both PMV and SPMV in two experiments PV01103 (1103) and PV01104 (1104). There were no plants from PV01104 infected with PMV only.

Persistence of viruses from 2012 to 2013

Some (10 or less) plants sampled in 2012 that were negative for PMV, positive for PMV only, or positive for PMV+SPMV were resampled in 2013 and tested for the presence of the viruses(Table 4). Among the 10 plants that had no virus in 2012, two acquired PMV only or PMV+SPMV in 2013. All of the eight plants with PMV+SPMV in 2012 retained the virus combination in 2013. In contrast, plants that were positive for PMV in 2012 were found to be either negative for PMV or positive for PMV+SPMV in 2013.

2012 No PMV			2012 PMV			2012 PMV+SPMV		
10			9			8		
2013			2013			2013		
No PMV	PMV	PMV +SPMV	No PMV	PMV	PMV +SPMV	No PMV	PMV	PMV +SPMV
8	1	1	4	0	5	0	0	8

Table 4: Detection of PMV and PMV+SPMV in the same plants sampled in 2012 and 2013. Numbers are numbers of plants in each category.

DISCUSSION

This is the first study in which the spatial extent and severity of disease caused by PMV in switchgrass in the field was documented. In addition, this is the first time in which SPMV was found in association with PMV infecting in switchgrass in the field. Mottling and stunting of switchgrass by PMV was reported over 50 years ago (Sill and Picket, 1957) but its occurrence on switchgrass in the field has not been recognized since. We have shown in this study that infection of switchgrass by PMV, or the combination of PMV and SPMV, can occur in significant numbers in switchgrass even in fields that are only in their second year of growth. The only established method of transmission for PMV and SPMV is mechanical transmission (Batten and Scholthof, 2004). This raises the question of whether or not mowing border grasses that could potentially harbor PMV or PMV+SPMV could potentially contribute to the spread of these viruses in and out of switchgrass field. Further identification of potential hosts for PMV will be important to develop management strategies for these viruses based on weed/alternate host management.

Experiments PV1103 and PV1104 exhibited high incidences of plant with virus symptoms in 2012 despite the plants being only in their second year of growth in the field and the plots not being harvested in the autumn of 2011. The experiment plots, however, were subjected to mechanical trimming in spring 2012, and this might have been responsible for the transmission of the viruses from nearby inoculum sources. Surveys of other switchgrass field experiments in the general location which were direct seeded over five years prior to this study also revealed virus symptomology and PMV infection (G.Yuen, personal communication), providing evidence local sources of virus inoculum are present. But because all of the plants in experiments PV1103 and PV1104 were first planted and grown in a greenhouse prior to field transplantation, the possibility that mechanical transmission of PMV and SPMV occurred in the greenhouse growth phase cannot be discounted.

The wide range of symptom severity observed in the field experiments, even among plant within a half-sibling family, suggests that there is great genetic diversity among switchgrass populations and among plants within populations as to resistance (i.e. resistance to virus transmission) and/or tolerance (i.e., ability to suppress symptom expression following virus transmission). In the comparison of five switchgrass strains in experiment PV0910 for the frequency of infection by PMV and PMV+SPMV, we found rates of single- and dual-virus infection varied considerably among the switchgrass strains. This was in line with results from experiments PV1103 and PV1104. Although K x S-derived families tested in PV1103 were not identical to the K x S strain used in experiment PV0910, they exhibited similar infection rates, and each was lower than the respective Summer-derived populations in PV1104 and PV0910. This is further evidence

that populations differ in resistance or tolerance to PMV and SPMV. It is also possible, however, that variations virus infection incidences observed in the field experiments were due to non-uniform exposure to virus inocula rather than variation in resistance in transmission. It will require further investigations involving uniform delivery of virus inoculum to all plants to confirm whether resistance to transmission of PMV or PMV+SPMV does exist in switchgrass. It should also be noted that at this time it is unknown if there are strain differences in PMV and therefore the differences in symptom development may also be due to strain differences.

We found that plants infected with PMV+SPMV in experiments PV1103 and PV1104 exhibited a wide range of symptom severity including no symptoms and only trace mottling. This finding is more direct evidence for the existence of tolerance. On the other hand, high symptom severity levels of 4 or 5 were observed in plants with the dual infection, whereas symptom levels did not exceed 3 in plants infected only by PMV. This supports findings that SPMV can act synergistically with PMV to heighten symptom expression in other graminaceous hosts (Batten and Scholthof, 2004). The fact that there also a considerable proportion of dual infected plants that exhibited mild or no symptoms suggests that the synergism effect is host plant-dependent.

In the analysis of plants collected in 2013, we found that the vast majority of plants infected by PMV also contained SPMV. This may indicate that the PMV+SPMV combination is more easily transmitted than PMV alone or that SPMV is easily spread to plants already infected with PMV. The finding that plants infected with PMV alone in 2012 appeared to either lose the single virus in or acquired SPMV 2013 suggests an alternative explanation, that switchgrass plants can overcome infection with PMV alone,

and that if plants are co-infected with PMV+SPMV the infection may be more effectively maintained. The finding that all 2012 samples that were positive for PMV+SPMV contained both viruses in 2013 supports the supposition that the dual infection is more effectively retained than the single infection by PMV. The number of plants sampled for analysis of virus persistence, however, was too small to draw definitive conclusions. To gain a better understanding of the dynamics PMV and PMV+SPMV infection within switchgrass, it would be necessary to conduct experiments with larger sampling sizes and carry out the study over a number of years.

Because switchgrass is perennial and because these viruses overwinter in crown and root tissues, infected hosts potentially will suffer stress from viral infection each year, which, over time, could result in decreasing growth and survival. Research conducted over a longer time span is needed to assess infection impacts on different switchgrass strains over the long-term, specifically the projected 8- to 10-year productive life span of a biofuel switchgrass crop. Because these viruses are mechanically transmitted, mowing and other mechanical operations will spread the viruses further and, thus, the proportion of infected plants within a field also will likely increase over time. Unless new switchgrass cultivars are selected for resistance or tolerance to PMV and SPMV, these viruses potentially could affect biomass yield.

Chapter 3:

GREENHOUSE STUDY OF RESISTANCE AND TOLERANCE IN SWITCHGRASS STRAINS

INTRODUCTION

As reported in Chapter 2, the frequency of infection by PMV or PMV+SPMV varied among switchgrass strains planted in breeding field experiments in Nebraska. The levels of symptom expression expressed in infected plants varied as well. These results mirror those reported by Sill and Pickett (Sill and Pickett, 1957) who inoculated switchgrass with PMV under greenhouse condition. It is unknown, however, whether plants found to not be infected with PMV or PMV+SPMV in the breeding field experiments or those plants exhibiting no symptoms in the study by Sill and Pickett were resistant to infection or had escaped mechanical transmission. Furthermore, it is uncertain whether differences in symptom expression among infected plants were related to differences in physiological tolerance or to variation in such factors as the time when infection occurred. To address these questions, a greenhouse experiment was conducted in which different strains of switchgrass were inoculated with PMV alone or the PMV+SPMV combination under controlled conditions and their response to viral inoculation was assessed on the basis of direct detection of virus in the plants, in addition to symptom development. The specific objectives of the experiment were: 1) to determine whether or not the switchgrass strains differ in susceptibility to infection by PMV or PMV+SPMV; and 2) to determine whether or not co-infection with SPMV affects infectivity by PMV.

MATERIALS and METHODS

Switchgrass strains and growth conditions

The four strains of switchgrass compared for disease response in this experiment were among the five planted in field experiment 910 and investigated in Chapter 2. The switchgrass strains used in this study were: 'Kanlow', a lowland cultivar; 'Summer', an upland cultivar; 'Kanlow' x 'Summer' (KXS) High Yield; and KXS Seed Increase. KXS High Yield is a selection from the newly released biofuel cultivar 'Liberty' (Vogel, et al., 2014), which originated from a crossing of 'Kanlow' and 'Summer'. Seed lots for 'Kanlow' and 'Summer' used in this experiment were the same as planted in field experiment 910, but seed lots for the other two strains were different between this experiment and field experiment 910.

Three to five seeds of a seed lot were placed in 2 in diameter by roughly 8 in in length sized conetainers (Ray Leach single cell Cone-tainers with UV stabilizers, Hummert's International, Mound City, MO) containing a potting mix (pasteurized soil mix at 1:1:1:1 soil: sand: peat moss: vermiculite). Planted conetainers were placed in a growth chamber kept at 18 h light/6 h dark with low pressure sodium lights, fluorescent lights, and incandescent lights, and at constant 21◦C. The plants in each conetainer were thinned to one per conetainer. Plants were watered once every Tues., Thurs., and Sat. and fertilized every Thurs. The fertilizer was 250 ppm of nitrogen (Peters General Purpose fertilizer at 20-10-20; this also contained micronutrients).

Viral inoculation methods

Prior to inoculation with virus (when plants exhibited approx. 5 cm of growth), leaf samples were obtained from each plant with gloved hands and assayed for the presence of PMV. Only plants confirmed to be PMV-free were used in the experiment. Plants were inoculated with virus when 8-15 cm tall. For each switchgrass strain, 25 plants were inoculated with PMV, 25 plants were inoculated with PMV+SPMV, and 25 plants were mock inoculated. Virus inoculum in the form of pearl millet (*Pennisetum glaucum*) leaves infected with PMV or PMV+SPMV was provided by K.-B. Scholthof, Texas A&M University, who produced the inoculum by inoculating greenhouse-grown pearl millet with RNA transcripts of PMV and PMV+SPMV from cDNA (Turina et al., 1998). As reported in Turina et al., 1998, "The cDNA constructs were made by 10 cDNA clones that had been either polyadenalated and primed with an oglio dT primer, primed with random oglionucleotides, or primed with specific oglionucleotides complementary to internal regions of PMV RNA. Each cloned insert was sequenced on both strands and the sequence was confirmed on the full-length infectious cDNA clone after it was constructed." To inoculate switchgrass plants in this study, roughly 2 g of virus-infected leaf tissue was ground in 100mL virus inoculation buffer (0.05M potassium phosphate monobasic and 1% celite in distilled/deionized water) that was previously autoclaved. A 10uL aliquot of the ground tissue extract was then rubbed onto each plant while wearing gloves, which were changed between PMV and PMV+SPMV inoculations. Mock inoculated plants had the virus inoculation buffer applied only (no leaf material) in the same method as the non-mock inoculated plants. After inoculation plants were grown for 30 days and observed for symptom development.

Assessment of plant response to viral inoculation

Following inoculation, plants were monitored every 6 days for the occurrence of symptoms (chlorosis, stunting, or necrosis). At 30 days post inoculation (dpi), plants were cut with sterile sheers at the base. A separate set of shears was used to cut plants given the same virus treatment and were wiped with sterile water and dried after cutting each plant to minimize virus transmission between plants. The tops were placed in -20◦C until processing. After 48 to 72 hrs storage, each sample was weighed and then tested for PMV and SPMV presence via RT-PCR described below. After harvest of tops 30 days dpi, plant crowns were kept in their conetainers under the same conditions as mentioned above. Following completion of the viral assay, plants that were virus inoculated but tested negative for the inoculated virus were returned to the greenhouse. When sufficient top regrowth had occurred, these plants were reinoculated with the respective virus treatment and then reassayed for virus 30 dpi.

For statistical analysis, the number of infected plants, i.e. those which harbored inoculated virus(es) and the total number of plants subjected to the virus treatment were used to calculate the percent infected plants (infection frequency). Corresponding data relating to symptom development was used to determine symptom frequency. Plant top weight measurements were subjected to factorial ANOVA with virus treatments and switchgrass strains as the factors. Treatment means were separated using Fisher's LSD test.

Virus Detection

Samples collected preinoculation and 30 dpi were ground in PBST buffer (1 to 2 g of tissue, same protocol as that outlined in Chapter 2) and then 50uL of each extract was

added to 400uL of phenol for RNA extraction. All subsequent steps were as outlined in the RNA extraction kit (Direct-Zol RNA MiniPrep Zymo Research). cDNA was prepared using SuperScript III Reverse Transcriptase (Life Technologies) following manufacturers instructions. First-strand cDNA synthesis reactions used primers specific for either PMV capsid protein (PMV-CP) or SPMV capsid protein (SPCP) (see Table 1). The generated cDNA samples were used as templates for standard Taq polymerase PCR amplification, using primers specific for PMV-CP and SPCP. Sequences for primers used in PMV/SPMV RT-PCRs are listed in Table 1.

Primers for PMV and SPMV RT-PCR	
PMV p26 Forward Primer	ATGAATCGCAATGGAGCTAC
PMV p26 Reverse Primer	TTATGCGCTAACCCCACTGA
SPMV 87 Forward Primer	ATGGCTCCTAAGCGTTCCA
SPMV 297 Reverse Primer	ATACAGGCGCGCGTTATACATC

Table 1: List of primers used for either PMV or SPMV RT-PCR (primers provided by Karen-Beth G. Scholthof at Texas A&M University)

RESULTS

Effects of SPMV on infection by PMV

When plants encompassing all four switchgrass strains were considered together, the frequency of infection by PMV alone, i.e. PMV detected, versus infection with the combination of PMV and SPMV, i.e. PMV and SPMV detected, were the same. 95% of all plants inoculated with either PMV alone or with PMV+SPMV became infected with the respective virus(es) after the first inoculation. When the inoculated plants that were

negative for the inoculated virus treatment were reinoculated, all of them tested positive for the respective virus(es).

There also were no significant differences in symptom development between inoculation with PMV and inoculation with the PMV+SPMV combination. For either virus treatment, fewer than five plants across the four switchgrass strains (i.e., <5%) showed any symptoms associated with viral infection. Otherwise, PMV- and PMV+SPMV-inoculated plants were similar in appearance to mock-inoculated plants.

In the factorial ANOVA for top weights, there was no significant virus treatment X strain interaction, but the virus treatment factor was significant at $P = 0.001$. Biomass averaged across all strains was lower in plants inoculated with PMV or PMV+SPMV as compared to the mock inoculation; the two virus treatments, however, reduced biomass to the same extent.

Differences among switchgrass strains

There were no significant differences found in infection frequency among the four tested switchgrass strains. The four tested switchgrass strains were equally susceptible to infection by PMV and dual infection by PMV+SPMV. There also were no significant differences in symptom development among the switchgrass strains. Very few plants in any switchgrass strain expressed symptoms (no more than 3 out of 25, or 12%).

There was a significant switchgrass strain effect ($P < 0.001$) for top biomass in the factorial ANOVA and no interaction with virus treatment. Top weight for strains 2700 and 2785b average across the virus treatments were higher than those for the other two strains.

DISCUSSION

One key finding from this experiment is that the different switchgrass strains are equally susceptible to infection by PMV. Furthermore, it appears that each switchgrass strain is completely susceptible, i.e., all switchgrass plants of a given strain can become infected with PMV given sufficient exposure of damage tissue to PMV inoculum. A third key finding is that the presence of SPMV confers no advantage to PMV in infecting switchgrass plants, i.e. PMV can be mechanically transmitted to a switchgrass plant with the same ease regardless of whether SPMV is present or not. These findings are key because they allow us to better understand the basis for our observations made in Nebraska breeding field experiments regarding the incidences of infection by PMV and PMV+SPMV (Chapter 2). First, if all switchgrass plants are susceptible to infection, then those plants that were found to be uninfected in the field experiments (exhibiting no symptoms and no presence of PMV) were uninfected not because they because they were resistant but because they escaped mechanical inoculation with PMV. This could have resulted from the plants being exposed to cytosol released from previously cut plants that contained no PMV virions or a virus titer too low for effective infection. Second, given that the strains tested in this experiment were equally susceptible to PMV infection then differences in PMV-infection incidence observed in the field experiments between different switchgrass strains (e.g. <10% in 'Kanlow' vs. >75% in 'Summer' in field experiment PV0910) can be explained by each population being planted in separate blocks and then each block of plants being exposed to different sources of plant cytosol, some with high titers of PMV, others with little or no PMV. It is important to note, however, that the PMV strain used in this experiment was derived from archived material

from the Sill and Pickett, 1957, study conducted in Kansas. It is unknown if the same or different strains were causing disease in Nebraska field experiments, and thus, the possibility of variation in infection frequencies among switchgrass strains in the field reflecting differential strain response to less infective PMV strain(s) cannot be discounted. Finally, the predominance of plants in the field experiments exhibiting dual infection with PMV+SPMV over plants infected with PMV alone was not due to SPMV aiding PMV in the infection process. Instead, it may reflect a higher number of previously-infected switchgrass or alternate host plants that served as viral inoculum sources carrying both viruses than carrying only PMV.

The very small number of plants exhibiting symptoms associated with viral infection in this experiment contrasts with numbers we observed in the field experiments (Chapter 2) and numbers reported by Sill and Pickett (1957) resulting from inoculation. One possible explanation for this discrepancy might be growth chamber conditions in this experiment being suboptimal to plant growth, and thus, keeping viral replication to a relative low level. The discrepancy also can be explained by the short time period between inoculation and observations for symptoms (30 days) in this experiment and the much longer incubation periods occurring in the field and used in the Sill and Pickett study. The fact that PMV and PMV+SPMV plants exhibited reduced biomass development within the 30 day period compared to the control indicates that the virus inoculum was indeed virulent, and thus, the incidence of symptom expression would likely have been higher had the plants been grown under more natural conditions or if the plants were kept growing longer after inoculation. The finding that most of the plants inoculated with PMV or PMV+SPMV did not exhibit symptoms does supports the supposition that individual

plants within various switchgrass populations have tolerance, the ability to suppress symptom expression despite being infected. This is in line with our observations from field experiments and with results from greenhouse inoculations reported by Sill and Pickett . The very small percentage of symptomatic plants in this experiment, however, does not allow any conclusion to be made as to whether or not switchgrass strains differ in the frequency of plants possessing tolerance. A definitive conclusion also cannot be made as to whether or SPMV acts synergistically with PMV to cause earlier or heightened symptom development. Nevertheless, the results suggest that there is no dramatic synergistic effect. This brings into question the nature of the synergistic relationship of PMV+SPMV within different host plant species. In previously studied hosts (St. Augustinegrass, centipedegrass, *Brachypodium distachyon*, and pearl millet), the PMV+SPMV combination demonstrated a synergistic relationship both in symptom expression as well as in virus accumulation or titer. In our field experiment observation, we found the highest symptom levels in PMV+SPMV-infection plants but not in plants infected solely with PMV; we also found, however, PMV+SPMV-infected plants with only mild or no symptoms (Chapter 2). These observations suggest that the synergistic relationship does occur in switchgrass but its occurrence is host plant dependent. To better answer these questions relating to strain differences or synergism, it will require repeating this experiment with a greater number of plants.

Chapter 4:

THESIS CONCLUSION

THESIS CONCLUSION

The overall goal of using switchgrass as a biofuel feedstock has numerous far-reaching benefits spanning improvement of the environment to strengthening of the US economy. The development of switchgrass for this truly new purpose presents the pathology community with a unique opportunity to address potential problems proactively while the crop is still in the development stage, before the problems impact growers. The threat of viruses such as PMV and SPMV in switchgrass, if not faced by the breeding and pathology community before switchgrass production is implemented, will demand attention after its launch in a large scale.

Field and greenhouse studies conducted in this thesis provide insight into the epidemiology of disease in switchgrass caused by the PMV complex and have implications as to future management strategies. First, PMV was found to infect all inoculated plants regardless of the genetic background of the plant. This indicates that immunity (resistance to infection) does not exist in switchgrass against PMV or it will be difficult to find. The implication is that management of the disease cannot be dependent on the use of resistance to prevent transmission. Second, results from greenhouse inoculation with the virus combination showed no synergistic effect as to transmission from co-infection by SPMV with PMV, while infection of plants in the field experiments by PMV+SPMV was much more prevalent than infection with PMV alone, suggesting perhaps that SPMV may enhance PMV establishment in switchgrass or simply that most sources of field inoculum contain both viruses.

It was also found that although severe viral symptoms that might impact biomass development were found in the field experiments, this effect is host-dependent. There

were plants in the field experiments that appeared to be symptom-free despite being infected with PMV or PMV+SPMV, an indication that some plants have greater tolerance to infection by the single virus or to the combination than others. Such plants could be the basis for breeding and selecting populations with higher tolerance to PMV overall. Such population could useful in achieving high yields in area in which PMV is indigenous. The use of PMV tolerant strains cannot be relied upon as the sole management procedure, however. Even highly selected populations of switchgrass will have genetic diversity among plants within the populations. So even populations selected for high overall tolerance to PMV would still have a portion of the population having low tolerance. If an indigenous local reservoir of virus inoculum is present within or near that population, the entire population could become infected, with the low-tolerance members contributing little to overall yield. In addition, the tolerant plants could become symptomless carriers of inoculum that potentially could be spread to any agricultural crop species growing nearby. Therefore, strategies that prevent dissemination of PMV inoculum into healthy switchgrass field and strategies that inhibit mechanic transmission of the virus among plants within a field need to be identified and implemented along with host tolerance. Currently a main established method of transmission of PMV and SPMV is mechanical transmission. The viruses could be spread easily via mowing, which is how switchgrass is harvested. However with switchgrass for use as a biofuel feedstock in this study, it is most optimum to harvest switchgrass after senescence, when the leaves are very dry. This is likely to reduce the chances of transmission as compared to what would occur if mowing were done on green tissue, which would have much more moisture in the leaves.

With mowing it is also important to consider any potential plants near the boarders of a switchgrass field that could also be infected with PMV or PMV+SPMV. PMV has been found to infect the following in nature: St. Augustinegrass (*Stenotaphrum secundatum*), switchgrass, and centipede grass (*Eremochloa ophiuroides*). Millet plants (*Setaria italic* L., *Panicum miliaceum* L., and *Pennisetum glaucum* L.) can be used to propagate PMV and its satellites. The virus has also been mechanically transmitted to maize (*Zea mays* L.) and some wheat cultivars (*Triticum aestivum* L.). (Batten and Scholthof, 2004) It is possible that other plant species that can be infected with PMV but have not been shown to be susceptible. The other concern in not having a more complete host list for PMV and PMV+SPMV is that the planting of biofuel switchgrass into a new area might result in the inadvertent introduction of PMV and SPMV into the new switchgrass crop. As to management, the best option would be to plant resistant or tolerant plant material. The former type of strategy would include identifying and removing virus-infected weed hosts. An example of the second type of strategy would be harvesting switchgrass when the plants are senescent and thus less prone to infection via mechanical transmission.

Although PMV infection in switchgrass was previously known, there is still much to learn about PMV and PMV+SPMV within switchgrass, especially as new strains of switchgrass are being developed. Several key areas relating to the epidemiology and management that should be explored include: management tactics that best reduce incidence of PMV and PMV+SPMV; sources of true resistance (immunity) in switchgrass; strains of switchgrass with the most tolerance, and the most effect strategies for deploying resistant or tolerant material. In addition, the nature of the interactions of

PMV and PMV+SPMV within switchgrass needs to be investigated further at a molecular level within switchgrass. Important questions at the molecular level include the mechanisms behind tolerance and resistance, if available; and the fate of the viruses in tolerant plants. Also it is important to remember that the field experiments described here are from two seasons. Given it is expected that a biofuel switchgrass crop will provide nearly a decade of yield, it is important to make sure whether or not conclusions drawn from two years remain consistent over a ten year timespan.

Results from this thesis have not produced evidence in the greenhouse of any resistant plant material. Data from field experiments suggests that some switchgrass strains have lower infection rates than others. Because all the plants in the field experiments were hand planted, it is possible that there was PMV and PMV+SPMV spread in the greenhouse and that some switchgrass strains experienced some sort of escape at this point. However, PMV has been found in naturally seeded switchgrass fields also at the Mead, NE location (unpublished Gary Yuen). Also because all field experiments were randomized in planting and were mowed, it can be argued that all plants were exposed. Field experiments seem to indicate that response to PMV and PMV+SPMV infection in both frequency and severity can differ from switchgrass strain to switchgrass strain. This indicates that tolerant material is available and suggests that there may be resistant plant material, or at least a chance that it could be developed.

This thesis provides a diagnostic method for the detection of PMV and SPMV in switchgrass that can be used to screen for resistance and/or tolerance or to gain better diagnostics in the future. This diagnostic method can be used by breeders to ensure the development and deployment of switchgrass strains that maintain high yields if exposed

to PMV or SPMV. This diagnostic method can also be used in the future for growers to determine if they have PMV or SPMV within their fields.

Recommendations to growers in terms of sanitation to limit the spread of PMV and SPMV include mowing switchgrass after the leaf material has become senescent. This reduces the amount of moisture within leaves and thus reduces the likelihood of viral mechanical transmission. Growers should also consider cleaning equipment between fields to reduce the likelihood of introducing PMV or SPMV to another field. Cleaning could include ensuring the removal of plant material and spraying equipment with a bleach solution. However the effectiveness of these methods would need to be verified in future work.

If resistance or tolerance is found, it is important to understand how stable it is. This is dependent on PMV and perhaps SPMV. If the PMV were to mutate easily, it may be able to overcome tolerance or resistance in switchgrass. If resistance was found, knowing whether or not other plants, namely weeds, were able to be infected management of those would be important. If alternative hosts were allowed to be in close contact with resistant switchgrass plants, it is possible that the virus could mutate on the alternative host to the point where it could become infectious on the switchgrass. However this has never been confirmed in switchgrass. If the mutation rate of PMV and SPMV are low within switchgrass, this should make resistance or tolerance not only effective but also perhaps long lasting. Thus to understand how best to deploy resistance or tolerance, a basic understanding of how the virus interacts with switchgrass must be studied more thoroughly.

Chapter 5:

Literature Cited

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