SOME PHYSICAL PROPERTIES OF PANICUM MOSAIC VIRUS

by

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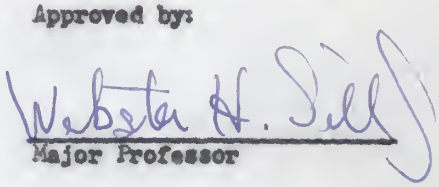

Major Professor

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INTRODUCTION

Panicum mosaic, a virus disease on switchgrass, Panicum virgatum, L., was first observed in 1953 in a 2-year-old breeding nursery at the Kansas Agricultural Experiment Station (Sill and Pickett, 1957).

According to Sill and Talens (1962) accumulating evidence indicates that this virus is probably endemic in the Great Plains States. However, the disease has not been reported from any State except Kansas. Although not important in Kansas now, the disease can cause such extreme forage and seed reduction in individual plants that it must be considered as potentially severe (Sill and Pickett, 1957).

This virus is manually transmitted in the greenhouse and does not appear to be seedborne (Sill and Desai, 1960). Attempts to transmit the virus using the apple grain aphid, Rhopalsiphum prunifoliae (Fitch), failed (Sill and Pickett, 1957).

Panicum mosaic virus appears to be a new virus and this study was initiated to determine some physical properties for further characterization of the virus. Digitaria sanguinalis L. (crabgrass) was used as the host because it has broader and more tender leaves, and is easier to grow than P. virgatum (switchgrass).

REVIEW OF LITERATURE

The virus of switchgrass, Panicum virgatum, given the name Panicum mosaic virus (PMV) by Sill and Pickett (1957) was first

seen in 1953 in a 2-year-old breeding nursery at the Kansas Agricultural Experiment Station. Then in 1955 the disease was observed in a 3-year-old breeding nursery with about 4 percent of the plants being infected (Sill and Pickett, 1957). It was severe in several selection in the variety Blackwell.

In the field, some plants were badly stunted but some were not, even though diseased (Plate 1). Primary symptom expression was usually a mild green mosaic and mottle which did not develop until July and occasionally not until early August. In advanced stages, a yellow or light green blotchy mottle, mosaic and stunting of the leaves were characteristic and the entire plant or sectors of it were chlorotic (Plates II and III).

Further investigations by Sill and Pickett (1957), Sill and Desai (1960), and Sill and Talens (1962) established a list of hosts, namely: Panicum virgatum L., P. miliaceum L., P. ramosum L., P. capillare L., P. scribnerianum Nash, P. hallii Vasey, P. decompositum R. Br., P. turgidum Forsk., Setaria italica L., S. lutescens W., S. verticillata L., Digitaria sanguinalis L., and Echinochloa crusgali L., a symptomless carrier.

So far all tested varieties of wheat, oats, rye, barley, corn and sorghum have been immune (Sill and Pickett, 1957).

Incubation periods varied in different hosts at varying temperatures but generally were shorter at the more ideal warm temperatures (Sill and Talens, 1962). This was also observed in this study. In the greenhouse, symptoms were more pronounced during summer months when the temperature was a high as 85^{0} to

95°F. This is further evidence that Panicum mosaic virus is a warm temperature virus as mentioned earlier by Sill and Talens (1962). The fact that it occurs naturally in an important native perennial grass, Panicum virgatum, Sill and Talens (1962) suggests, that it may be an endemic virus in the high Great Plains States since high summer temperatures are characteristic in this area.

No studies have been made of the physical and chemical properties of panicum mosaic virus. This study deals with the physical properties such as dilution end-point, thermal inactivation point, and longevity in vitro. These properties may be most useful in differentiating panicum mosaic virus from other small grain viruses.

MATERIALS AND METHODS

Digitaria sanguinalis was used as the host for this study because it has a broader leaf which showed the symptoms very clearly, softer leaves to inoculate, and is much easier to grow than the common host, Panicum virgatum.

This study was conducted with crude and/or clarified or chloroplast-free extracts of infected plants. All virus preparations were obtained from fresh cultures in the greenhouse which were started from dessicated leaf tissue. Plants used were about 1 to 1½ months old. The plants used for the thermal inactivation and longevity in vitro experiments were transplanted about 1 month from sowing into square flats and then after 15 days were inoculated. Healthy control plants were

grown beside treated plants but were given no treatment.

Preparation of Inoculum

Fresh infected leaves showing marked symptoms were picked, cut into small pieces and weighed. For every gram of tissue 9 ml of distilled water was added to make approximately a 1 to 10 dilution. This was blended in a Waring Blendor for about 2 minutes or until a homogeneous mixture was obtained. The extract was collected by squeezing the mixture through a cheesecloth. Then this was refiltered through another piece of cheesecloth and was labeled "crude sap".

Equal amounts of crude sap were centrifuged at 4,500 rpm for 10 minutes. The supernatants were decanted very carefully so as not to disturb the sedimented chloroplasts and other extraneous materials. These decanted supernatants were centrifuged at 6,500 rpm for 10 minutes and again the supernatants were decanted and finally spun at 9,500 rpm for another 10 minutes. The third supernatant was almost clear and was collected and labeled "clarified sap". Fresh preparations of crude and/or clarified sap were used in all experiments.

Dilution End-Point

A series of sterilized test tubes containing 9 ml of distilled water were prepared. From the prepared 1 to 10 diluted stock solution of inoculum, 1 ml was transferred to 9 ml of water by a pipette, making the dilution 1 to 10^2 dilution was transferred to 9 ml of water, making a 1 to 10^3 dilution.

Subsequent dilutions of 1 to 10^4, 1 to 10^5 and 1 to 10^6 were prepared. These dilutions were done separately on crude and/or clarified sap.

Each dilution was well shaken before subsequent dilutions were made. These series of dilutions were then used to inoculate plants by the mechanical or abrasive inoculation method. Inoculations started with the highest dilution (1:10^6) and proceeded down to the lowest dilution (1:10).

Thermal Inactivation Point

Thermal inactivation was accomplished using the 1 to 10 inocula stock solutions both in crude and/or clarified sap. The infective sap was placed in glass tubing 24 cm in length and 4 mm in outer diameter. These tubes were filled with corks at one end so that they would float vertically in the water bath. The water baths used were the Fisher Unitized Constant Temperature Bath with a micro-set thermoregulator and a power stirrer attached to a Fisher Unitized Bath Control and the Constant Temperature Water Bath built by Precision Scientific Company.

Transfer of the desired amount of sap into the glass tubings was done by means of a syringe and needle which made it easier to adjust the level of sap just below the cork. This was very important to assure that the whole inoculum solution was under the water level of the bath. The bottom end of the tubes were plugged with corks. These tubes containing the extract were then placed in a wire screen in an orderly fashion so that the

fitted corks were suspended on the screen. This saved a lot of time and diminished possible errors in timing. It eliminated also the possibility of the tubes touching the copper heating wire and the power stirrer in the water bath. The power stirrer was provided to maintain a uniform temperature in the water bath. After 10 minutes of heat treatment the glass tubings containing the extract were removed from the water bath and immersed immediately in cool running water for about 5 minutes. The thermal inactivation tests were conducted at 60, 65, 70, 75, 80, 85 and 90°C ± 0.2°C.

After the heat treatment and subsequent cooling, the corks plugged on the bottom end of the tubes were removed to release the heat treated inoculum and plants were inoculated immediately.

Longevity In Vitro

Both crude and/or clarified sap at a dilution 1 to 10 were used in these experiments. Equal amounts of these inocula stock solutions were placed in two separate flasks and stored at a room temperature of about 25-30°C. Corks were used for the experimental trials. Inoculations were made on the same day stock solutions were prepared and after 2, 4, 8, 10, 12, 14, 16, and 20 days. Flasks were shaken very well each time inoculum was taken for inoculation.

Inoculation Technique

The carborundum-rubbing method was used in all inoculations (McKinney, 1930). In this method the thumb and index finger are

dipped into the inoculum to which 400 mesh carborundum powder had been added. The leaves were rubbed gently, but with enough pressure to make only slight injury to the epidermal cells. After rubbing, plants were immediately washed with water. The plants were about 1 to 1½ months old when inoculated. This time of inoculation varied because the seeds took more time to germinate during winter than summer months. The inoculations for the dilution end-point started with the highest dilution (1 to 10^6) and proceeded to the lowest dilution (1 to 10). In the thermal inactivation tests, inoculations were made first with the clarified sap and then with the crude sap for each treatment. The same schedule was followed for the longevity in vitro experiments. All inoculated plants were maintained between about 25-35°C in the greenhouse. Final readings were made after 4 to 5 weeks from the day of inoculation in order to give all plants adequate time to develop symptoms.

During the experimental work all utensils contacting the virus were sterilized in a steam sterilizer for one hour without pressure. Prior to each inoculation, hands and fingers were washed thoroughly with soap and water and dried with paper towel.

EXPERIMENTAL RESULTS

Preliminary Trials

The purpose of the preliminary trials was to find the probable or approximate dilution end-point, thermal inactivation point and longevity in vitro of PMV.

For dilution end-point, crude and/or clarified sap were used to inoculate individual plants with dilutions from 1 : 10 to 1 : 10^6. Dilutions were made with distilled water. The mechanical or abrasive inoculation method was used. After 4 weeks from the day of inoculation, readings were made. Results in Table 1 show that no infection occurred at dilution 1 : 10^5 for crude sap and for clarified sap it was at 1: 10^6 dilution.

For the thermal inactivation point, a preliminary trial on the comparison between crude and/or clarified sap was conducted. Each treatment was done separately using freshly prepared inoculum. Heat treatments were conducted in a Fisher Unitized Constant Temperature Bath. Results in Table 2a show that the infectivity of panicum mosaic virus was not affected at 60° but diminished at 65°C for crude sap, and no infection was observed for clarified sap. Both crude and clarified sap showed no infection was observed for clarified sap. Both crude and clarified sap showed no infection at 70°C. There was no significant difference in infectivity between crude and/or clarified sap.

The preliminary test for longevity in vitro of the virus also was done with crude and clarified sap. Equal amounts of crude and clarified sap were placed in separate flasks and then covered with corks. These flasks were kept at room temperature at about 25-30°C. Sets of plants were inoculated on the same day the inocula were prepared and after 2, 4, 8, 10, 12 and 16 days. Results indicated that the infectivity of the virus decreases gradually with an increase in the number of days the inoculum-source was kept at room temperature. After 16 days,

the infectivity was totally lost (Table 3). There was no apparent difference between crude and clarified sap.

Experimental Trials

Based upon the results of the preliminary trials on the dilution end-point, thermal inactivation point, and longevity in vitro of panicum mosaic virus, three additional trials for each were conducted. The number of plants inoculated for each treatment ranged from 9 to 62. Readings were made 4 to 5 weeks after the day of inoculation during cool months and 10 to 15 days after during summer months.

Dilution End-Point : Table 4a presents data showing the dilution end-point for crude sap to be somewhere between 1 : 10^4 and 1 : 10^5. For clarified sap there were 2 of 59 plants infected at 1 : 10^5. A gradual decrease of infected plants with increased dilution was shown in all three trials for crude sap. The same was true with clarified sap except for the first trial where there were fewer infected plants at 1 : 10^2 than at 1 : 10^3 and 1 : 10^4 dilutions and for the third trial where there were fewer infected plants at 1 : 10 than at 1 : 10^2 dilution. Table 4b shows clearly that the percent infectivity of both crude and/or clarified sap decreases as dilution increases and that clarified sap tends to be slightly more infective.

Thermal Inactivation Point: There were 2 aspects to the thermal inactivation determinations : (1) a comparison between crude and/or clarified sap infectivity after heat treatment and (2) determination of the thermal inactivation point of panicum

mosaic virus both in crude and/or clarified sap.

For the comparison between crude and/or clarified sap infectivity after heat treatment, the virus inocula used were freshly prepared for each treatment. Results in Table 2b show that the difference in infectivity between crude and clarified sap was not significant and that the virus is still infective at 70°C.

For the determination of the thermal inactivation point, the freshly prepared virus inocula were used in all treatments for each trial. Results in Table 5 show that the thermal inactivation point of panicum mosaic virus in both crude and clarified sap is about 90°C. The infectivity gradually decreased as the temperature became higher.

Longevity In Vitro : Three trials were conducted in this determination and each trial were done separately. Table 6 shows that panicum mosaic virus started to lose its infectivity after the 14th day except for the third trial with clarified sap where it started after the 8th day. Infectivity was being lost rapidly and was very low by the 16th day and was completely lost by the 20th day in all trials. There seemed to be no difference between crude and/or clarified sap.

Table 1. Preliminary trial on dilution end-point of panicum mosaic virus.

Dilutions	Crude sap	Clarified sap
1:10	9/14*	6/30
$1:10^2$	7/17	8/15
$1:10^3$	8/18	6/25
$1:10^4$	4/26	6/28
$1:10^5$	0/19	7/26
$1:10^6$	0/20	0/18

*Numerator indicates number of plants infected. Denominator indicates number of plants inoculated.

Table 2a. Preliminary trial on the comparison between crude and/or clarified sap infectivity after heat treatment.

Temperature (°C)	Crude sap	Clarified sap
60	19/20*	18/20
65	2/17	0/23
70	0/19	0/19

*Numerator indicates number of plants infected. Denominator indicated number of plants inoculated.

Table 2b. The comparison between crude and/or clarified sap infectivity of Panicum mosaic virus after heat treatment.

Temperature (°C)	Crude sap Trials*			Clarified sap Trials		
	I	II	III	I	II	III
60	16/18	17/20	43/47	22/25	21/23	49/52
65	3/22	3/22	2/49	5/20	0/15	0/26
70	0/19	1/36	5/62	1/18	0/24	11/55

*Numerator indicates number of plants infected. Denominator indicates number of plants inoculated.

Table 3. Preliminary trial on the longevity in vitro of panicum mosaic virus.

Inoculum age (days)	Crude sap	Clarified sap
0	14/15*	11/14
2	18/19	19/20
4	16/17	13/15
8	12/16	9/15
10	3/17	6/13
13	2/15	4/19
16	0/18	0/16

*Numerator indicates number of plants infected. Denominator indicates number of plants inoculated.

Table 4a. Experimental results on dilution end-point of panicum mosaic virus.

Dilutions	Crude sap Trials*			Clarified sap Trials		
	I Jan.	II Oct.	III Mar.	I Jan.	II Oct.	III Mar.
1:10	15/22	17/18	14/15	11/22	18/19	11/14
$1:10^2$	8/14	15/17	12/14	3/20	15/18	15/16
$1:10^3$	4/14	12/18	5/10	9/25	13/20	19/25
$1:10^4$	3/17	4/20	3/12	8/30	5/20	4/17
$1:10^5$	0/15	0/18	0/19	1/27	0/20	1/12
$1:10^6$	0/18	0/17	0/20	0/22	0/18	0/12

*Numerator indicates number of plants infected. Denominator indicates number of plants inoculated.

Table 4b. Percent infectivity of individual dilution end-point trials.

Dilutions	Crude sap Trials*			Clarified sap Trials		
	I	II	III	I	II	III
1:10	68.2	94.4	93.3	50.0	94.7	78.6
$1:10^2$	57.1	88.2	85.7	15.0	83.3	93.8
$1:10^3$	28.6	66.7	50.0	36.0	65.0	76.0
$1:10^4$	17.7	20.0	25.0	26.7	25.0	23.5
$1:10^5$	0	0	0	3.7	0	8.3
$1:10^6$	0	0	0	0	0	0

*Figures rounded to the first decimal place.

Table 5. Thermal inactivation point of panicum mosaic virus.

Treatment (°C)	Crude sap Trials*			Clarified sap Trials		
	I	II	III	I	II	III
60	10/10	10/10	9/10	10/10	10/10	9/10
65	5/10	5/10	5/10	9/10	4/10	4/10
70	8/10	4/10	3/10	8/10	3/10	3/10
75	3/10	2/10	3/10	4/10	2/10	2/10
80	1/10	1/10	2/10	2/10	1/10	1/10
85	1/10	0/10	0/10	2/10	0/10	0/10
90	0/10	0/10	0/10	0/10	0/10	0/10

*Numerator indicates the number of plants infected. Denominator indicates the number of plants inoculated.

Table 6. Longevity in vitro of panicum mosaic virus.

Inoculum age (days)	Crude sap Trials*			Clarified sap Trials		
	I	II	III	I	II	III
0	10/10	10/10	20/20	10/10	10/10	20/20
2	20/20	20/20	25/25	20/20	20/20	20/20
4	20/20	20/20	20/20	20/20	20/20	20/20
8	20/20	20/20	19/20	20/20	20/20	20/20
10	18/20	16/20	12/20	20/20	18/20	5/20
12	10/10	10/10	10/10	10/10	9/9	6/10
14	10/10	9/10	10/10	10/10	10/10	0/10
16	6/20	5/20	2/10	1/10	1/10	0/10
20	0/10	0/10	0/10	0/10	0/10	0/10

*Numerator indicates number of plants infected.
Denominator indicates number of plants inoculated.

PLATE I

Susceptible clump of *Panicum virgatum*, switchgrass, plants infected with the PMV. Notice extreme stunting, chlorosis, and necrosis compared with the healthy clump of plants at the background.

PLATE I

PLATE II

Leaf symptoms caused by Panicum mosaic virus on *Panicum virgatum*. The leaf at the far left is healthy. Early symptoms may be light green to yellow spots or streaks (3 leaves at left). Later a general blotchy, irregular light green-to-yellow mottle and mosaic is more characteristic (leaves toward the right). Older infected leaves may be a bright yellow.

PLATE II

PLATE III

Panicum mosaic virus symptoms as they appear on Digitaria sanguinalis, crabgrass. Note the green-to-yellow mosaic and mottle and the dark green irregular eyespots with yellow borders. Notice the gradual dying of diseased leaves from the tip. Healthy leaf at far right.

PLATE III

DISCUSSION AND SUMMARY

Studies on the host range of panicum mosaic virus (PMV) by Sill and Pickett (1957), Sill and Desai (1960), and Sill and Talens (1962) indicate that it is a distinct grass virus. In an attempt to characterize this virus further, this study was initiated to determine some physical properties such as dilution end-point, thermal inactivation point and longevity in vitro. The fact that only grasses can be infected and no local lesion semiquantitative hosts suitable for virus assay have been found as yet, this study was conducted on the basis of relative infectivities in a systemic host.

Standard methods for determining physical properties were followed. Digitaria sanguinalis (crabgrass) was used as the host or indicator plant. The inoculation technique used was the carborundum-rubbing method. All experiments were conducted with both crude and/or clarified sap so that comparisons could be made.

Characteristic symptom expression was usually a mild green mosaic in the early stage. In advanced stages, a yellow or light green blotchy mottle, mosaic and stunting of the leaves were very pronounced. Generally, the entire plant or sectors of it were chlorotic and stunted (Plates I,II and III). These were the primary bases for determining infection in this study and if in doubt, subinoculations were conducted.

The dilution end-point of crude sap was between $1:10^4$ and $1:10^5$ and for clarified sap appeared to be very near $1:10^5$

(Table 4a). This small difference could be due to a slightly higher concentration of the virus in the clarified sap or to the presence of a mild inhibitor in the plant extract. Bawden (1964) mentioned that some viruses are rapidly inactivated by components of sap, particularly by oxidizing enzymes, but more general importance is the fact that extracts of many plants contain substances that although not virus inactivators inhibit infection. Tannins are common components of plants and their presence in sap often explains failure to transmit a virus by mechanical inoculation. The data obtained however, was not sufficient to draw a definite conclusion. A gradual decrease in the percent of infectivity was observed for both crude and/or clarified sap as dilution increased (Table 4b). There was also an indication that more plants were infected during the October and March trials than the January trial. This could be due to environmental conditions such as temperature, light intensity, and daylength. Kassanis (1957) stresses that almost any change in the conditions under which plants are grown affects their susceptibility to infection, the way in which infected plants respond to infection, and the extent to which viruses accumulate in them.

A comparison between crude and/or clarified sap infectivity after heat treatment was conducted before the thermal inactivation point was determined. A preliminary trial for treatments at 60°, 65° and 70°C indicated that crude sap was still infective after heat treatment at 65°C while the clarified sap showed no infected plants (Table 2a). However, the next trials

showed inconsistent results (Table 2b). Trial 1 for the treatment at 65°C showed that there were more plants infected by clarified sap than with crude sap. Trials I and II for treatment at 70°C showed not much difference whereas in Trial III, there were more plants infected by clarified sap than with crude sap and more than at 65°C. These inconsistencies could be due to the differences in concentration of virus, non-homogeneity of the testplants or some other factor.

Consequently, to determine the thermal inactivation of panicum mosaic virus, three more carefully controlled trials were conducted. Table 5 presents data for treatments from 60° to 90°C with an interval of 5°C. Results indicated that the thermal inactivation point of the virus was near 85°C. These trials were conducted during the rather ideal months of May and June and symptoms were observed 7 days after inoculation. The readings were made 10 days after inoculation.

The preliminary trial on the longevity in vitro of panicum mosaic virus indicated that both crude and/or clarified sap lost infectivity after the 16th day (Table 3). However, experimental trials (Table 6) showed that both inocula were still infective at low levels after the 16th day except for the third trial with clarified sap. This could be due again to differences of virus concentration. It appeared however, that the virus was resistant to aging for 16 days at room temperature. The data in Table 6 showed a 100 percent infectivity even after 14 days and a rapid drop in infectivity by the 16th day.

The high thermal inactivation point and rather high dilution

end-point would make one think that this virus might be related to tobacco mosaic virus. However, the relatively short period in which the virus is inactivated in vitro and the narrow host range reported by others would eliminate this possibility. So far PMV does not appear to be closely related to any other known plant virus.

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Panicum mosaic, a virus disease of Panicum virgatum L. (switchgrass), was first observed in 1953 in a 2-year-old breeding nursery at the Kansas Agricultural Experiment Station. All evidence indicated that it was a new virus disease. Hence, it was named Panicum mosaic virus (PMV).

It has been reported that this virus is probably endemic in the Great Plains States. However, the disease has not been reported from any state except Kansas.

This virus was easily transmitted in the greenhouse by manual inoculation. It was also found that it is not seed-borne and not transmitted by the apple grain aphid, Rhopalsiphum prunifoliae (Fitch).

Incubation periods varied with temperatures but generally were shorter at the more ideal warmer temperatures of 85° to 95°F.

The studies reported in this paper involved both greenhouse and laboratory work. Standard procedures for the determination of dilution end-point, thermal inactivation point and longevity in vitro were followed. Three experimental trials plus preliminary trials were conducted for each physical property. Digitaria sanguinalis L. (crabgrass), was used as host plant in all experiments.

The dilution end-point for crude sap was found to be between $1:10^4$ and $1:10^5$. For clarified sap it was very near $1:10^5$ dilution. The percent infectivity of both crude and/or clarified sap decreases as dilution increases.

The difference between crude and/or clarified sap in-

fectivity after heat treatment was not significant. The thermal inactivation point was found to be between 85° and 90°C for both.

Longevity in vitro experiments showed that both crude and/or clarified sap were resistant to aging at room temperature until the 16th day. Occassionally the virus was still infective at the 16th day but never at the 20th day.

The differences found in different trials were probably due to environmental conditions in the different seasons such as temperature, light intensity, and daylength. The inocula also probably varied in virus concentration even though leaves of similar age and symptoms were picked. Another probable variable was the lack of complete homogeneity of the testplants.