CHARACTERIZATION OF WHEAT STREAK MOSAIC VIRUS SEROTYPES AND THEIR EFFECT ON WHEAT (*TRITICUM AESTIVUM* L.)

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Submitted to the Faculty of the
Graduate College of
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
December, 1995

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ACKNOWLEDGEMENTS

I would like to express my sincere heartfelt gratitude to my major academic advisor, Dr. Robert M. Hunger, who accepted me as his student in June of 1992. I am also grateful for his allowing me the self determination of discovering which research I wished to conduct and then allowing me to work in this area even though it did not correlate very well with his program. Also, my chosen area of research required substantial financial input without which this project could not have been accomplished and I sincerely appreciate his acceptance of that financial burden.

Special appreciation is also extended to Dr. Hassan A. Melouk and Dr. Larry L. Singleton both of which constantly provided words of optimism and encouragement that helped me to fulfill the final seven months of Ph.D. requirements. I will remember both of them fondly for their upbeat attitude and humor.

Special thanks are extended to Dr. John L. Sherwood for the provision of an area in his lab where the majority of this work was conducted.

I also sincerely thank Dr. James A. Webster for

graciously serving on my committee as a replacement for Dr. David L. Weeks and to Dr. Alex Filonow for providing the financial assistance in the spring of 1991 without which I could not have continued my Ph.D.

My deepest appreciation is reserved for Mae E. Rankin who became my best friend and provided both psychological and emotional support, without which this long journey could not have been accomplished.

Finally, the degree of Doctor of Philosophy granted to me by Oklahoma State University this year of 1995, is dedicated to my parents: Joe and Rickie, whose affection, support, and understanding allowed me to complete this degree.

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CHAPTER I

INTRODUCTION

Wheat is an important grain crop throughout the world and is a member of the Graminaceae, tribe Hordeae and genus *Triticum*. Wheat is widely cultivated and has been a primary source of food since man changed from a hunter/gatherer to cultivating crops to obtain food. Many pests and diseases affect the growth and development of wheat and ultimately its yield. Some of the most important of these diseases are caused by viruses. In North America, these are barley yellow dwarf virus (BYDV), wheat soilborne mosaic virus (WSBMV), wheat spindle streak mosaic virus (WSSMV), and wheat streak mosaic virus (WSMV). (Brakke, 1987b; Cook, 1991; Hatchett et al., 1987; Hunger et al., 1992; Moorhead, 1959; Wiese, 1987).

Losses associated with wheat streak mosaic (WSM) can be large, and losses in Kansas alone have exceeded 30 million dollars in some years (Wiese, 1987). WSM is common throughout the central and western portions of North America with the central Great Plains of the U.S. being the area where most significant losses have been reported. The area affected by WSM can range from small localized areas to thousands of acres

with corresponding yield decreases ranging from insignificant to complete.

WSMV is transmitted by the wheat curl mite (WCM), *Aceria tulipae* (Kiefer) [syn. *Eriophyes tulipae*] (Connin, 1956). Thus, the occurrence and spread of WSM is related to this mite (Slykhuis, 1955, 1962. 1965, 1980). *Aceria tulipae* feeds by piercing plant cells and then sucking the contents (Brakke, 1971, 1987b). While doing so, the virus is transmitted to host plants. With severe mite infestations, WSM also may increase.

Characteristic symptoms of WSM are stunted plants with yellow mottled and/or streaked leaves. Infections most often occur in the fall with symptoms appearing the following spring. Symptoms become more severe as temperature increases (Hunger et al., 1992; Slykhuis et al., 1957). Since infected plants are often symptomless in the fall, the incidence of disease and its projected severity are difficult if not impossible to predict. Inspection of plants periodically for the presence and population levels of the WCM may be more reliable than visual symptoms for predicting severity. However, it must be remembered that not all mites are viruliferous and therefore WCM numbers must be considered with care.

Alternative plants that serve as hosts to either the virus or mites are critical to the persistence and spread of

WSMV (Hatchett et al., 1987; Wiese, 1987). Before wheat has fully matured in spring, the mite spreads to volunteer wheat seedlings or alternative hosts. Volunteer wheat seedlings may result from the harvesting procedure or after hail has knocked seed to the soil where it germinates. WSM is most severe when such a "green bridge" (Cook, 1991), i.e., a host continuum, exists for both the mite and the virus between wheat harvested in the spring and the emergence of wheat seedlings in the fall.

Symptoms produced on specific wheat cultivars have been used to separate WSMV isolates. Such isolates have been separated based on severity of symptoms with basically only two types reported , i.e., virulent and very mild strains. Although, serological reactivity of WSMV isolates was examined by Moorhead (1959) and Slykhuis (1963), different serotypes of WSMV were not reported.

Therefore, because of the importance of wheat production in Oklahoma, the increasing interest in dual usage of wheat for grazing animals and for grain production, the importance of WSM, and preliminary reports of serological reactivity of WSMV, the objectives of this research were:

1. To determine the effect of time of infection and clipping of plants to simulate grazing, on development and severity of WSM expressed on selected wheat cultivars with possible sources of resistance.

2. To determine if serotypes of WSMV exist, in Oklahoma as well as in other areas of the United States where wheat is cultivated.
3. To characterize WSMV isolates, collected from Oklahoma or obtained from surrounding states, by protein fingerprinting, serological assays, and level and type of disease expression.

CHAPTER II

LITERATURE REVIEW

Importance of Wheat Streak Mosaic

The four viral pathogens of cereals that cause the most prominent yield losses in wheat in North America are barley yellow dwarf virus (BYDV), wheat streak mosaic virus (WSMV), wheat soilborne mosaic virus (WSBMV), and wheat yellow mosaic virus [(WYSV) (also called wheat spindle streak mosaic virus (WSSMV)] (Cook and Veseth, 1991; Wiese, 1987). BYDV is transmitted by species in three genera of aphids (*Sitobion*, *Schizaphis*, and *Rhopalosiphum*) (Matthews, 1991; Ranieri et. al, 1993) and WSMV by a mite [Acarina, *Acerica tulipae* (Syn. *Eriophyes tulipae*)]. WSBMV and WSSMV are transmitted by the soilborne fungus, *Polymyxa graminis* (Brakke et al., 1965; Campbell et al., 1975; Cook and Veseth, 1991; Eversmeyer et al., 1983; Hunger et al., 1991; Matthews, 1991).

Yield losses from wheat streak mosaic (WSM) depend on time of infection and result from a reduced number of seed heads or production of sterile heads. Infections of wheat most often occur in the fall with symptoms appearing the following spring. Characteristic WSM symptoms develop in the

spring as temperature increases and include stunted plants with green-mottled and/or streaked leaves. Typically fall infected plants are symptomless and therefore the incidence of disease and its projected severity are difficult if not impossible to predict at this time.

Losses associated with WSM can be large and losses in Kansas alone exceed 30 million dollars in some years (Wiese, 1987). WSM is common throughout the central and western portions of North America with the most significant losses occurring in the central Great Plains. WSM can occur in a few localized areas to thousands of acres with corresponding yield decreases ranging from insignificant to complete. A suitable level of resistance to WSMV is not available in acceptable agronomic wheat cultivars, but cultivars with some resistance to the vector of WSMV are available (Harvey et al., 1990).

Epidemiology

In addition to wheat, WSMV infects other monocotyledoneae such as barley, oats, corn, rye, millet and many wild grasses. WSMV was reported to infect the dicotyledon *Chenopodium quinoa* (Somsen and Sill, 1970), although this was the only report and could not be reproduced by J.R. Montana in 1993 (unpublished). Some wild grasses that are hosts of WSMV are *Buchloe dactyloides*, (buffalo grass); *Alopecurus* sp., foxtail; and

Bouteloua sp., (grama grass) (Brakke, 1987b; Cook and Veseth, 1991; Sill and Fellows, 1953; Somsen and Sill, 1970; Wiese, 1987).

The population of the wheat curl mite (WCM) on wheat in the fall may not be more reliable than visual symptoms for predicting WSM severity since not all mites are viruliferous. WSM is most severe when a green bridge exists for both the mite and the virus between the crops harvested in the spring and planted in the fall or when alternative hosts are present in the field periphery (Somsen and Sill, 1970; Wiese, 1987). After the wheat has been harvested, the mite vector can only persist if it spreads to volunteer wheat seedlings or alterative hosts. Other green bridges occur in areas where both spring and winter wheats are grown e.g., western North Dakota and the neighboring area of Montana, or where wheat is planted in late summer/early fall for the purpose of pasture. In southwestern Kansas and the panhandle of northwestern Oklahoma, the green bridge results when corn or other alternative hosts are grown, or weed control is not properly conducted between wheat harvest and planting in the fall.

Delaying the planting date reduces the possibility of virus infection. However, late planting of wheat to break the green bridge effect can result in winterkill of seedlings and thereby limit yields. Thus, fall planting should be done after a two to three week period of "clean-till", to reduce

the possibility of both a virus-infected host and the vector being present when the seedlings develop. Where winter wheat is grown for livestock grazing and grain, the wheat is sown early and elimination of the green bridge may not be possible.

Virus

WSMV is a member of the *Potyviridae* and consists of flexuous rod shaped particles (15 X 700 nm) found in relatively low amounts within infected plants (Shukla, 1984). While WSMV is vectored by *Aceria tulipae* in nature it is also mechanically transmissible. The genome of WSMV is composed of a single strand of positive sense RNA with a relative migration (Mr) of $\approx 2.8 \times 10^6$ (Brakke et al., 1968, 1971, 1987, 1987b, 1990). Although the capsid most likely consists of a single species of coat protein four characteristic bands composed of two doublets of ≈ 31 and 46 kDa are seen in a 12% acrylamide SDS-PAGE gel, (Brakke et al., 1990; Seifers, 1992; Sherwood et al., 1985, 1987, 1990). The intact or nondigested capsid will form a doublet at 44-46 kDa while the other commonly observed doublet, especially in older leaves, at 31-33 kDa is considered to be the trypsin resistant core (TRC). The ≈ 14 kDa peptide(s) above those of the TRC, are suspected to have various biological functions and are strain specific (Brakke et al., 1990; Shukla et al., 1988). Strain

differences of WSMV have been observed by comparing the molecular weight of the capsid. The type strain PV-57, and the mild strain PV-91 (Brakke, 1971) available from the American Type Culture Collection, (ATCC, Rockville, MD), both have a capsid 1000 daltons larger than that of many other WSMV isolates collected (Brakke, 1987b).

WSMV is closely related to agropyron mosaic virus (AgMV), ryegrass mosaic virus (RMV), oat necrotic mottle virus (ONMV) and hordeum mosaic virus (HMV) (Shukla et al., 1984; Slykhuis and Bell, 1966; Wiese, 1987). In the last 20-30 years these four viruses have been sufficiently characterized by host and vector differences to allow their separation into distinct viruses. An even closer relationship between AgMV, ONMV, and WSMV was once suggested using serological assays and resulted in the proposal that WSMV, ONMV and AgMV were serologically related (Van Regenmortel, 1982). AgMV was even suggested to be a serotype of WSMV. However, improved immunological procedures have shown that while AgMV is serologically related to WSMV, it is not a serotype. Its relatedness is as a member of the *Potyviridae*. Serologically it is inter-virally related (= genus) rather than intra-virally related (= species, strain, or serotype). WSMV also produces cylindrical "pinwheel" inclusion bodies which are often seen in the infected host associated with the plasma membrane and at sites that overlie plasmodesmata (Andrews and Slykhuis, 1958; Brakke

et al., 1987; Langenberg, 1986, 1987; Lawson et al., 1971). When viewed in cross section these inclusions contain a central tubule with radiating curved arms, thereby giving the appearance of a pinwheel. These inclusions are composed of an aggregate of a protein monomer, and consist of a series of plates and curved scrolls with a finely striated substructure containing a periodicity of about five nm. They have a molecular weight of ≈ 66 kDa (Matthews, 1991) and amounts of ≈ 100 μg/g of leaf tissue are found (Brakke et al. 1987). The purpose of these inclusions may involve cell to cell virus movement (Langenberg, 1984, 1986, 1987) or RNA replication (Carrington and Dougherty, 1987, 1988; Dougherty and Hiebert, 1980). As pinwheel inclusions expand into the cytoplasm of the host cell, the tubule core and its arms expand but later become disassociated from the plasmodesmata, eventually lying free in the cytoplasm. Virions are associated, especially during early stages of infection, with these pinwheel arms. Normally occurring or structures altered in plant cells due to the preparation for electron microscopy might be mistaken for these inclusions or viral particle aggregations.

Vector

WSMV is transmitted by *Aceria tulipae* (Keifer) [syn.

Eriophyes tulipae].[1] These arthropods are members of the mite family Eriophyidae, of the class Arachnida. *A. tulipae* is thought to also transmit wheat spot mosaic virus (WSV) and can be found in double viral infections in wheat along with WSMV (Slykhuis and Bell 1962, 1966, 1980; Wiese, 1987). Double infections can result in an additive or synergistic increase in symptom expression. Members of the Eriophyidae are known to vector six viruses, three of which are potyviruses.

These mites have stylets that are located within the groove of the rostrum having two pads that function as ducts for their saliva. *A. tulipae* feeds by piercing plant cells and then sucking its contents. These eriophyid mites are approximately 0.2 mm in length. They are usually white and cylindrical in appearance, and have four frontal legs which do not provide an extensive ability for independent movement. These front pairs of legs of *A. tulipae* possess a "feathery claw," which enables the mite to firmly attach to a leaf. When they release their grip, these mites are spread primarily by the wind in a random manner (Hatchett et al., 1987; Slykhuis, 1955, 1962, 1965, 1980).

A. tulipae populations, commonly referred to as wheat curl mites, (WCM) are quickly reduced in number by

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[1] Note: In Yugoslavia this same mite is called *A. tosicheller* (Keifer) and therefore, caution should be exhibited in reviewing the literature as the WCM can be listed by three different names (Tosic, 1973).

desiccation, lack of available host plants, and by predation. WCM cannot survive extended periods away from a host plant, and although wheat is the primary host, WCM are able to either survive for short periods on nonhost plants or may increase in number on alternative host plants. Mites cannot survive on ripened grain or on grass that has gone into senescence. When conditions are optimum for their survival and reproduction, mites can complete a life cycle in 14 days and are capable of laying 12-20 eggs during their lifetime. Therefore, each mite theoretically could have 3×10^6 descendants in 60 days (Slykhuis, 1974). Their developmental period is completed within a 6-14 day period with two nymphal instars followed by a resting psuedopupa. Some species of eriophyidae have two types of female, with one type a specialized version for the purpose of hibernation. Male mites are never a significant proportion within the mite population.

WSM is often seen first at the margins of wheat fields, as the vector is passively blown in from adjoining wild grasses or volunteer wheat. Because the mites are randomly dispersed by the wind, a distance of one mile from wild grasses harboring the mites is considered sufficient to provide escape from the mite and, consequently, the virus (Brakke, 1987b; Cook and Veseth, 1991; Somsen and Sill, 1970; Wiese, 1987).

Plants infested with high populations of WCMs have leaves

that remain erect with the lateral margins of the leaves rolled toward the upper midrib. This is due to the feeding habit of mites, which feed preferentially on the upper surface and near the margin of leaves. Only when mites are present in extremely high numbers does this type of feeding damage occur, while low mite populations may still be destructive due to virus transmission.

WCMs at the nymph stage can acquire WSMV after 15 min of feeding on an infected plant in a persistent (circulative) manner. Upon movement to another host and feeding, nymphs or adults mites can transmit the virus in as little as 15 min. The virus is retained in the mite through molting (transstadial transmission), and infectivity can be retained for up to nine days after acquisition (Matthews, 1991; Slykhuis, 1980). Viruliferous mites can maintain their infectivity however, for more than two months, when kept at 3 C on host plants immune to the virus. Approximately 30% of the total mite population sampled from host plants can be infected with WSMV (Orlob, 1966; Slykhuis, 1955). However, 30% of a WCM population that may increase in number from a few mites to 3 X 10^6, comprises an enormous number of infective mites. Although WSMV does not replicate in the vector, Paliwal (1980) found viral particles of WSMV in the midgut, body cavity, and salivary glands of the WCM. WSMV is carried in the midgut and hindgut of all larval and adult mite stages

but is not passed from the adult mite to progeny through the eggs (non-transovarial) (Brakke, 1971; Takahashi and Orlob, 1969; Wiese, 1987).

Serological Identification and Methodology

Serology is a subdivision of immunology concerned with *in-vitro* antigen/antibody (Ag/Ab) reactions (Stryer, 1988; Van Regenmortel, 1966, 1967, 1970; Voet and Voet, 1990). A serotype is determined by the antigenic determinant which the antibody recognizes and results in the production of specific antibodies. Isolates with different antigenic sites are termed serotypes because they differ in their binding of different monoclonal antibodies (MAb's) or polyclonal antibodies (PAb's). For example, *Salmonella* species are rich in their antigenic diversity, which means that vaccination for protection against one serotype may provide little or no protection to a different serotype. There are over 1,000 serotypes, isotypes, and serovars known in the genus *Salmonella* with each serotype defined by their "H" or "O" antigens. Such non-protection by a vaccine demonstrates the importance of correctly identifying the serotype(s) prevalent in a particular region in order to provide the proper vaccine and monitor the spread of the disease (Begg, et al., 1990; Throns et al., 1994; Goethals et al., 1994; Smith et al.,

1993; Yancy, 1993; Lindberg et al., 1993; Helander et al., 19992; Gallego et al., 1992).

Determination of serotypes of a particular plant virus has been accomplished (Adams and Barbara, 1982; Dekker et al., 1988; Halk et al., 1984; Hughes and Thomas, 1988; Huss, 1987; Koenig, 1981; Koenig and Burgermeister, 1986; Rao et al., 1982;) using a variety of serological tests including serologically specific electron microscopy (SSEM) using immunogold labeling (IGL) to determine these serotypes (Dore et al., 1988; Friguet et al., 1983; Hiboyuki et al., 1974; Hitchborn, 1965; Langenberg, 1986).

The use of specific antibodies (Abs) allows the determination of a serotype and biochemical characterization follows to elucidate how the original (termed type strain) and the newly discovered serotype differ. Identification and then differentiation of serotypes is accomplished by using PAbs and MAbs (Clark and Adams, 1977; Hampton et al., 1990; Lommel et al., 1982). Proteins of viruses that are closely related may be distinguished by these Abs. Antibodies are exquisitely specific analytic reagents and therefore may be used in quantitative analysis of antigenic determinants and separation of viral strains.

In solid-phase immunoassay the antibody specific for the antigen of interest may be attached to a polymeric support or conversely, the antigen is attached to the support and then

after a period of incubation, followed by the antibody. The enzyme-linked immunosorbent assay (ELISA) technique provides useful procedures to differentiate viral strains (Lommel et al., 1982; Sherwood, 1987; Stoddard et al., 1987; Torrance and Dolby, 1984). These may be in various forms e.g., indirect or direct double antibody sandwich ELISA (DAS-ELISA) or direct antigen plating ELISA (DAP-ELISA). Although these and many other methods are used to accomplish a serological assay, eventually an antibody which carries a radioactive, fluorescent, or enzyme tag is included, (alkaline phosphatase), in the procedure in order to quantify (ELISA), or visualize (western blot, Ouchterlony double diffusion, or precipitation assay), the antibody/antigen complex. When an enzyme tag is used, it rapidly converts an added colorless substrate into an intensely colored product. The intensity can be measured at the appropriate wavelength. These ELISA techniques have a high level of sensitivity with the ability to detect < 1 ng of protein (Hampton et al., 1990).

Another immunologic test that can be used, and which has high Ag/Ab avidity is western blotting. In this procedure, proteins are first separated according to their molecular weight by SDS-PAGE then transferred to a polymer sheet, such as nitrocellulose. The sheet is then probed with the PAbs or MAbs in a manner similar to ELISA to detect the protein bands. A western blot provides more information than just immunologic

recognition. The Mr of the immuno-reactive bands provide information about possible degradation products and use of specific MAbs can provide information about the number of epitopes.

In SDS-PAGE, all proteins that have migrated are stained using a dye such as Coomassie Blue R-250, Fast Stain® or a metal such as silver stain on the original gel. This is done in order to visualize all the proteins and subsequent banding patterns of the viral serotypes, not just the proteins which would react with Abs. The bands from each serotype can then be compared to the original strain. This is a useful technique to visualize the differences in protein size (Mr) and whether there is a single or multiple proteins associated with each serotype. Such a technique has extremely high resolution and can resolve as little as 10 ng of protein per band depending on the thickness of the gel, the quality of the buffers and reagents used and the running conditions.

Another technique that separates proteins using electrophoresis, and may also be used for distinguishing serotypes, is isoelectric focusing. In this technique proteins are separated on the basis of relative acidic and basic residue content. When electrophoresis is conducted on proteins, in a gel without the presence of SDS, their movement through the pH gradient is such that each protein will move until it reaches the position in the gel where its pI is equal

to a specific pH. Such a gradient is produced by running an electrophoresis with a gel containing a mixture of polyampholytes, which are small polymers that contain a multiple of charges and therefore many pI values. This technique is able to resolve proteins that only differ by 0.01 in pI value or, put another way, proteins differing by one net charge.

Using isolectric focusing in combination with SDS-PAGE results in extremely high protein resolution. The proteins of interest are first run on a gel by isolectric focusing then placed horizontally on the top of another SDS-PAGE gel and conducting a vertical electrophoresis. This technique will yield a two dimensional pattern of spots corresponding to the various proteins. Such separations produce proteins differentiated by isoelectric point (horizontal direction of electrophoresis) and by molecular weight (vertical direction of electrophoresis).

To maintain the conformational status and thus the antigenic character of a protein, important in the identification of the epitopes which make up a serotype, other biochemical techniques can be utilized. Purification of proteins in their native state (active form) can be accomplished on the basis of size, solubility, charge, or specific binding affinity. In contrast, SDS-PAGE only provides estimates of the mass of dissociated polypeptide

chains under denaturing conditions.

Once the existence of serotypes are confirmed then it is imperative to determine the amino acid sequence of the antigenic sites in order to locate the protein(s) that confer the MAb specificity. A technique that is useful for this purpose is enzymatic cleavage. Specific cleavage can be accomplished by the use of particular enzymes and/or chemicals. For example, a chemical such as cyanogen bromide (CNBr) will cleave polypeptide chains specifically on the carboxyl side of methionine residues. Therefore, if a particular protein contains five methionines, six peptides may result after treatment with CNBr. Cleavages of proteins with enzymes such as trypsin and chymotrypsin also is well documented (Sherwood et al., 1990; Brakke et al., 1990; Daniels and Campbell, 1992).

Finally, an Ouchterlony agar double diffusion test may be used for serotype determination. This test involves placing virus isolates in peripheral wells surrounding a central well with either MAbs or PAbs. As the Abs from the central well and the antigen (Ag) from the peripheral wells migrate out in all directions eventually the Abs and Ags will meet somewhere between the wells. If a continuous or confluent precipitation line forms between two peripheral wells with different isolates this suggests a optimum absorption of the diffusing antibodies with both sets of antigens. Such a result

indicates that the two virus isolates are closely related serologically. In contrast if a spur forms, this suggests that one of the antigens is not precipitating with some of the antibodies, which are diffusing from the central well. Because of this lack of precipitation some of the antibodies have passed through the precipitation lattice. However, the antigenic proteins diffusing from the adjoining well are able to precipitate the antibodies resulting in the creation of the spur. Development of such a spur indicates that the two viruses share at least one common antigenic protein but their other antigenic proteins are not identical. Thus, the two viral isolates can be considered related but serologically distinct. It is important to test these serological differences with both PAB (heterogenous) and MAB (homogenous) antisera because, if only the MAB antiserum is utilized, no spur may form.

Viral Serotypes

Serological procedures have been used extensively to identify and estimate viral infection. Serological tests only detect and measure the viral protein and not the amount of infective virus. Still, serology is utilized to investigate viral structure, the relationships of the virus with the host, and virus activity in host cells.

Serological testing is a beneficial procedure to determine if two isolates are related. Determining the existence of serotypes is accomplished by collecting isolates of a virus, and testing using the antisera created to the type strain. If the isolate(s) react differentially to this antisera then serotypic differences have been observed between the type strain and these isolates. Then for further characterization antisera must be produced to these isolates and tested against the type strain which would demonstrate that each reactive antisera indeed only reacts with its own antigen (Matthews, 1991). The delineation of viral serotypes is a particularly important part of a program which purports to be attempting to produce resistant cultivars.

Published Examples of Viral Serotypes

There are many examples of the use of these serological techniques to identify a virus and differentiate viral serotypes (Atreya et al., 1990; Gill, 1976; Goding, 1983; Jaegle and Van Regenmortel, 1985). In an early paper Moorhead (1959) used electrophoresis and serological techniques to study the antigenic characteristics of WSMV by the production and use of PAbs. Since then these techniques have been greatly improved resulting in the ability to differentiate serotypes. McDaniel (1992) used indirect ELISA,

electroblotting, immunological analysis of SDS-PAGE, peptide mapping analysis, and finally enzymatic proteolysis to characterize tobacco streak virus. McDaniel also used electron microscopy to visually examine the purified viral particles previously stained with uranyl acetate. He felt that the use of these techniques allowed the demonstration of serological differences between these isolates as well as characterization of coat protein composition.

Tests for serological differentiation of alfalfa mosaic virus (AMV) was accomplished by the use of polyclonal antibodies (Hajimorad and Franki, 1991) and the use of a serological differentiation index (SDI). Others have conducted similar research with different viruses (Adams, 1978; Bahrani et al., 1988; Ball and Brakke, 1968; Boonekamp et al., 1990; Campbell, 1984; Christe and Edwardson, 1977; Clark and Adams, 1977; Dekker et al., 1989; Zettler et al., 1993). There are many reports of serological differentiation followed by biochemical characterization. Hill et al., (1992) described a serologically distinct strain of maracaja mosaic virus using several of these techniques. PAS-ELISA was the principle serological technique utilized by Miller et al., (1991) to identify, record the prevalence, and, note the distribution of WSMV, WSBMV, and WSSMV. Seifers (1992) partially characterized an isolate of AgMV found in Colorado by comparing it to the AgMV type strain and to WSMV isolates

using peptide mapping, SDS-PAGE, viral nucleic acid analysis, serological tests, symptom rating, and dry weight analysis of four wheat cultivars.

There are many other examples of the use of such techniques for identification and characterization of isolates, strains, and serotypes of a particular virus e.g., beet soilborne virus (Barbarossa, et al., 1992); cucumber mosaic virus isolates from California (Daniels and Campbell, 1992); and various ilarviruses i.e., prunus necrotic ringspot virus (Crosslin et al., 1992; Mink et al., 1987; Smith and Skotland, 1986), apple mosaic virus (Halk et al., 1984), tobacco streak virus (McDaniel, 1992), and alfalfa mosaic virus (Hajimorad et al., 1990; Hajimorad and Francki, 1991; Halk et al., 1984).

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CHAPTER III

EFFECT OF PLANTING DATE, CLIPPING TO SIMULATE GRAZING AND INOCULATION DATE ON SEVERITY OF WHEAT STREAK MOSAIC IN HARD RED WINTER WHEAT

Abstract

Three cultivars of hard red winter wheat (Chisholm, Triumph 64, and Rall) were inoculated with the OSU isolate of wheat streak mosaic virus (WSMV). Three inoculations were made, one in the fall and two at different times the following spring. The purpose was to determine the effect of mechanical clipping to simulate grazing on the severity of wheat streak mosaic (WSM) on the three wheat cultivars. Presence of WSMV was evaluated using a double antibody sandwich ELISA (DAS-ELISA). Severity of WSM was determined by visual assessment of symptoms, and measuring total yield, thousand kernel weight (TKW), fertile tiller number, and plant height. Results of DAS-ELISA values from Rall confirmed the presence of WSMV and increasing WSMV titer occurred in some replications during the late spring, but Rall displayed a form of field resistance based on symptoms, plant growth, and yield measurements. WSM was not as severe on wheat inoculated in the spring as wheat inoculated in the fall determined by the above parameters. Final tissue sampling on 6 May, 92 showed that some early

spring inoculations had approximately equal DAS-ELISA values to the fall inoculation. However, spring inoculation appeared to result in inconsistent symptoms and titer values while the fall inoculation was very consistent. Clipping did not have a significant effect on WSM.

Introduction

Wheat streak mosaic (WSM), an important disease of wheat worldwide, is caused by a flexuous, rod-shaped potyvirus called wheat streak mosaic virus (WSMV). Transmission of this virus is by a mite vector, *Aceria tulipae* Keifer (syn. *Eriophyes tulipae*), which is called the wheat curl mite (WCM) (Cook and Veseth, 1991; Matthews, 1991; Slykhuis, 1955; Wiese, 1987). When feeding on infected wheat or various alternative hosts, the virus is ingested by WCMs in as little as 15 min (Matthews, 1991). WCMs are spread by the wind, and approximately 30% of WCMs have been shown to be viruliferous in any given field (Orlob, 1966; Slykhuis, 1955).

Previous studies (Fellows and Sill, 1955; Hansing et al., 1950; Hunger et al., 1992; Sill, 1953; Slykhuis, 1952; Willis, 1984) have documented that planting date and the time of infection (i.e., fall verses spring) affect the severity of WSM. In Oklahoma and surrounding states, wheat is often used for the dual purpose of grazing and grain production which can

only be accomplished by planting wheat earlier in the fall. However, in potential WSM prone areas wheat should be planted late in the fall to escape or reduce WSM (Cook and Veseth, 1991; Hunger et al., 1992, Wiese, 1987). Thus, as wheat is planted early and used for a dual purpose in Oklahoma, control of WSM by late planting becomes more difficult.

Early infection of seedling wheat results in the greatest WSM symptoms the following spring and the most extensive yield reductions (Hunger et al., 1992). This correlation with the age of the plant when infected and disease raises the question regarding the possible affect of grazing WSM. Grazing causes wheat plants to regenerate foliar tissue that is less mature physiologically, and may be more susceptible to WSMV.

This study was therefore initiated to determine whether clipping, to simulate grazing, affects the severity of WSM when compared to plots not clipped, and secondly, to determine if there is a correlation between the time of year that infection occurred (i.e., fall, early or mid-spring) and the clipping of wheat.

Materials and Methods

Location and Experimental Design

A field test was conducted approximately one mile west of the OSU Small Grains greenhouses in Stillwater OK. The plot was fertilized according to local recommendations based on soil tests to obtain a 3.34 metric tons/ha yield goal.

The experimental design was a split-plot (Gomez and Gomez, 1984; Steel and Torrie, 1966) with three factors (sub-subplot = inoculation date = 3, main plots = cultivars (Rall, Triumph 64, Chisholm) and a non-inoculated control = 4, and subplot = clipped or not clipped = 2 and a 3 X 4 X 2 ($a^3b^4c^2$) factorial arrangement of treatments within the six replications (= blocks). Seeds were planted on 10 Oct 91 at the rate of 60 seeds/ 1.5 m row, at a depth of 2.5 cm with 9 rows/cultivar/replication (clipped vs. non-clipped/cultivar in each replicate = six subplots/replicate. Between the subplots the wheat cultivar Vona was planted as an indicator plant to detect wheat soilborne mosaic or wheat spindle streak mosaic. The field was sprinkler irrigated on 06 Oct 91 with subsequent irrigation applied as required in the early fall and late spring to maintain plant growth and development.

Eight weeks after planting Glean® (chlorsulfuron) was

applied at 13 g a.i./187 L/ha to control weeds. The fungicides Bayleton® (triadimefon) at 140 g a.i./187 L/ha and Tilt® at 12 ml/7.6 L were applied in the spring to control fungal pathogens. Malathion was applied at the label rate (180 ml/84 L) as needed to control aphid infestations.

WSMV Inoculum, Dates Applied, and Cultivar Reaction

The isolate of WSMV used in this study was originally provided by Dr. Emil Sebesta (USDA Stillwater, OK) and is termed the OSU isolate. This isolate reacts to both polyclonal and monoclonal antibodies, (PAbs and MAbs respectively), produced to the intact virions of the OSU isolate, and has a virulence level equal to that of the WSMV type strain PV-57 obtainable from the American Type Culture Collection (ATCC, Rockville, MD) collected in 1932 by H.H. McKinney.

Inoculum was produced and applied as reported previously (Hunger et al., 1992). However, in this study inoculum was applied at 90 ml/m of row. Rows within a clipped or non-clipped subplot were randomly assigned an inoculation date. The fall inoculation was conducted on 12 Nov 91, the first spring inoculation on 20 Feb 92, and the final spring inoculation on 18 Mar 92. At each inoculation date, two flats of the wheat cultivar Blue Jacket also were inoculated to

insure that the OSU isolate used was viable and applied in sufficient quantity to produce WSM symptoms. These wheat plants were assessed visually for WSM symptoms and the presence of WSMV was confirmed using the double antibody sandwich ELISA (DAS-ELISA) assay [(Sherwood, 1987; Sherwood et al., 1990; (Figure 1)]. Foliage was collected randomly from each row, seven times during the spring. These samples were placed on ice until transferred to a -20 C freezer for storage until tested by DAS-ELISA. At the same time that wheat tissue was collected for the DAS-ELISA assay, the subplots were visually rated for severity of WSM. The rating scale was as follows: 0 = no symptoms evident (= to controls); 1 = no stunting present, leaves mostly light green with a few yellow streaks; 2 = plants slightly stunted, leaves with mixed green and yellow streaks; and 3 = plants stunted, leaves with severe yellow streaking and a few green streaks or green islands.

Plots were clipped on 10 Dec 91; 03 Jan 92, 20 Jan 92, and 31 Jan 92. This allowed the clipped plants to grow so that enough plant tissue was available for the spring inoculations. On 20 Jan 92, all cultivars were rated as ≤ 1 for WSM symptom expression. On 20 Feb 92 wheat tissue of Vona, and from the border rows of the three cultivars, was randomly harvested from within rows and between subplots and tested by DAS-ELISA for the presence of wheat soilborne mosaic virus.

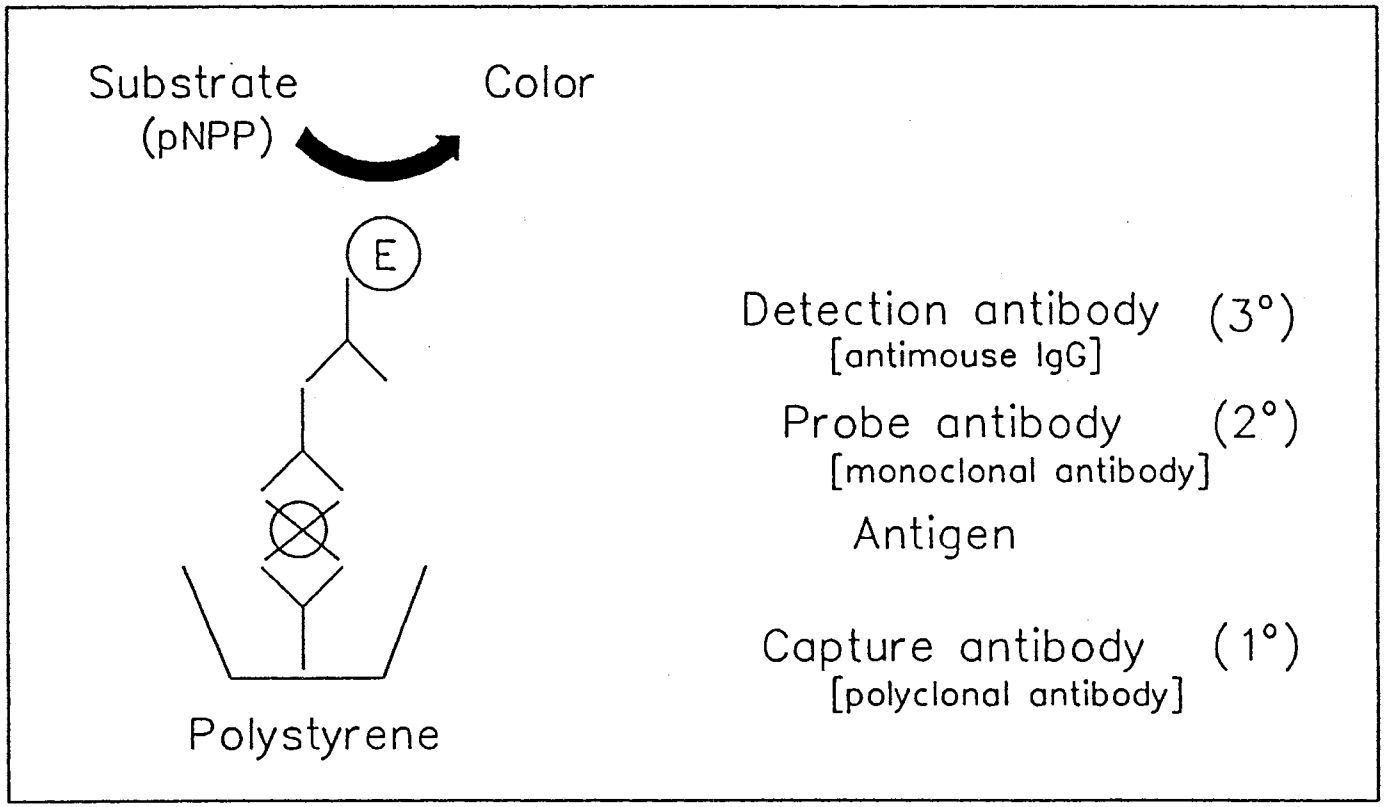

Figure 1. Schematic representation of indirect double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

Plant height (soil surface to highest point of the plant) and fertile tillers were randomly sampled in a 0.3 m area of each row/treatment combination. The middle one meter of each row was harvested on the 12 Jun 92, placed in a paper bag and dried in the glasshouse. The wheat was threshed, the grain cleaned, and thousand kernel weight and total yield determined.

Based on previous studies (Hunger et al., 1992; Sherwood et al., 1990) the positive/negative thresholds for DAS-ELISA was considered to be ≥ 0.100 and < 0.100 respectively.

Results and Discussion

Neither WSBMV nor WSSMV were detected in this study. Also, random sampling of the non-inoculated controls and the wheat to be inoculated in the spring revealed no WSMV from natural infection.

On 19 Feb 92, the first tissue samples from wheat inoculated in the fall and from the cv Blue Jacket inoculated in the flat were tested by DAS-ELISA for WSMV (Table I). DAS-ELISA values from WSMV-infected Rall tissue were as low as the non-inoculated controls while foliage of fall inoculated Chisholm and Triumph 64 had positive DAS-ELISA values throughout the six replications. In the second through the fourth sampling of tissue from the field plots (06 Mar to 30

Table I. Values from DAS-ELISA for three hard red winter wheat cultivars following inoculation with wheat streak mosaic virus in 1991.

Cultivar Inoculation[1]	Tissue Sampling Date (1992)		
	Feburary 19	March 6	March 21
	ELISA[2]	ELISA	ELISA
Chisholm			
Check	0.004 ± 0.007	0.008 ± 0.010	0.021 ± 0.026
Fall	0.233 ± 0.124	0.327 ± 0.144	0.459 ± 0.231
Early Spring		0.022 ± 0.021	0.020 ± 0.020
Late Spring		0.009 ± 0.012	0.065 ± 0.209
Triumph 64			
Check	0.002 ± 0.012	0.010 ± 0.014	0.002 ± 0.006
Fall	0.201 ± 0.113	0.332 ± 0.117	0.499 ± 0.166
Early Spring		0.012 ± 0.011	0.010 ± 0.033
Late Spring		0.007 ± 0.014	0.000 ± 0.006
Rall			
Check	0.001 ± 0.002	0.011 ± 0.011	0.006 ± 0.011
Fall	0.016 ± 0.010	0.018 ± 0.021	-0.002 ± 0.005
Early Spring		0.010 ± 0.016	0.001 ± 0.005
Late Spring		0.013 ± 0.019	0.000 ± 0.005

(Table I. continued on next page)

Table I. (continued) Values from DAS-ELISA for three hard red winter wheat cultivars following inoculation with wheat streak mosaic virus in 1991.

Cultivar Inoculation[1]	Tissue Sampling Date (1992) March 30 ELISA[2]	April 25 ELISA	May 6 ELISA
Chisholm			
Check	0.012 ± 0.014	0.012 ± 0.021	0.121 ± 0.257
Fall	1.035 ± 0.365	0.531 ± 0.184	0.774 ± 0.430
Early Spring	0.045 ± 0.076	0.380 ± 0.293	0.631 ± 0.376
Late Spring	-0.002 ± 0.008	0.140 ± 0.164	0.222 ± 0.159
Triumph 64			
Check	0.003 ± 0.012	0.000 ± 0.014	0.011 ± 0.013
Fall	1.014 ± 0.373	0.476 ± 0.189	0.716 ± 0.343
Early Spring	0.010 ± 0.020	0.161 ± 0.214	0.267 ± 0.271
Late Spring	-0.002 ± 0.006	0.065 ± 0.090	0.081 ± 0.074
Rall			
Check	0.005 ± 0.018	-0.005 ± 0.009	0.010 ± 0.006
Fall	-0.006 ± 0.006	0.008 ± 0.062	0.058 ± 0.196
Early Spring	0.000 ± 0.013	-0.005 ± 0.017	0.002 ± 0.005
Late Spring	-0.002 ± 0.006	0.024 ± 0.053	0.061 ± 0.039

[1] Seeds were planted on 01 Oct 91, and inoculations with the OSU serotype of WSMV were conducted on 11 Nov 91, 28 Feb 92, or 15 Mar 92.

[2] Each value is the mean absorbance (i.e., optical density) at 405 nm of three replications with three readings/replication. Values ≥ 0.100 are considered positive and values < 0.100 are considered negative.

Mar 92) Rall was both visually and by DAS-ELISA negative for WSMV (Table I). By 04 Apr 92, WSM symptoms were obvious and severe on the fall inoculated Chisholm and Triumph 64. Although DAS-ELISA would continue on these two cultivars for the fall inoculation treatment, it had become apparent that it was no longer required for verification of the presence of WSMV. Because of the obvious presence of WSM all subsequent DAS-ELISA assays were conducted only after both spring inoculations had been performed. Samples collected in mid to late March, showed that the early inoculation in the spring, required approximately 31 days for DAS-ELISA values to be considered positive i.e., in the range 0.100 to 0.200 for Chisholm and Triumph 64. By the sixth and final sampling of Chisholm and Triumph 64, the DAS-ELISA values for the first spring inoculation were $\geq$ the DAS-ELISA values of their fall inoculated counterparts (Table I) in some replications. These results however, were inconsistent between treatment/rep combinations, which was also seen to a lesser degree for the late spring inoculation where sporadic positive values occurred.

Clipping did not significantly affect, in a positive or negative manner, the visual expression of WSM or the DAS-ELISA values of WSMV in any of the cultivars (Table II). Conversely, while clipping did not statistically increase disease expression or titer values as hypothesized, it also

Table II. Severity of wheat streak mosaic on three hard red winter wheat cultivars under field conditions in a 1991/1992 simulated grazing (clipping) study.

Cultivar Inoculation[1]	25 April 1992 Symptom rating[2]	6 May 1992 Symptom rating[2]
Chisholm		
Check	0.75	0.92
Fall	2.92	2.95
Early Spring	1.17	1.34
Late Spring	1.17	1.42
Triumph 64		
Check	0.42	0.67
Fall	2.92	3.00
Early Spring	1.50	1.75
Late Spring	1.50	1.58
Rall		
Check	0.17	0.42
Fall	1.00	1.25
Early Spring	0.58	0.83
Late Spring	0.67	0.75

[1]Seeds were planted on 01 Oct 91, and inoculations with the OSU serotype of WSMV were conducted on 11 Nov 91, 28 Feb 92, or 15 Mar 92.

[2] The parameter "clipped" was not statistically significant. Therefore, each mean value is from six replications (12 observations) rated as 0 = no symptoms; 1 = no stunting present, 2 = leaves mostly green with a few yellow streaks; and 3 = plants stunted, leaves with severe yellow streaking and a few green streaks or no green islands.

did not reduce the apparent ability of the cultivars to reach their yield potential when the non-inoculated clipped and non-clipped control plants were compared (Table III). This supports the use of wheat for a dual purpose in Oklahoma if WSM is not a factor. However, in areas where WSM is a problem early planting of wheat to obtain forage may exacerbate the relative amount and severity of WSM because early planting enhances infection by WSMV.

The results obtained in this and other studies (Fellows and Sill, 1955; Hansing et al., 1950; Hunger et al., 1992; Willis, 1984) demonstrates that planting wheat in the early fall with subsequent early infection results in the greatest losses from WSM. However, the cultivar Rall exhibited a high degree of tolerance to the OSU serotype of WSMV in terms of severity determined by the parameters measured. Only late in the spring of 1992 did positive values from DAS-ELISA began to occur, and by then the virus did not appear to seriously affect Rall as compared to the non-inoculated checks. Sill (1953) stated that if wheat were infected (inoculated) at the early stages of tillering, then severe damage from WSM could be expected. These results are supported by this study, since the fall inoculation produced the highest level of WSM severity for Chisholm and Triumph 64. Conversely Rall which was also inoculated in the fall did not display WSM, nor did DAS-ELISA record the presence of WSMV for most of the growing

Table III. Reaction of three hard red winter wheat cultivars to wheat streak mosaic virus isolates under field conditions in a 1991/1992 simulated grazing (clipping) study.

Cultivar Inoculation[1]	Thousand[2] Kernel weight (g)	Yield[2] (g)	Fertile[2] tillers (no./0.3 m)	Plant[2] Height (cm)
Chisholm				
Check	25.30	63.78	34.75	28.46
Fall	13.58	13.51	18.83	18.88
Early Spring	21.75	41.87	33.75	28.05
Late Spring	21.44	43.15	35.50	27.45
Triumph 64				
Check	26.27	56.51	36.58	36.86
Fall	15.32	18.89	20.83	27.77
Early Spring	24.40	53.41	38.33	36.54
Late Spring	22.93	53.78	41.83	36.84
Rall				
Check	23.74	59.09	51.17	39.49
Fall	22.60	58.18	48.00	39.05
Early Spring	22.68	52.45	45.92	39.06
Late Spring	21.23	55.82	51.33	38.83
LSD alpha = 0.05	1.791	9.51	6.726	1.723
LSD alpha = 0.01	2.372	12.60	8.909	2.282

[1] Seeds were planted on 01 Oct 91, and inoculations with the OSU isolate of WSMV were conducted on 11 Nov 91, 28 Feb 92, or 15 Mar 92.

[2] The parameter "clipped" was not statistically significant. Therefore, values from "clipped" and "nonclipped" were combined. Significant parameters were inoculation date and the cultivar/inoculation date interaction.

season. Sill further stated (1953), that the response to WSMV in wheat inoculated after early tillering was inconsistent, that some wheat escaped infection, and in the infected plants disease symptoms developed more slowly. Our data tend to confirm such a reaction pattern with the fall inoculated Chisholm and Triumph 64 severely affected by infection of WSMV. Others have described variation in HRWW tolerance to WSMV or to the mite vector (Harvey et al., 1990; Martin et al., 1976; Martin, 1978; Miller et al., 1991; Seifers and Martin, 1988; Shahwan, and Hill, 1984). Reports have also been made of differences in tolerance to WSMV in spring wheat (Edwards and McMullen, 1988; McNeal and Carroll, 1968) and in agrotricums as well (Pfannenstiel and Niblett, 1978).

Tolerance is defined by Cook and Veseth (1991) as the ability of a cultivar to yield well in spite of infection. They further stated that no adequate resistance or tolerance to WSMV existed in North America and felt that adequate resistance to WSMV would probably not come about by conventional breeding, but may be developed in the future through nonconventional breeding programs utilizing new biotechnology approaches. Meanwhile, wheat growers have to rely on cultural practices such as control of volunteer wheat, control of weedy plants especially those that might act as alternative hosts, and proper choice of planting date in order to control losses to WSM.

It was suggested (Hunger et al., 1992) that plant maturity at time of infection (inoculation) affected virus replication and development of WSM with older plants being more resistant than younger plants. However, in this study both fall and early spring inoculations produced high titer values in Chisholm and Triumph 64. Also, Rall did not display high titer values throughout the study with only mean values approximately 0.200 at the sixth and final sampling just prior to harvest (10.5 on the Feekes scale). Hunger et al., (1992) found that while Rall displayed tolerance to WSMV in their studies, the virus was replicating as was shown in their DAS-ELISA values, and for a much longer portion of their experiment.

These results showing highest degree of WSM in fall infected (inoculated) wheat, again stress the avoidance of early wheat planting (excluding Rall) in areas where WSMV is a known pathogen. Simulation of grazing with electric grass clippers did not have a significant effect on WSM expression. Forcing the wheat plants to regenerate the clipped tissue did not produce plants more susceptible to inoculated virus. Conversely, the wheat plants were mechanically inoculated and the amount of virus placed into the plant may have been so high as to mask the effects that clipping might have had.

By extrapolation then, perhaps in a natural system where the mite is the source of wheat infection rather than the

mechanical inoculation used in this study, herbivore grazing may diminish the amount of tissue in a field available to the mite. Also, since the mite is wind blown and lands on susceptible host plants including weedy species and wheat in a uncontrolled manner, reduction of weeds along the periphery of the field and diminished wheat tissue by grazing, should result in a reduction of WSM. This might be especially so if the grazing kept the wheat low to the ground and alternative hosts were also controlled.

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CHAPTER IV

CHARACTERIZATION OF SEROTYPES OF WHEAT STREAK MOSAIC VIRUS

Abstract

Wheat suspected to be infected with wheat streak mosaic virus (WSMV) did not react with monoclonal antibodies (MAbs) made to the Oklahoma State University (OSU) isolate of WSMV but did react to polyclonal antibodies (PAbs) made to the OSU isolate. Additional WSMV isolates reacted similarly, indicating the existence of WSMV serotypes. Serological and electrophoretic comparisons were made of the coat proteins of nine WSMV isolates and one agropyron mosaic virus (AgMV) isolate. Partial and/or complete chemical or proteolytic digestion of the capsid proteins followed by electrophoretic analysis in 4-20% pre-cast gradient gels and reaction with either PAbs or MAbs indicated that defined grouping of the nine WSMV isolates is possible. This work will directly affect wheat growers, breeders, and epidemiologists.

INTRODUCTION

Wheat streak mosaic (WSM) is a destructive disease of wheat, (*Triticum aestivum* L.), caused by wheat streak mosaic

virus (WSMV) (Wiese, 1987). This virus is transmitted by the wheat curl eriophyid mite *Aceria tulipae* (syn. *Eriophyes tulipae* Keifer) (Slykhuis, 1962, 1980; Whitmoyer et al., 1972). When juvenile wheat plants are infected WSM can cause significant forage and yield losses (Brakke, 1971, 1987b; Hunger et al., 1992; Seifers, 1992; Wiese, 1987). Other viruses including wheat soilborne mosaic virus (WSBMV), barley yellow dwarf virus (BYDV), and agropyron mosaic virus (AgMV) can occur as mixed infections with WSMV (Montana et al., 1993; Wiese, 1987). Therefore, identifying the causal virus in symptomatic plants is critical for accurate diagnosis and for formulating appropriate control and cultivar recommendations.

Serological procedures such as the enzyme-linked immunosorbent assay (ELISA), serologically specific electron microscopy (SSEM), and immunodiffusion tests have been used for detection, identification and characterization of viruses (Bashir, 1992; Edwards and Cooper, 1985; Clark and Adams, 1977, 1987; Langenberg, 1986, 1986b, 1987, 1989; Lommel, et al., 1982; Sherwood et al., 1985, 1990, Sherwood, 1987). Hajimorad and Franki (1991) characterized biologically distinct but antigenically similar alfalfa mosaic virus (AMV) strains using polyclonal antibodies (PAbs) in ELISA and immuno-diffusion tests. Seifers (1992) included serology in his partial characterization of AgMV, and Nelson and Wheeler

(1978) used both biological and serological characters to identify potyviruses that infect peppers. Daniels and Campbell (1992) differentiated cucumber mosaic virus isolates collected in California, by serology, proteolytic cleavage of the viral coat protein, and by comparing the relative migration of these cleavage products in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ranieri et al., (1993) recently used ELISA and a panel of monoclonal antibodies (MAbs) and PAbs to assess the occurrence of BYDV serotypes in Mexico. Diaco et al., (1986) and Forde (1989) also used PAbs and MAbs to demonstrate serological relationships among isolates of BYDV.

Methods other than serology also have been used to separate viral isolates into distinct groups. For example, Slykhuis and Bell (1966) used disease symptoms, vector differences, and host range to differentiate a new virus, hordeum mosaic virus (HMV), from AgMV and WSMV. HMV previously was considered a strain of AgMV or WSMV. Symptoms of WSM on wheat vary widely and have been attributed to temperature, cultivars, environment and strain of WSMV (McKinney, 1937; Somsen and Sill, 1970; Sill, 1959). Strains of WSMV that have been classified based on symptoms, include the type strain (PV-57, ATCC, Brakke, 1971) and a mild strain of WSMV (PV-91, ATCC, 1956). Additional characterization of WSMV isolates has been limited (Brakke, 1971), but Carroll et

al., (1982), used disease expression on the wheat cultivar Michigan Amber to compare eight isolates of WSMV collected in Montana to PV-57 and PV-91. They reported that two classes of variants or strains existed, with seven isolates similar to PV-91 and one resembling PV-57, which they termed a severe variant.

Recently, serological differences between isolates of WSMV were reported (Montana et al., 1993) as was variation in the sequence of the coat protein region of WSMV isolates based on RAPD analysis (French and Robertson, 1994). Identification of WSMV serotypes has important implications in breeding for resistance to WSM and in studying the etiology and epidemiology of WSM because differences in antigenic character, could be associated with vector transmissibility (Atreya et al., 1990) host range, and degree of virulence (Barnett, 1992; Matthews, 1991; Shulka et al., 1988, 1988b, 1989, 1989b, 1989d, 1989e, 1992; Vincent et al., 1990). Thus, the objective of this study was to characterize isolates of WSMV using ELISA, the Ouchterlony double diffusion test (ODD), competitive binding assays utilizing monoclonal antibody (MAb), serologically specific electron microscopy (SSEM), peptide fingerprinting and their corresponding western blots.

MATERIALS AND METHODS

Virus Isolates and Maintenance

Nine isolates of WSMV and one of AgMV were used (Table IV). All isolates were increased and maintained on the hard red winter wheat cv. Blue Jacket. Seed (10-15 seeds/pot) were planted in 11 cm diameter pots and seedlings fertilized with an initial application of 14-14-14 Osmocote (6 g/pot) and lightly fertilized (1/6 rate of Peters' soluble fertilizer) every third watering thereafter. Light ($900\mu E \bullet m^{-2} \bullet s^{-1}$; 12/12 photo period) was provided by a metal halide lamp placed 112 cm directly above the pots on a laboratory bench. A rotating fan was used to dissipate heat generated by the halide lamp. Twenty days after planting, seedlings (four pots/isolate) were mechanically inoculated using 1 part infected tissue ground in 10 parts of inoculation buffer, (0.2 M monobasic sodium phosphate, 0.2 M dibasic sodium phosphate, diluted to 0.01 M @ pH 7.0) with 1 g celite plus 1 g 225 μm corundum. Inoculated seedlings were then covered with water saturated paper towels for 18-24 hours. Fourteen days after inoculation, foliage was harvested and stored at −20 C until used.

ELISA

Two ELISA methods were used to differentiate the isolates using PAbs and MAbs previously prepared (Sherwood et al.,

Table IV. Origin, host plant, and contributor of wheat streak mosaic virus isolates.

Isolate Designation	Year Obtained[1]	Plant Species	Source	Contributor
OSU	< 1970	wheat 'Blue Jacket'	USDA/ARS/OSU	Emil Sebesta
COLO	1992	wheat 'Stephens'	Fruita, CO	Bob Hammond
COLO13	1992	[2]oats		
OK994	1992	wheat	OK Panhandle Texas County	Diagnostic Lab O.S.U.
OK964	1992	wheat	OK Panhandle Texas County	Diagnostic Lab O.S.U.
TAMU	1992	wheat 'Mustang'	Bushland, TX	Gretchen Heidel
PV-57	1992 (1937)	wheat	[3]ATCC, Salina, KS	H. H. Mckinney
PV-91	1992 (1956)	wheat	ATCC, Ellis Co., KS	H. H. McKinney
PV-106	1992 (1965)	wheat (corn)	ATCC, (from Ohio)	L.E. Williams
AgMV	1992	wheat	Univ. of Nebraska	Roy French

[1] These are for the date, the person, or the company from which the isolate was received. Information in parenthesis is original source, if known.
[2] This was the COLO isolate that was inoculated onto oats, infected tissue harvested and then inoculated onto wheat before using to avoid confounding of results by two suspected viruses.
[3] ATCC = American Type Culture Collection, Rockville, MD

1987, 1990). The indirect double antibody sandwich ELISA (DAS-ELISA) was used with PAb as the primary antibody (Ab) (Figure 2) diluted in coating buffer, [0.05 M carbonate buffer @ pH 9.6 with 5% sodium azide (NaN_3)], and WSMV MAb as the secondary Ab diluted in phosphate buffer saline plus Tween 20 (PBS-T @ pH 7.4).

DAS-ELISA Procedures:

Primary Ab in coating buffer was placed (100μl) in each of three wells of a Dynatech Immulon II™ plate for either a two h incubation at 25 C or 30-60 min at 37 C. Plates were washed three times with PBS-T, followed by a blocking step of 2% bovine serum albumin (BSA) or 5% carnation milk in PBS for 30 min at 37 C. Stored tissue samples of the WSMV isolates was removed from -20 C and sap from this infected wheat was extracted from 1 g of leaf tissue into 10 ml of PPBS-T using a leaf squeezer (Piedmont Machine and Tool, Box 250, Six Mile, S.C.) (Sherwood et al.). Samples were incubated for at 37 C for 30 min. After three PBS-T washes, 100 μl of MAb (1:1000) was added to each well, plates were incubated at 37 C for 30 min, washed three times with PBS-T, and then 100 μl of rabbit antimouse IgG (1:5000) conjugated to alkaline phosphatase as the tertiary Ab (Sigma A-4312) was added to each well to incubate for 30 min. After three PBS-T washes p-nitrophenyl phosphate at 1 mg/ml [(pNPP), (Sigma N-2765)] in substrate buffer [97 ml Diethanolamine, (Sigma D-8885), 0.2 g

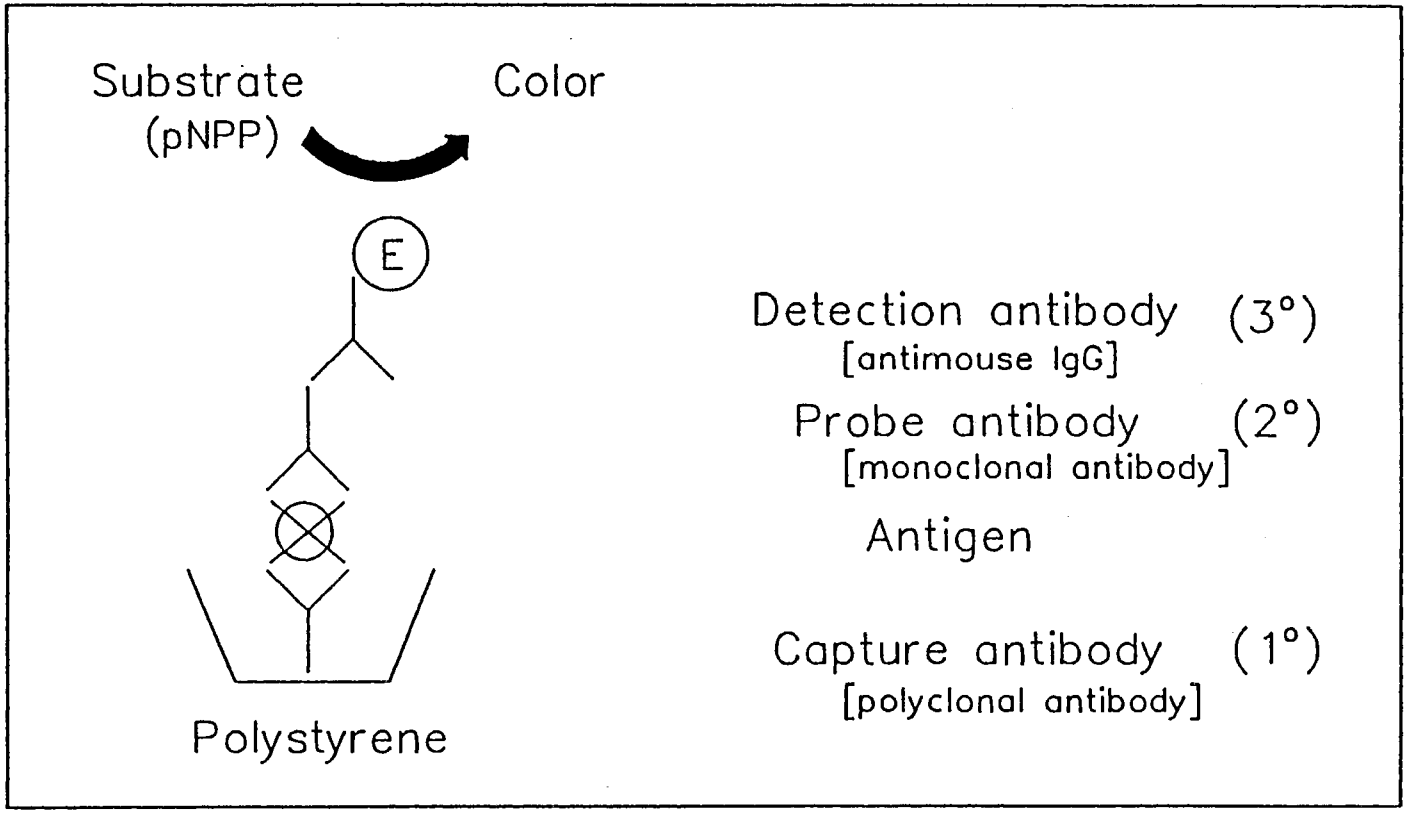

Figure 2. Schematic representation of indirect double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).

NaN3, 800 ml water @ pH 9.8] was applied and incubated for 1 h. Prior to reading the absorbance, the reaction was terminated with 100 μl of 5 M NaOH. Color change within the wells was recorded on a Bio-Tek EIA plate reader® (model EL-307, Bio Tek Instruments Inc., Burlington, Vt).

DAP-ELISA Procedures:

The other method used to detect virus was a direct antigen plating ELISA (DAP-ELISA; Figure 3). Sap from 1 g of virus infected leaf tissue was ground in coating buffer (pH = 9.6) and added (100μl/well) directly to the wells of the Immulon II™ plate. The primary Ab (whether WSMV or AgMV) was PAb (1:1000), and the secondary Ab was goat antirabbit IgG conjugated to alkaline phosphatase (1:5000, Sigma A-3687). After these steps, pNPP in substrate buffer was added and then the reaction was terminated with NaOH prior to reading the absorbance level as described above.

Whenever either ELISA was conducted, three wells were used for each isolate. Additionally, three wells of non-infected plant sap were included for each plate as a control and to provide a zero baseline, and three wells of wheat infected with the OSU isolate (both PAb and Mab reactive) were included as the positive control. A positive ($\geq$ 1.000) and negative ($<$ 0.100) reaction threshold was set based on previous evaluation of material for WSMV (Hunger et al., 1992; Montana et al., 1993, Sherwood et al., 1990).

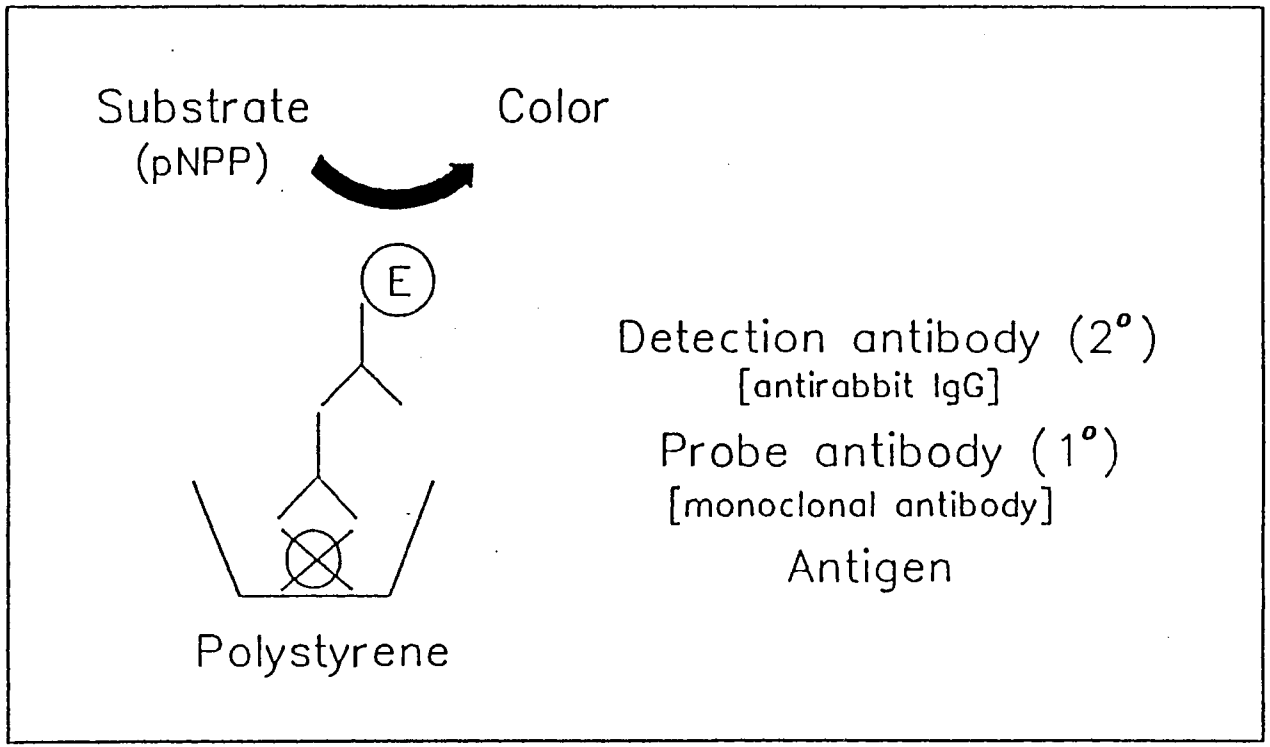

Figure 3. Schematic representation of direct antigen plating enzyme-linked immunosorbent assay (DAP-ELISA).

Initial Isolate Analysis by Western Blot

The ten virus isolates (nine WSMV and one AgMV) were initially tested in western blot analysis probed by either PAbs to WSMV or AgMV, or MAbs to WSMV. Duplicate gels wererun, one for the western blot and the other for total protein staining with Coomassie blue. Three WSMV MAb clones were produced as previously described (Sherwood et al., 1985, 1990). These three clones were designated 32C-1, 32C-6, and 33A-1. Isolates were ground in PPBS-T (PVP + PBS-T) buffer, (1 gm tissue/10 ml buffer), and 1 ml was centrifuged at 10,000 rpm for 10 min. A 30 μl aliquot of supernatant was prepared with 30 μl of SDS-PAGE sample buffer and 15 μl of each isolate was loaded to a SDS-PAGE gel (Sherwood et al., 1987, 1990). Conditions for electrophoresis, transfer to nitrocellulose, and western blot procedures were conducted as described (Deutscher, 1990; BioRad, 1994; Promega, 1995.

The two sheets of nitrocellulose were washed once with Tris buffered saline (TBS) for three 10 min intervals and unbound sites blocked with a 5% solution of non-fat dry milk and TBS on a shaker for either 1 h at 20-25 C or overnight at 4 C. The sheets were then washed three times (10 min on a shaker) in TBS and placed in a 1:1000 solution of the appropriate primary antibody for 1-2 h, returned to the shaker, and then washed. The first wash was a 5% milk solution and then subsequent washes were with TBS for 30 min

each. The membranes were incubated for 1-2 hours in either goat antirabbit IgG (1:5000) or rabbit antimouse IgG (1:3000) as appropriate, washed three times with TBS for 30 min and then placed in a solution of 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium (BCIP/NBT) (BioRad manual) for development. Reaction was stopped by placing in distilled water for 1-2 min. Membranes were placed between Whatman filter paper, covered with aluminum foil, and left overnight to dry.

Since MAb 32C-1 did not react in the initial western blots, additional blots were done at the same time with isolates OSU or PV-57 (MAb reactive isolates) and the three MAbs. Alternate lanes of two gels loaded with OSU or PV-57, run as above, and then proteins in one gel transferred to nitrocellulose. Protein in the other gel was stained with Coomassie blue. After transfer, the nitrocellulose was washed with TBS and then blocked with 5% dry fat milk in TBS. Prior to probing individually with the three MAbs, the nitrocellulose sheet was cut into sections with each section containing a lane of OSU and PV-57. These sections were then placed one to a tray containing one of the three MAb (1:1000). After Ab incubation (2 h at 20-25 C), the solution was decanted, and the sections washed with TBS, (with the first wash in 5% milk). The strips were then returned to a single tray, the secondary Ab applied (1:5000), the strips incubated

for 2 h, then washed three times, and substrate added.

Ouchterlony Double Diffusion

Ouchterlony double diffusion (ODD) precipitation tests were run in 0.8% Noble agar (Difco Laboratories, Detroit, Michigan #D-142-01), 0.5% SDS (Sigma Chemical Company, St. Louis, Missouri), and 1% sodium azide (Sigma #S-2002) prepared in millipore purified water. Distances between all wells was 1 cm.

Extracted sap from WSMV infected plants (1:10 dilution) for all the isolates, was obtained as described for the western blot procedure. Sap from each isolate was placed adjacent to the sap from every other isolate at least once to detect reactions of partial identity. The center well contained PAbs produced to the intact virion of the OSU isolate. AgMV was not tested since it did not react in ELISA or in western blots to WSMV PAbs or MAbs. Plates were placed overnight in plastic bags at 25 C.

Protein Fingerprinting

Seven isolates, (OSU, COLO, OK964, TAMU, PV-57, PV-91 and PV-106) were chosen for further characterization. COLO13, and OK994 were omitted because of the identical serologic reaction patterns produced by these isolates to COLO and OK964 respectively. Virus was isolated from infected tissue as previously described (Sherwood et al., 1990). The various WSMV isolates were placed in 0.01 M citric acid at pH = 8.0

Table V. Cleavage treatments used in the protein fingerprinting assay.

Treatment	Rate	Solution[1]
Purified virus (PV) + Sample buffer	15 µl/well	0.125 M Tris / HCL (pH 6.8)
PV + Cyanogen bromide (CNBr)	1 mg/ml	acetonitrile
PV + CNBr + acetonitrile (Control)		acetonitrile
PV + trypsin	1 mg/ml (1 ppm)	0.125 M Tris / HCL (pH 6.8)
PV + chymotrypsin	1 mg/ml (1 ppm)	0.125 M Tris / HCL (pH 6.8)
PV + papain	150 ppm	0.125 M Tris / HCL (pH 6.8)
PV + pepsin (protease V-8)[2]	10 ppm	0.125 M Tris / HCL (pH 6.8)
PV + N-chlorosuccinimide	0.15 M	0.125 M Tris / HCL (pH 6.8)
PV + Formic acid	75% solution	0.125 M Tris / HCL (pH 6.8)

[1] All of these cleavage agents were then added at the rate of 1:1 to sample buffer containing SDS and β-mercaptoethanol.
[2] This protease V-8 is from *Staphylococcus aureus* strain V-8, type XVII - B.

after isolation. For proteolysis, 10 μl of the chemical reagents or 1 μl protease was added to 50 μl of (1 mg/ml) purified virus (Table V).

Except for CNBr and acetonitrile all treatments were diluted in 0.125 M Tris/HCl buffer (pH @ 6.8) to obtain the 1 mg/ml concentration. For the CNBr treatment 225 mg of CNBr was first dissolved in the acetonitrile solution (100 μl) and adjusted to a final volume of 150 μl. Then 20 μl of this reagent was added to 200 μl of 0.6 N HCL plus 200 μl of 0.125 M Tris/HCl, (pH @ 6.8). Acetonitrile was not used for proteolysis but as a carrier solution for the CNBr and to prevent confounding of the resultant peptide digestion profile with the formic acid digestion. An acetonitrile control, as reported by Nikodem and Fresco (1979), was included.

All proteolytic/virus solutions were incubated for 1 h in tubes at 20-25 C or for the formic acid solution at 37 C. Proteolysis was terminated by adding an equal volume of gel electrophoresis sample buffer containing SDS and β-mercaptoethanol and heating for 5 min at 95 C. For the chemical digestion treatments, prior to the addition of sample buffer, 50 μl of acetone was added to each tube that were then placed at −20 C for 30 min. Tubes were then brought to 25 C, centrifuged at 10,000 rpm for 10 min and the solution removed. Tubes were air dried in a fume hood for 5 min prior to adding 50 μl of sample buffer to each tube for electrophoresis.

The major capsid protein band of the type member of WSMV has a Mr in an SDS-PAGE gel at ≈ 46 kDa with usually three bands from 31 kDa to this 46 kDa band (Brakke et al., 1968, 1990; Seifers, 1992; Sherwood et al., 1990). In the gel stained with the Fast Stain® solution, (ZOION Biotech Corp., Newton, MA, 02158-9883), many low Mr bands were apparent at the bottom of the discontinuous 12% T resolving gel after digestion. Consequently, 15% resolving gels and 4-20% pre-cast gradient gels (BioRad Corp., Hercules, CA, 94547) were used to retain these proteins.

Gels were dried in a "slab gel drying unit" (Ann Arbor Plastics / Scientific Products Department, Ann Arbor, MI, 48104) using a 10% glycerol solution to prevent rapid drying and cracking of gels and then scanned using the 'Quantity One'® software by PDI and the digitized information downloaded to a "Power Mac" computer, using the Macintosh SuperPaint® software, for production of photographic slides and prints.

Western Blot Analysis of Proteolyzed Capsid

Western blot analysis of proteolyzed capsid was conducted as described above except purified virions were used. Proteins in two pre-cast continuous 4-20% T gradient slab gels each containing a different isolate were transferred at a time utilizing the Mini Trans-Blot Cell® (BioRad Corp., model 200/20) at 100 volts constant voltage for 40 minutes. Western blots were probed with WSMV PAbs or MAb 32C-6.

SSEM Immunolabelling Protocol

Purified virions of the seven WSMV isolates were used for SSEM. AgMV and PAbs produced to AgMV (PAbs courtesy of Dr. Dallas Seifers, Fort Hays Branch Agricultural Experimental Station, Hays, KS) were included as a control (Matthews, 1991).

Procedures used in preparation for SSEM were essentially as stated by Westcott et al., (1993) with the following differences. Solutions, including the Abs, were kept on ice until needed. Antibodies used were PAbs to WSMV and AgMV, and the WSMV MAbs (33A-1, 32C-1 and 32C-6). These Abs were diluted at 1:10 using FPG (Fetal Bovine Serum-Glycine-PBS, Westcott et al., 1993) as the dilutant and grids allowed to remain for 30-40 min on the surface of the droplet, carrying over as little excess fluid as possible from the previous droplet.

The protein-A 15 nm gold labelled conjugate was prepared by diluting 1:15 in 5% FPG and 6 μl droplets for each grid placed on the parafilm. Grids were transferred to a 5% uranyl-acetate for 10 min and then dipped 15 times in a beaker of water prior to removing excess water using filter paper. The grids were air dried in a laminar flow hood prior to viewing on a JEOL-100 CXII scanning transmission electron microscope at 80 kV.

MAb Competitive Assay

In order to test whether only a single epitope or various epitopes were being recognized by MAbs 33A-1, 32C-6, and 32C-1), these antibodies were applied individually against the three MAb reactive isolates i.e., OSU, PV-57, and PV-91 in a 3 X 3 arrangement with primary Ab as the column and secondary Ab as the row. MAb 32C-6 was coded as "A", 33A-1 as "B", and 32C-1 as "C". The statistical design was a randomized complete block (RCB) (Gomez and Gomez, 1984; Steel and Torrie, 1960) with the three isolates and a control (sap from healthy wheat foliage) in each plate (block). Three plates/ELISA procedure and three ELISA procedures were tested with two replications/isolate/plate. Other variables were the MAb clone combinations, and number of times (readings) the absorbance level was quantified. Thus, the combination of row 1 column 1 could be "CA", row 1 column 2 "AB" etc., to produce the nine combinations which were randomized within each replication. The five variables were: ELISA procedure (3), plates/ELISA procedure (3), Ab combinations or treatments/isolate/rep (9), replications/isolate/plate (2), reading (3 or 4), and absorbance.

For two of the ELISA procedures Dynatech Immulon II® plates were directly coated with viral antigen, as stated in the DAP-ELISA procedure, using extracted plant sap or purified virus of each isolate. The third ELISA procedure was as

described for the DAS-ELISA using infected plant sap. All solutions were added to plates at 100 μl per well, with the nine possible Ab combinations for each virus considered a replication. The primary MAbs were applied at a concentration (1:300) previously determined to saturate the well (data not shown) (Stone and Nowinski, 1980). The two ELISA techniques were the same after the third step of a DAS-ELISA. The plates were covered with plastic and left overnight at 4 C. Plates were then washed with PBS-T, a 2 % BSA PBS-T solution added, incubated for 30 min at 37 C and then followed by two more PBS-T washes. The appropriate secondary MAb (1:1000) was applied at 100 μl/well and placed at 4 C for 12 h. Thereafter three washes were conducted followed by the addition of rabbit antimouse IgG either conjugated to alkaline phosphatase or β-D-galactosidase for 1 h at 37 C. Finally the addition of appropriate substrate [either pNPP or o-nitrophenyl-β-D galactopyranoside (ONPG, Sigma N-1127)] was applied and plates incubated at 20-25 C for 60 min. Plates were read several times at ten min intervals at 405 nm on either a Vmax computerized Kinetic microplate reader (Molecular Devices, CA, USA) or the Bio-Tek EIA plate reader to produce the absorbance curve.

Results

Initial ELISA and Western Blots

Differential reaction of isolates to Mabs was observed in both ELISA and western blots. Isolates OSU, PV-57 and PV-91 reacted with the WSMV MAbs and PAbs, but all other WSMV isolates reacted only with WSMV Pabs (Figure 4, Figure 5, Figure 6, Table VI). Initially, PV-57 and PV-106 were found to also contain AgMV but AgMV was removed by passage through oats (*Avena sativa*; Figure 4, lane c) a non-host for AgMV, (Brakke, 1971; Cook and Veseth, 1991; Wiese, 1987) prior to inoculating wheat cv. Blue Jacket to increase inoculum for the other tests. Thereafter, the PAbs of WSMV or AgMV did not cross react (data not shown). All MAbs reacted in DAS-ELISA to OSU, PV-91, and PV-57 (Table VI). Reaction and identical banding patterns also occurred in the western blots probed with MAb 33A-1 (Figure 5) or 32C-6 (Figure 6). However, MAb 32C-1 did not react to OSU, PV57, or PV-91 (the three DAS-ELISA reactive isolates) in the western blot (Table VI). This test was repeated three times with identical results.

Ouchterlony Double Diffusion

Precipitation was clearly observed a short distance from the peripheral wells for all isolates, except PV-91 which produced no or a weak precipitation, and the noninfected wheat tissue (Figures 7 and 8; Table VI). No precipitation lines

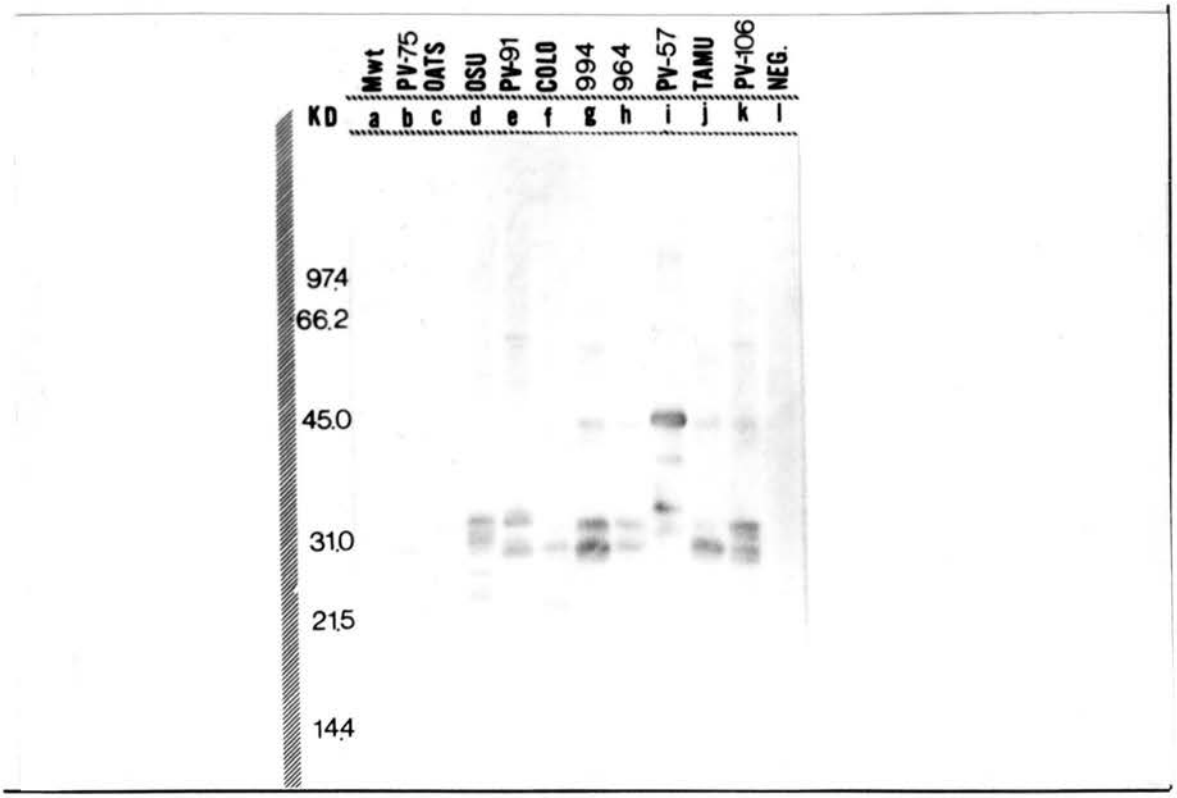

Figure 4. Preliminary western blot of isolates of wheat streak mosaic virus - probed with polyclonal antibodies.

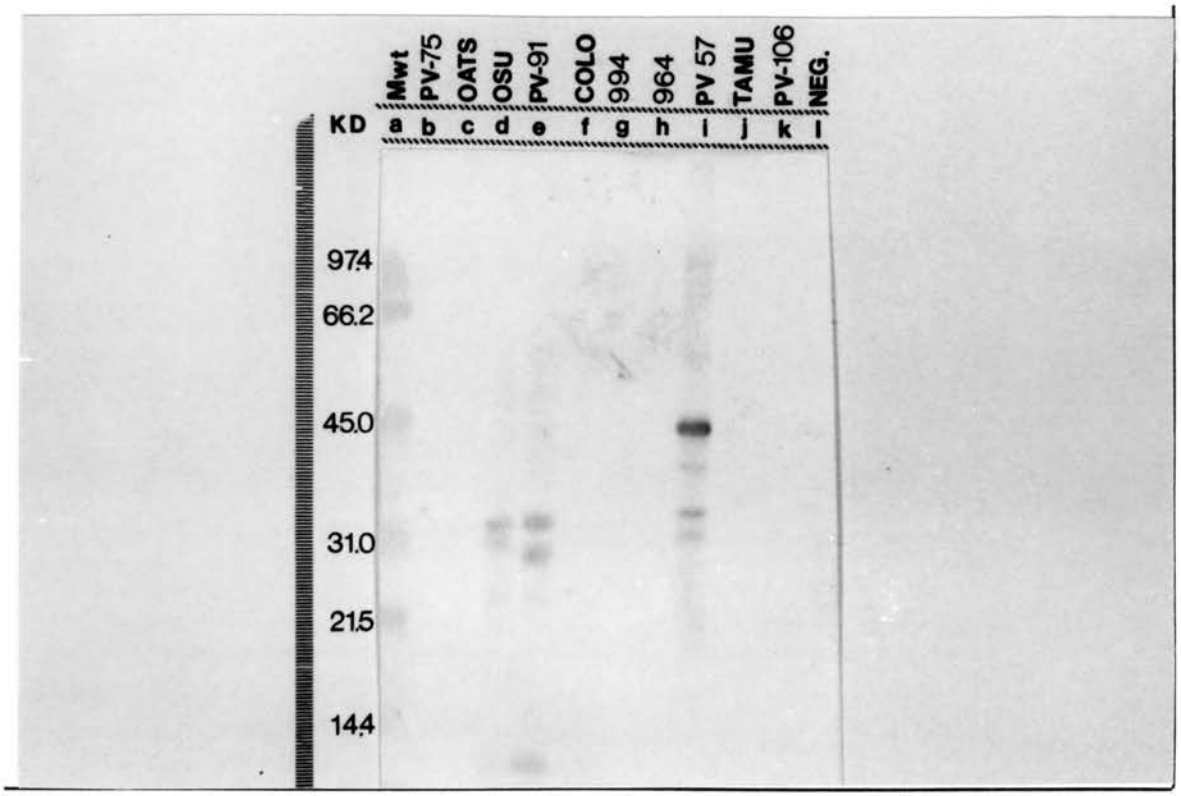

Figure 5. Preliminary western blot of isolates of wheat streak mosaic virus - probed with monoclonal antibodies from MAb clone 33A-1.

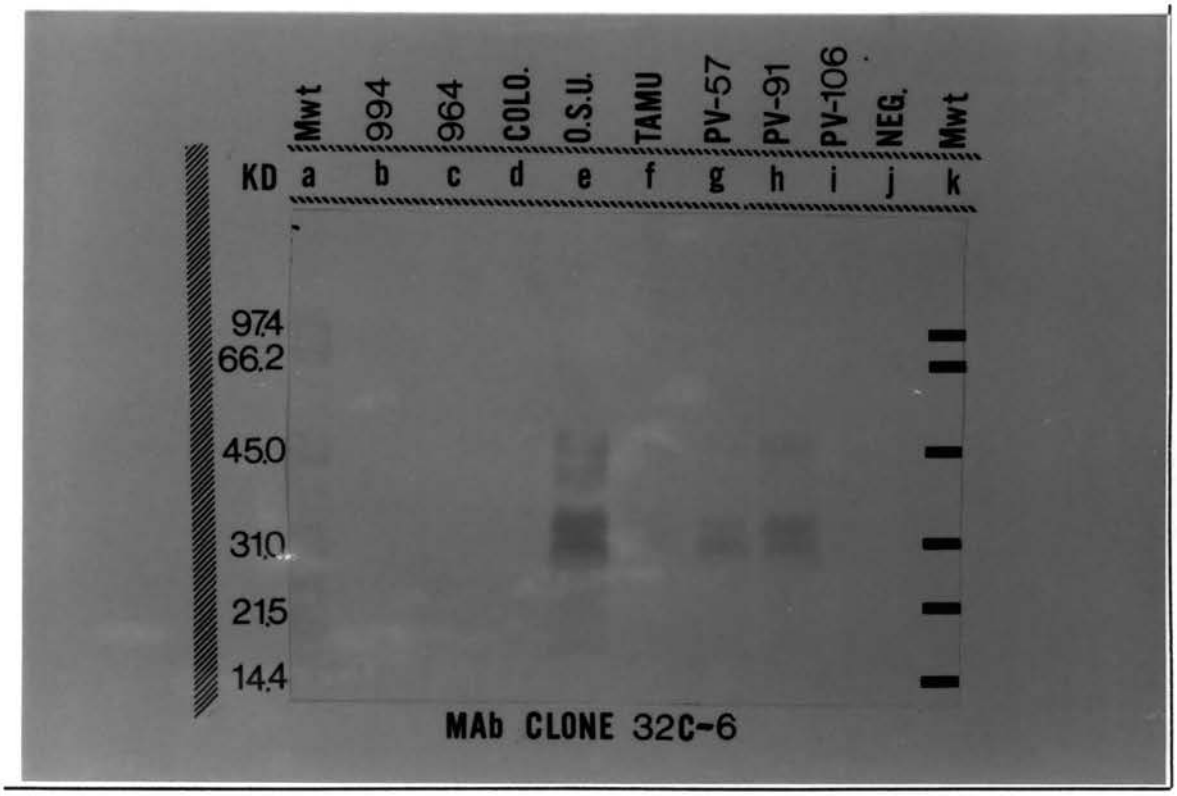

Figure 6. Preliminary western blot of isolates of wheat streak mosaic virus - probed with monoclonal antibodies from MAb 32C-6.

Table VI. Summary of antibody results showing that WSMV isolates contain at least three distinct reactive epitopes.

	MAb Western blot			MAb DAS-ELISA			PAb ELISA and Western blot	PAb ODD
	33A-1	32C-6	32C-1	33A-1	32C-6	32C-1		
O.S.U.	+	+	-	+	+	+	+	+
PV-57	+	+	-	+	+	+	+	+
PV-91	+	+	-	+	+	+	+	-[1]
Others	-	-	-	-	-	-	+	+

[1]Very weak or no precipitation reaction occurred.

Figure 7. Ouchterlony double diffusion assay using WSMV polyclonal antibodies in central well. The perimeter wells contain infected plant sap supernatant from WSMV isolates centrifuged (10 min @ 10,000 rpm) or non-infected control sap (well 1). Clockwise from well 1: OSU, COLO, PV-106, OK964, and PV-91.

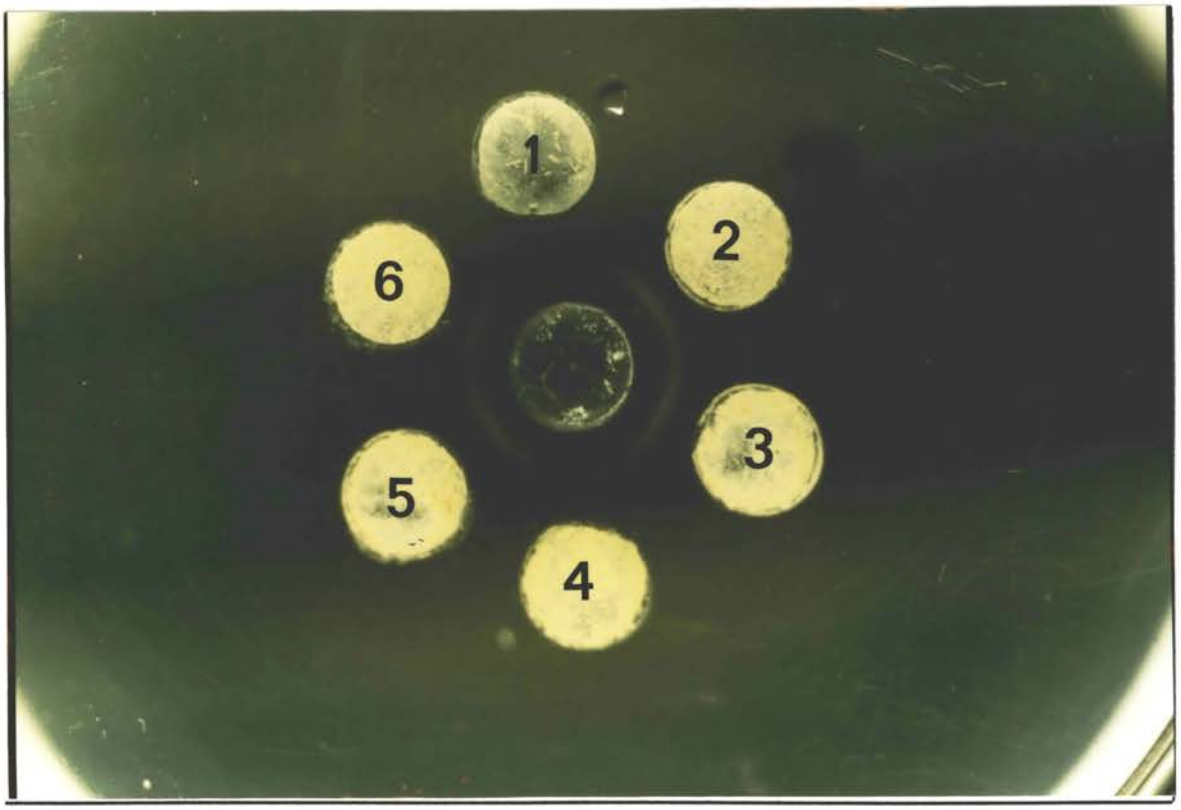

Figure 8. Ouchterlony double diffusion assay using WSMV polyclonal antibodies in central well. The perimeter wells contain infected plant sap supernatant from WSMV isolates centrifuged (10 min @ 10,000 rpm) or non-infected control sap (well 1). Clockwise from well 1: PV-106, COLO, PV-91, OK964, and TAMU.

were observed close to the antiserum well nor were spurs observed. No precipitation was observed in similar tests utilizing MAbs in the central well (data not shown).

SSEM Immunolabelling

Both PAbs and MAbs were found to bind along the length of the virion (Figure 9 and 10). Antibodies were also observed to bind to disrupted coat protein subunits (Figure 11) of the particular virus isolate. No binding was seen on control grids (Figure 12). The suspected difference in MAb reaction, as seen under the denaturing conditions of the Western blots, were not reproduced under SSEM. All three MAbs were found to bind to the OSU, PV-57, and PV-91 isolates whether as intact virions or when the virion was disrupted (Table VII).

MAb Competitive Assay

Results of the competitive assay with the three MAbs indicated significant differences in their reaction. The statistical model evaluated the MAb reactions based on both their inter-group (AX, BX, CX; where X = any other MAb) and intra-group differences (AB, AC above its AA baseline; BA, BC above BB; and CA, CB above CC). Results varied in significance by the time of reading of the plate and between the plates. Therefore, the RCB design was appropriate to accommodate this variability. Significant differences in avidity would be indicated by those MAbs that displayed a

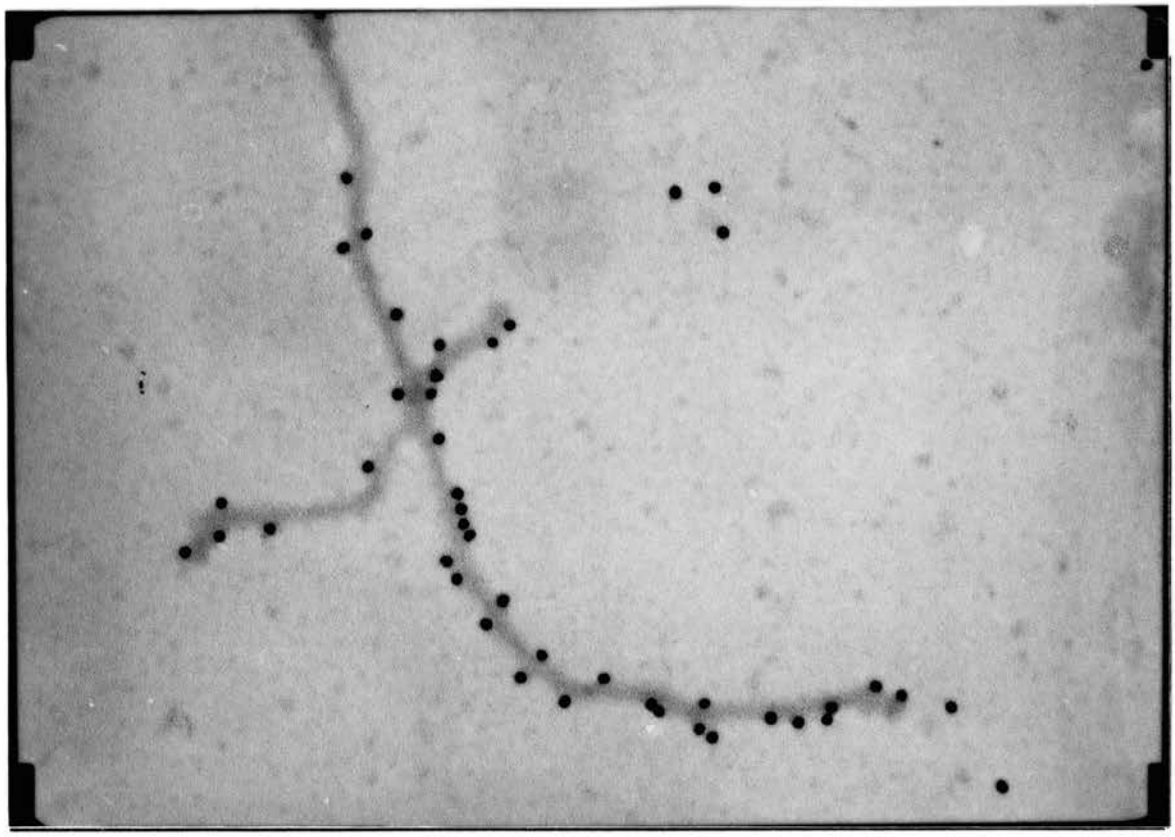

Figure 9. Serologically specific electron micrograph of WSMV-II serogroup member PV-57 probed with monoclonal antibody 32C-1. Note that 32C-1 also reacted with WSMV-II members in DAS-ELISA but not in western blots.

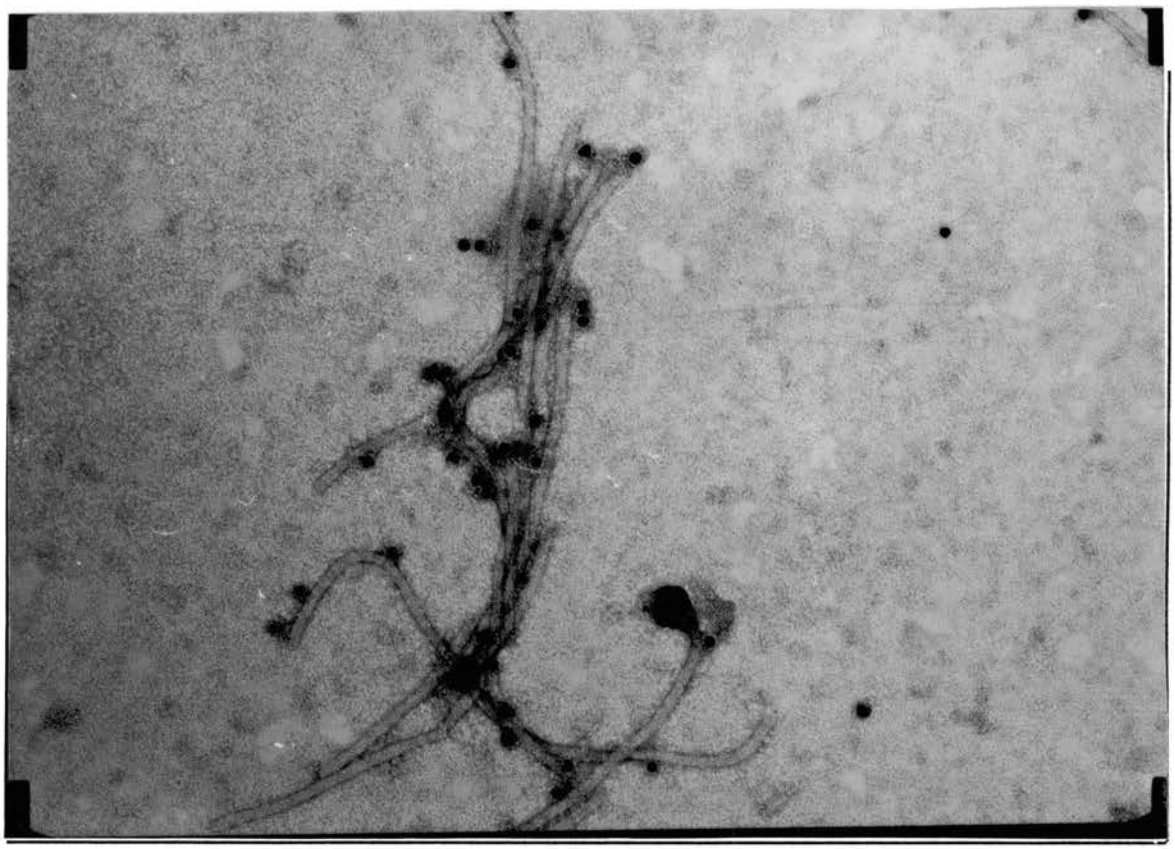

Figure 10. Serologically specific electron micrograph of WSMV-I serogroup member COLO probed with polyclonal antibodies.

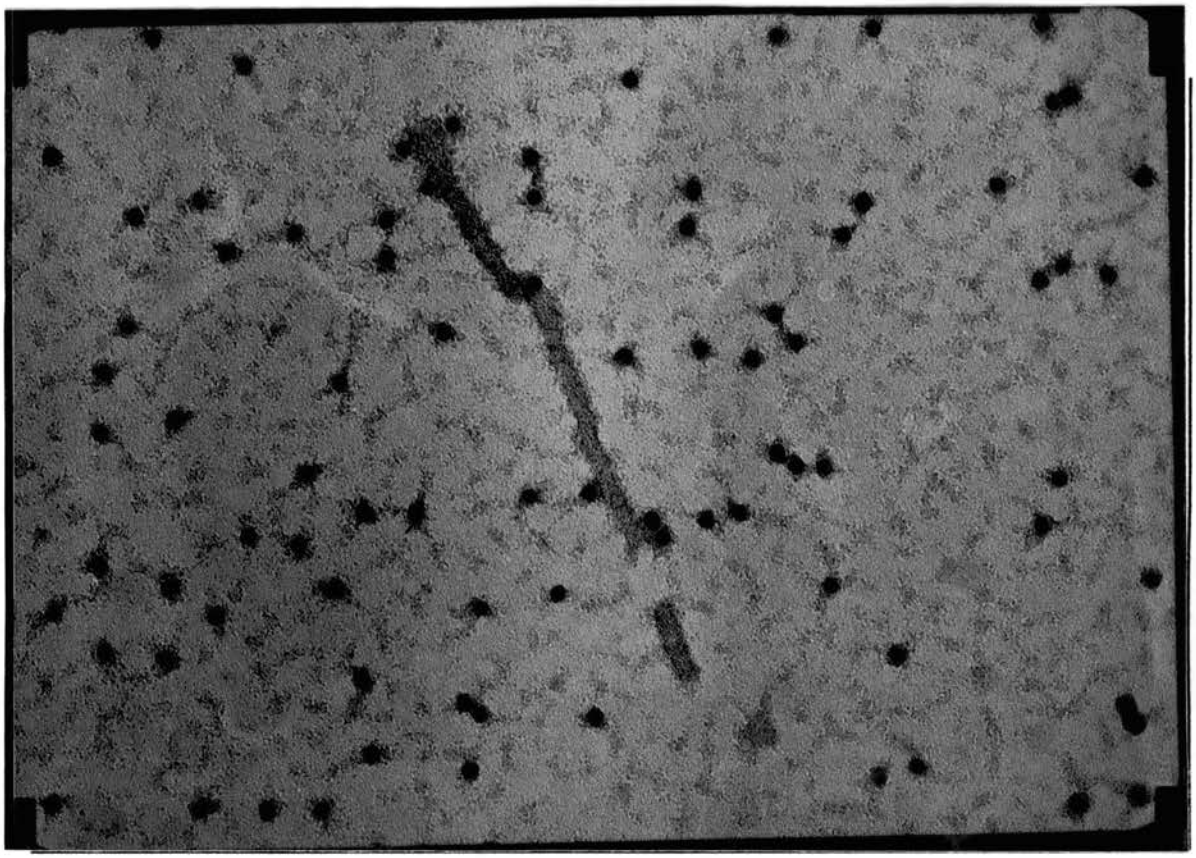

Figure 11. Serologically specific electron micrograph of disrupted subunits of WSMV-II serogroup member OSU probed with monoclonal antibody 32C-6.

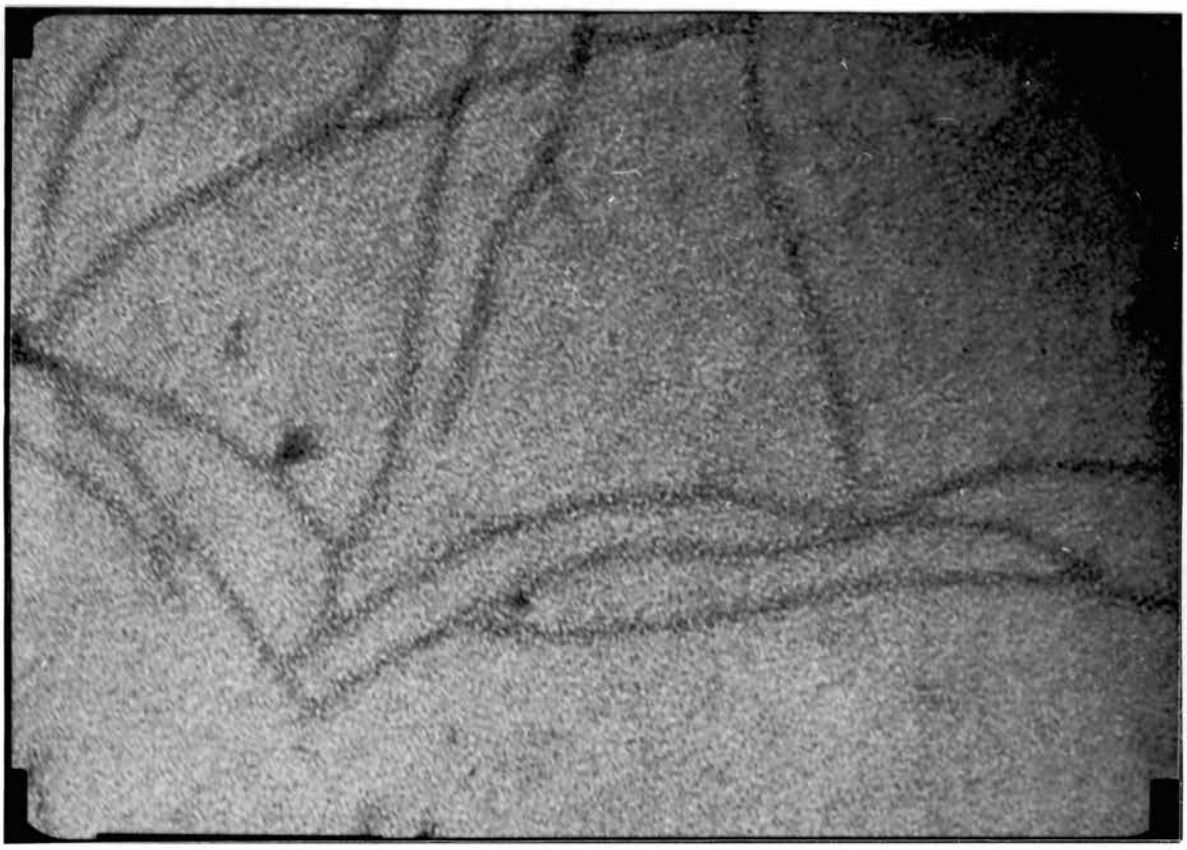

Figure 12. Serologically specific electron control micrograph of WSMV-I serogroup member COLO probed with monoclonal antibody clone 33A-1. The virions are not coated with the Protein-A gold/antibody complex.

Table VII. Summary of Serologically Specific Electron Microscopy (SSEM) probing with either polyclonal or three monoclonal antibodies.

	Wheat streak mosaic virus monoclonal antibodies			Polyclonal antibodies	
	32C-6	32C-1	33A-1	WSMV	AgMV
O.S.U.	+	+	+	+	-
PV-57	+	+	+	+	-
PV-91	+	+	+	+	-
All Others	-	-	-	+	-
Control Grids	-	-	-	-	-

consistently different level of absorbance regardless of competition. For example, if MAb "AX" has an absorbance of 1.5, "BX" 1.0 and "CX" 0.75 after 1 h substrate incubation,then these inter-group differences can be attributed solely to MAb avidity.

Competitive MAb results indicated that for the OSU isolate absorbance differences were due to differences in MAb avidity and not epitope competition between MAbs (Table VIII). The significant differences in absorbance were inter-group and not intra-group indicating that the three MAbs react to the same antigenic determinant. Results from assays with PV-57 indicated that all three MAbs also reacted to the same antigenic determinant (Table IX). The only possibly significant differences (LSMEANS, SAS Institute, Cary, NC) in intra-group reaction for WSMV isolate PV-57, was the addition of either MAb 32C-6 (A) or MAb 33A-1 (B) ($Pr > T = 0.0717$ and 0.0231 respectively) compared to the MAb 32C-1 (CC) baseline using purified virus and DAP-ELISA. However, the addition of MAb 32C-6 (A) would be significant only at the 10% level. This singular intra-group significant increase in absorbance only occurred 80 min after substrate was added. All other MAb reactions displayed a pattern similar to those of the OSU isolate.

As with the other virus isolates the majority of significant reactions for PV-91 were intergroup (Table X).

Table VIII. Monoclonal antibody competitive assay results for wheat streak mosaic virus isolate OSU.

	READING								
	DAP-ELISA[1]			DAS-ELISA[2]			DAP-ELISA[3]		
Treatment[4]	60[5]	70	80	60	70	80[6]	60	70	80
AA	0.534	1.140	1.433	1.035	1.851	*****	0.647	1.490	1.714
AB	0.687	1.270	1.524	1.135	1.998	*****	0.648	1.458	1.859
AC	0.644	1.220	1.485	0.989	1.747	*****	0.617	1.442	1.695
BB	0.425	0.970	1.180	0.885	*****	*****	0.559	1.513	1.702
BA	0.371	0.984	1.186	0.777	*****	*****	0.558	1.478	1.673
BC	0.510	1.057	1.300	1.069	1.907	*****	0.592	1.409	1.690
CC	0.527	1.085	1.397	0.867	1.818	*****	0.535	1.340	1.619
CA	0.561	1.200	1.471	0.956	1.872	*****	0.585	1.442	1.555
CB	0.581	1.214	1.452	0.868	1.793	*****	0.619	1.572	1.696
Significance[7] Level:	0.1437	0.0348	0.1289	0.3416	0.1332	******	0.0656	0.8205	0.3109

[1]Direct Antigen Plating ELISA (DAP-ELISA) using infected plant sap.
[2]Double Antibody Sandwich ELISA (DAS-ELISA) using infected plant sap.
[3]Direct Antigen Plating ELISA (DAP-ELISA) using purified virus.
[4]Where: "A" = 32C-6, "B" = 33A-1, and, "C" = 32C-1.
[5]Time in minutes after application of substrate.
[6]The absorbance level for this reading was > 2.000, the limit of the plate reader and therefore absorbance could not be statistically analyzed.
[7]Significance level signified is probability level of statistical model which incorporates both inter- and intra-group values. See text for intra-group Pr > T significance levels.

Table IX. Monoclonal antibody competitive assay results for wheat streak mosaic virus isolate PV-57.

	READING								
	DAP-ELISA[1]			DAS-ELISA[2]			DAP-ELISA[3]		
Treatment[4]	60[5]	70	80	60	70	80[6]	60	70	80
AA	0.623	1.550	1.780	0.921	1.811	*****	0.632	1.460	1.820
AB	0.665	1.640	1.840	0.825	1.845	*****	0.641	1.480	1.840
AC	0.792	1.570	1.800	0.930	1.732	*****	0.598	1.300	1.640
BB	0.714	1.440	1.740	0.968	1.800	*****	0.625	1.450	1.810
BA	0.618	1.400	1.650	0.930	1.706	*****	0.650	1.400	1.810
BC	0.741	1.450	1.720	0.867	1.654	*****	0.633	1.420	1.720
CC	0.715	1.550	1.810	0.843	1.624	*****	0.567	1.250	1.550
CA	0.663	1.590	1.870	0.812	1.749	*****	0.561	1.340	1.680
CB	0.675	1.460	1.780	0.917	1.624	*****	0.611	1.350	1.720
Significance[7] Level:	0.1088	0.0714	0.1037	0.9408	0.1685	******	0.2023	0.1124	0.0200

[1]Direct Antigen Plating ELISA (DAP-ELISA) using infected plant sap.
[2]Double Antibody Sandwich ELISA (DAS-ELISA) using infected plant sap.
[3]Direct Antigen Plating ELISA (DAP-ELISA) using purified virus.
[4]Where: "A" = 32C-6, "B" = 33A-1, and, "C" = 32C-1.
[5]Time in minutes after application of substrate.
[6]The absorbance level for this reading was > 2.000, the limit of the plate reader and therefore absorbance could not be statistically analyzed.
[7]Significance level signified is probability level of statistical model which incorporates both inter- and intra-group values. See text for intra-group Pr > T LSMEANS significance levels.

Table X. Monoclonal antibody competitive assay results for wheat streak mosaic virus isolate PV-91.

	READING									
	DAP-ELISA[1]			DAS-ELISA[2]			DAP-ELISA[3]			
Treatment[4]	60[5]	70	80	60	70	80[6]	60	70	80	90
AA	0.504	0.959	1.222	0.909	1.800	*****	0.062	0.150	0.226	0.366
AB	0.494	0.960	1.246	0.925	1.893	*****	0.062	0.162	0.243	0.402
AC	0.529	0.960	1.246	0.925	1.753	*****	0.061	0.161	0.244	0.391
BB	0.367	0.734	0.935	0.921	1.814	*****	0.048	0.121	0.185	0.297
BA	0.360	0.691	0.897	0.894	1.790	*****	0.055	0.133	0.199	0.324
BC	0.379	0.761	0.982	0.878	1.780	*****	0.050	0.135	0.208	0.330
CC	0.463	0.878	1.127	0.847	1.770	*****	0.073	0.178	0.265	0.428
CA	0.495	0.963	1.226	0.878	1.810	*****	0.063	0.166	0.246	0.411
CB	0.506	0.944	1.224	0.985	1.756	*****	0.064	0.168	0.231	0.406
Significance[7] Level:	0.0058	0.0061	0.0041	0.0074	0.2093	******	0.0002	0.0001	0.0001	0.0001

[1]Direct Antigen Plating ELISA (DAP-ELISA) using infected plant sap.
[2]Double Antibody Sandwich ELISA (DAS-ELISA) using infected plant sap.
[3]Direct Antigen Plating ELISA (DAP-ELISA) using purified virus.
[4]Where: "A" = 32C-6, "B" = 33A-1, and, "C" = 32C-1.
[5]Time in minutes after application of substrate.
[6]The absorbance level for this reading was > 2.000, the limit of the plate reader and therefore absorbance could not be statistically analyzed.
[7]Significance level signified is probability level of statistical model which incorporates both inter- and intra-group values. See text for intra-group Pr > T significance levels.

However, with the infected sap and DAS-ELISA procedure and at 60 min after substrate was added, significant intra-group differences were seen. Addition of MAb 33A-1 (B) significantly (PR > T = 0.0002) increased absorbance above the MAb 32C-1 (CC) baseline.

Significant inter- and intra-group differences occurred using the purified virus DAP-ELISA procedure. Sixty minutes after substrate addition, MAb 32C-6 (C) increased absorbance (Pr > T = 0.0226) above the MAb 33A-1 (BB) baseline. The addition of either MAb 32C-6 (A) or MAb 33A-1 (B) reduced absorbance below the MAb 32C-1 (CC) baseline. By 70 min after substrate addition, MAb 33A-1 (B) increased (Pr > T = 0.0402) absorbance above the MAb 32C-6 (AA) baseline and addition of MAb 32C-6 (A) or MAb 32C-1 (C) increased absorbance (Pr > T = 0.0324 and 0.019 respectively) above MAb 33A-1 (BB). Addition of MAb 32C-6 (A) or MAb 33A-1 (B) to MAb 32C-1 (CC) continued to decrease absorbance. By 80 min after addition of substrate, both MAb 33A-1 (B) and MAb 32C-1 (C) increased absorbance above the MAb 32C-6 (AA) baseline (Pr > T = 0.0431 and 0.0387 respectively. Addition of MAb 32C-1 (C) also increased absorbance above MAb 33A-1 (BB), and absorbance continued to be decreased by the addition of either MAb in comparison to the MAb 32C-1 (CC) baseline. Finally, 90 min after substrate was added, the above trends continued with addition of either MAb 33A-1 (B) or MAb 32C-1 (C) increasing

absorbance above the MAb 32C-6 (AA) baseline, addition of MAb 32C-6 (A) or MAb 32C-1 (C) increasing absorbance above MAb 33A-1 (BB), and the decrease in absorbance by either MAb compared to the MAb 32C-1 (CC) baseline.

Protein Fingerprinting

Fast stain

Migration of capsid protein in SDS-PAGE was affected when capsid was cleaved by formic acid, N-chlorosuccinimide and CNBr. Inhibition of migration caused by these chemical agents was believed to be due to the acidity of the solutions (pH < 1). Inhibition of migration was minimized by addition of acetone to precipitate the capsid proteins, however, upon addition of sample buffer, the buffer would sometimes still change from blue to yellow indicating that residual acidity remained.

Comparison of the banding pattern before and after digestion shows that the nondigested capsid proteins of the majority of WSMV were 45 kDa (Figure 13). Some variation was seen however with TAMU and PV-91 at 40 kDa. Several bands from 16-45 kDa were observed for PV-57, as well as a band at 66 kDa for PV-57 and OSU. A doublet at 33-35 kDa was observed for PV-106. No capsid cleavage was evident for any of the isolates by the acetonitrile (control) or for the formic acid agents (data not shown). The CNBr digestion (Figure 14)

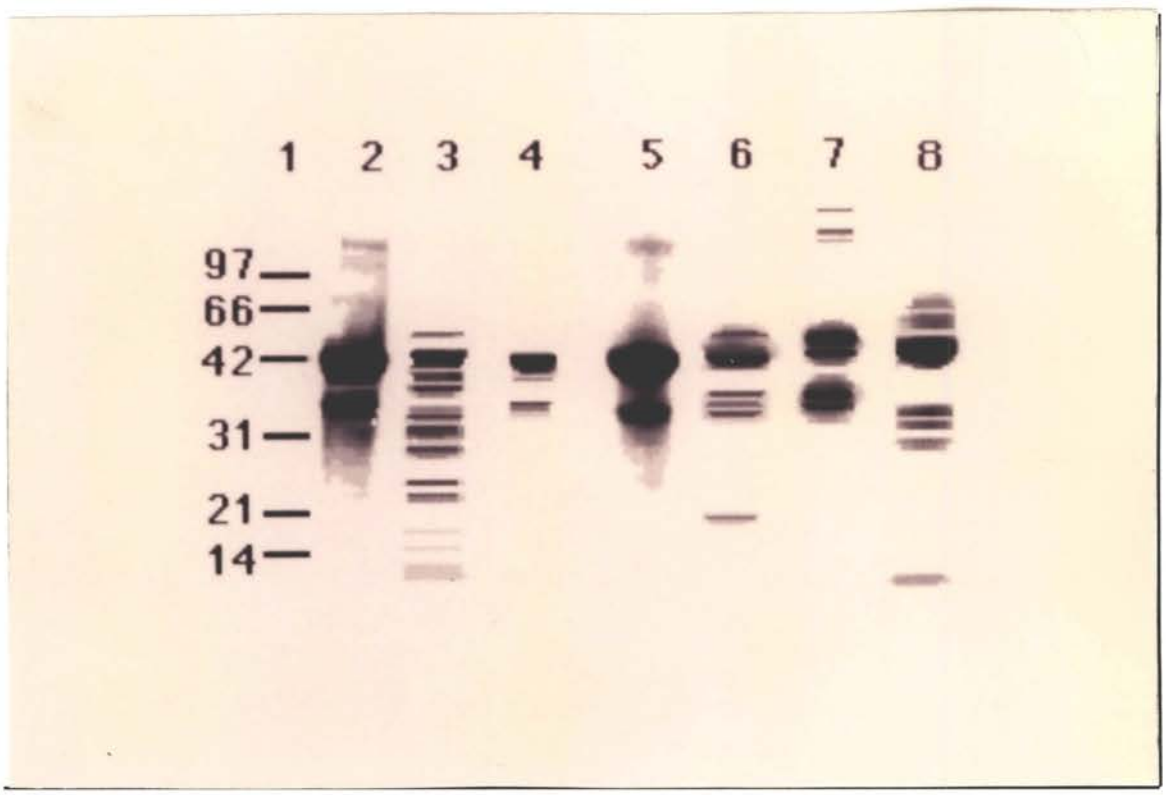

Figure 13. Migration pattern of non-digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel. Five μl of BioRad's low range standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

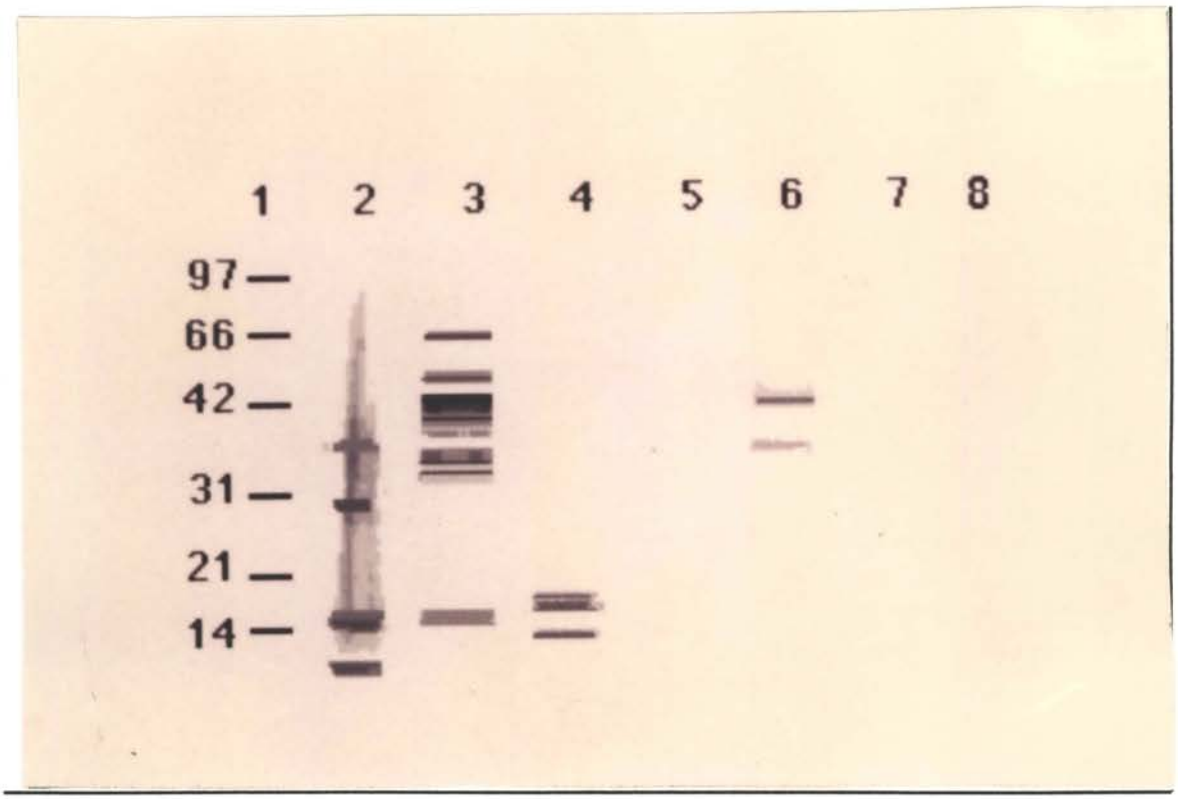

Figure 14. Migration pattern of CNBr digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel. Five μl of BioRad's low range standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

provided variable information from no bands present to a clear (PV-91, TAMU, OSU, and PV-57) affect due to CNBr. TAMU was not cleaved by CNBr, PV-57 had a single doublet at 16 kDa, OSU had two doublets appearing below 14 kDa and the capsid of PV 91 produced four bands between 12 and 21 kDa (Figure 14).

Trypsin digestion could not be used to separate the isolates since the banding pattern was similar between all seven isolates (data not shown). Proteolytic agents that produced distinctive banding patterns useful for serotype comparison included chymotrypsin which produced similar cleavage products for COLO and PV-91 showing loss of the typical non-digested 31-46 kDa capsid doublets (Figure 15). The other serotypes also had one or more of the typical capsid bands i.e., either the 31 kDa band (PV-57, PV-106), the 31-34 kDa doublet (OSU, TAMU), or a band at 31 kDa and a second doublet band located at 27 kDa (OK964). All isolates produced bands occurring from 6-8 kDa.

Papain cleavage (Figure 16) resulted in banding patterns that indicated five distinct groupings: TAMU and OK964; OSU and PV-91; COLO; PV-57; and PV-106. PV-106 produced the normal 31-46 kDa doublets indicating no papain cleavage. Cleavage of the COLO isolate by chymotrypsin or papain resulted in identical band production. This was unique to the COLO isolate.

For the V-8 protease digest (Figure 17) PV-106 produced

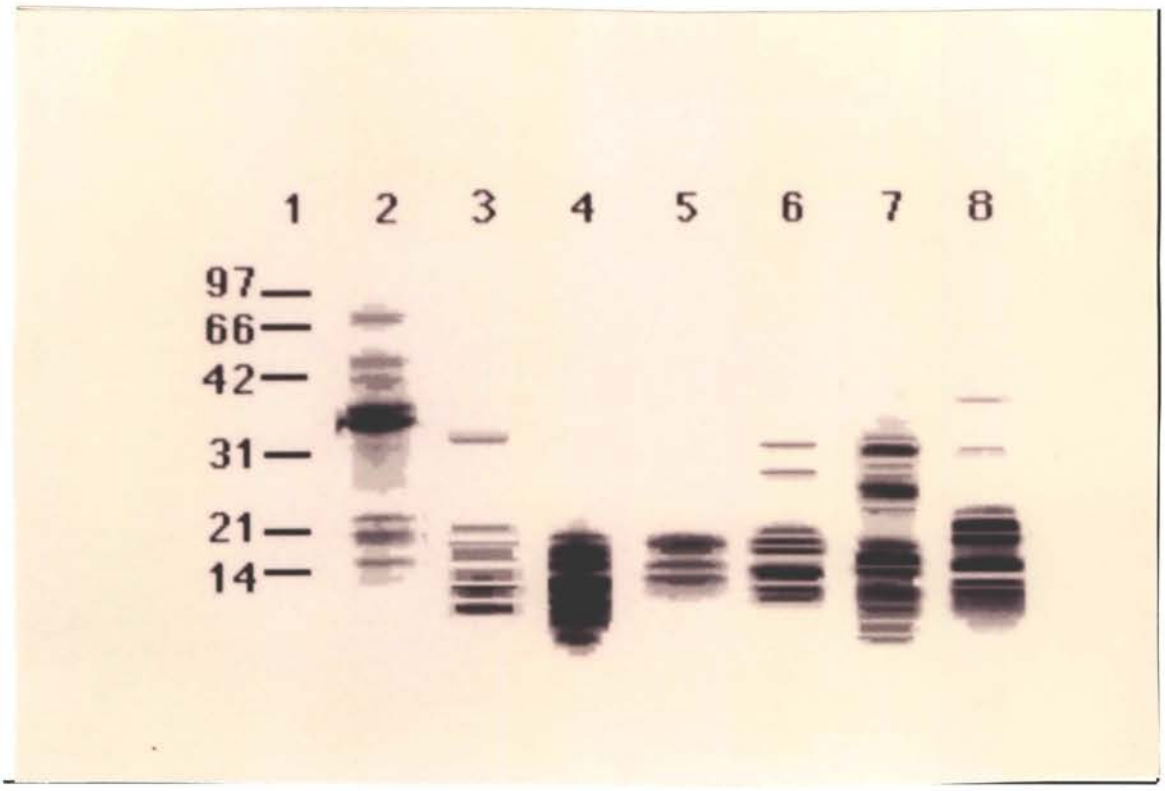

Figure 15. Migration pattern of chymotrypsin digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel. Five μl of BioRad's low range standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

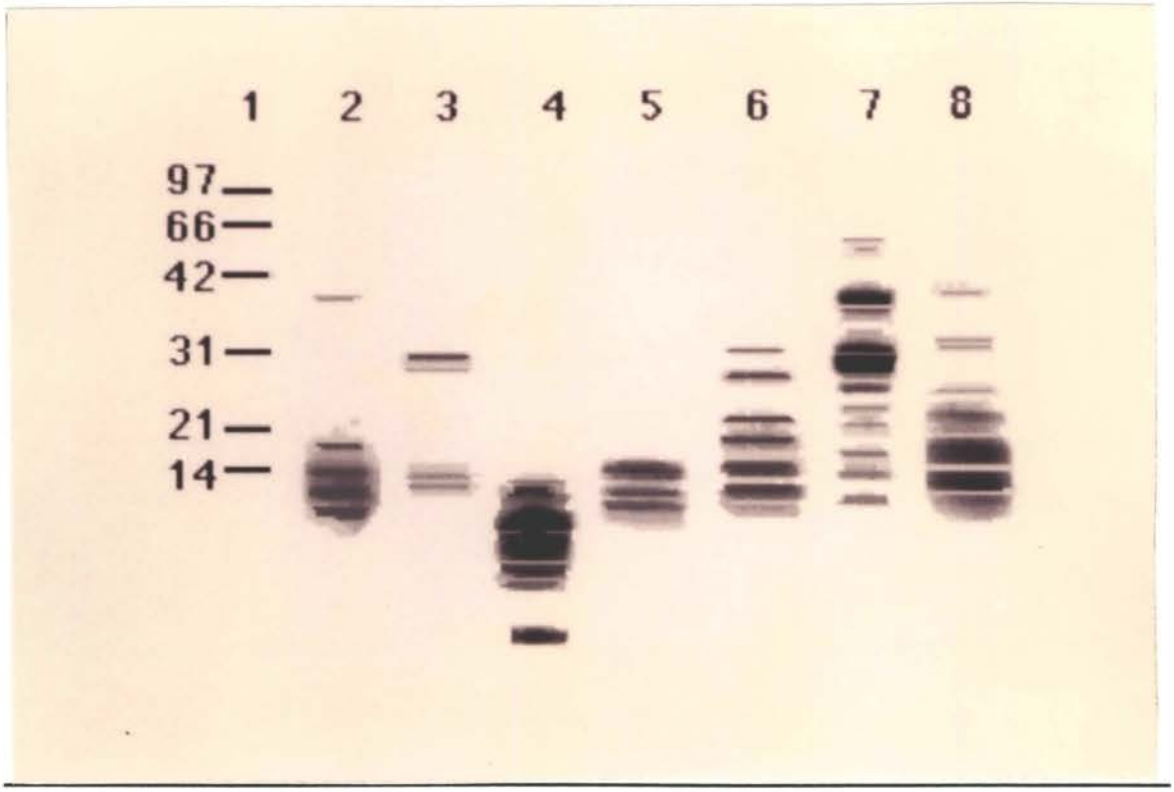

Figure 16. Migration pattern of papain digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel. Five μl of BioRad's low range standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

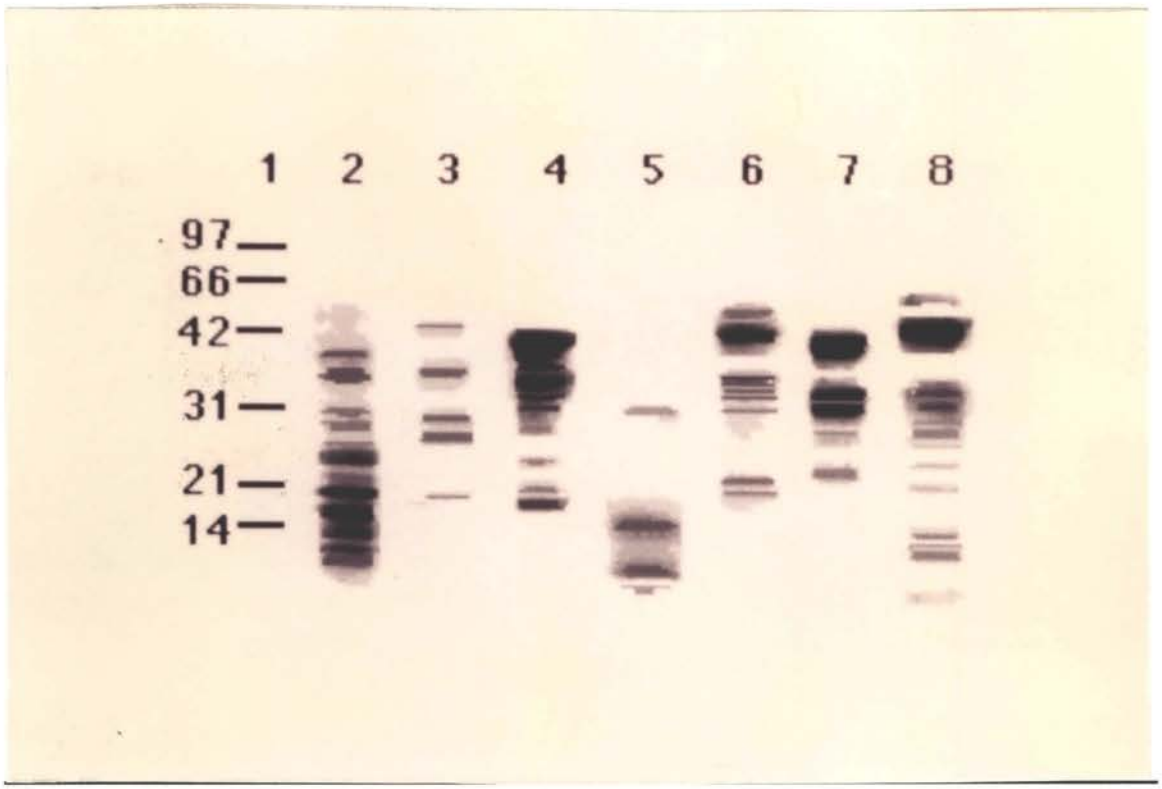

Figure 17. Migration pattern of protease V-8 digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel. Five μl of BioRad's low range standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

only a few bands ending with a doublet at 21 kDa, but had overall similarity to TAMU, PV-91 and PV-57. OSU and OK964 could also have been included in this grouping since the pattern is similar, but with other bands in the 6-21 kDa range. Only the COLO isolate was severely cleaved displaying many bands ≤ 6.5 kDa with one doublet remaining at 26 kDa.

When capsid was digested with N-chlorosuccinimide some protein migration problems, due to low pH were observed. However, similar digestion products for PV-57 and PV-91 with loss of the 31-46 kDa doublets and occurrence of bands of 14-25 kDa were observed (data not shown). TAMU had bands from 6-31 kDa and doublets at 31-46 kDa. Colo had three bands ≤ 21 kDa.

Western Blot Analysis of Proteolyzed Capsid with PAb

The non-digested capsid proteins were run in an 4-20 % gradient SDS-PAGE gel and probed with PAbs (Figure 18). For those chemical agents which produced protein bands that reacted with the PAb e.g., CNBr (Figure 19) no differences were observed between isolates. Trypsin produced no distinct PAb reaction differences between the isolates but did produce the same patterns reported earlier for the OSU isolate (Sherwood et al., 1990), and by others using the type strain (Brakke et al., 1990). This does confirm that either purified viral digestion within an extracted gel slice or in solution results in similar banding patterns. The chymotrypsin

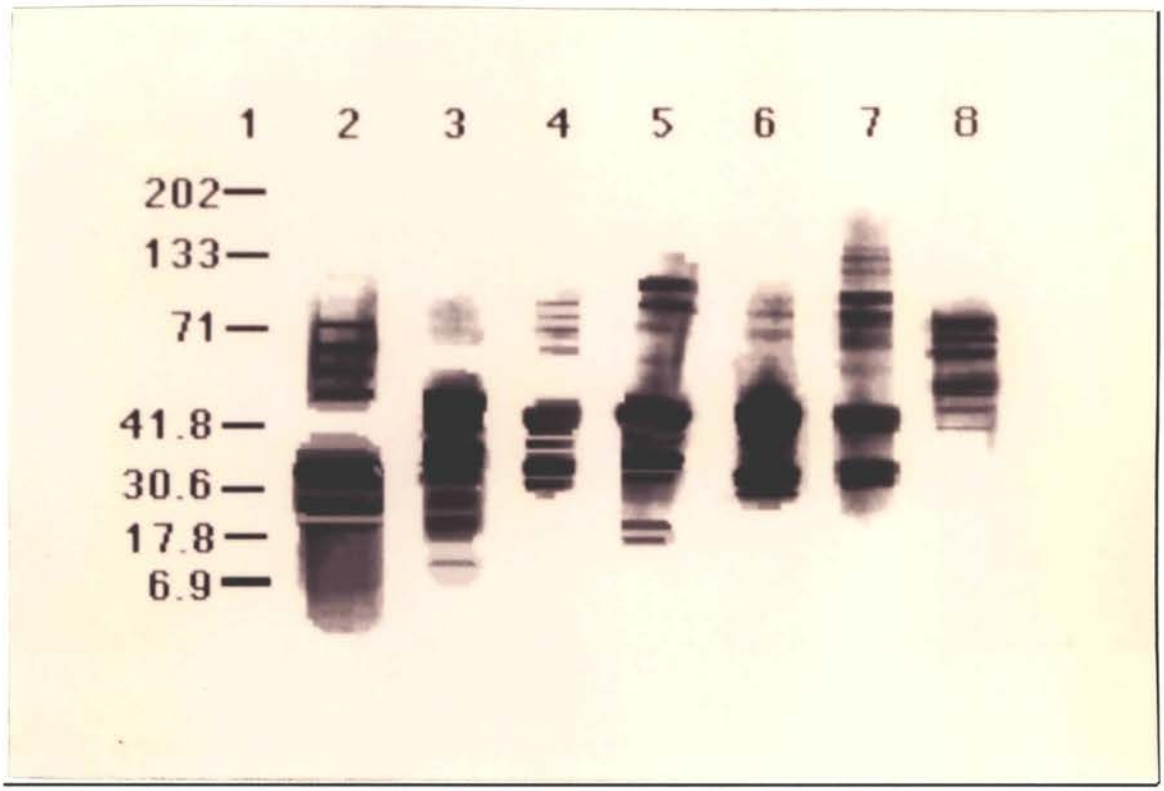

Figure 18. Migration pattern of non-digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with polyclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

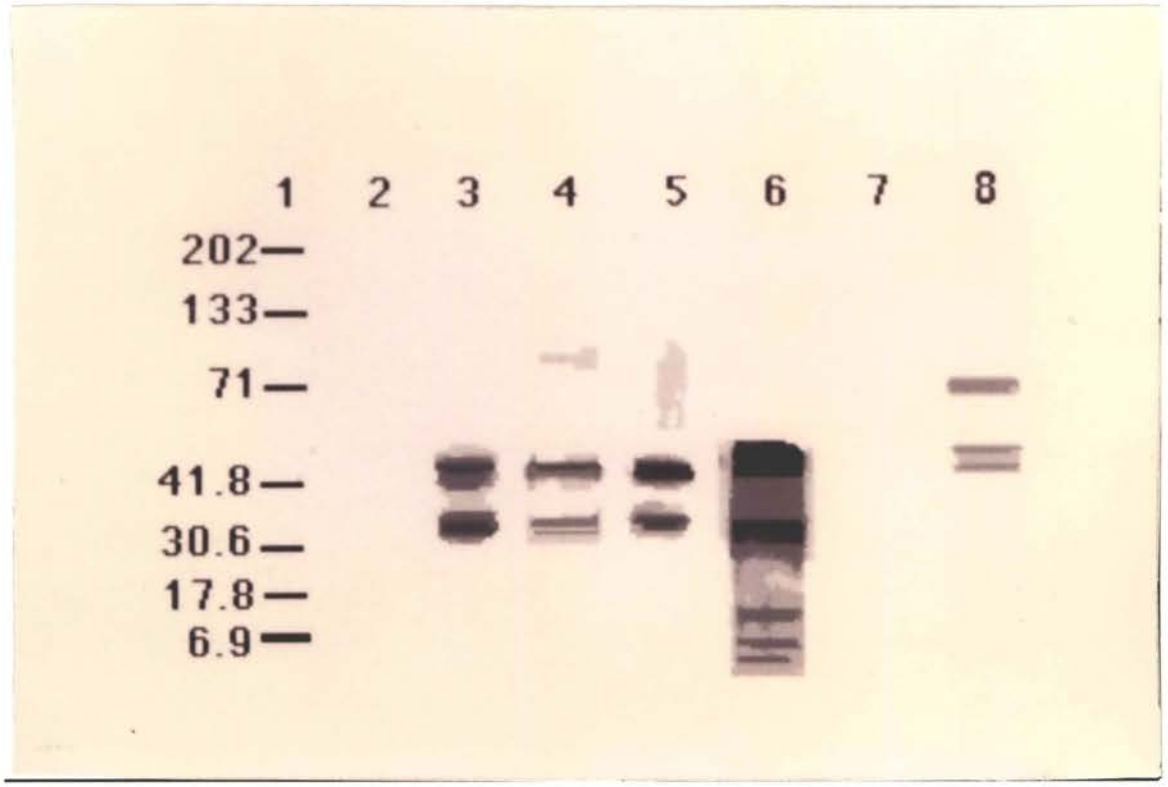

Figure 19. Migration pattern of CNBr digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with polyclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, PV-91, COLO, TAMU, PV-106, and OK964.

digest/PAb reaction was distinctive among the serotypes and displayed similar banding patterns for COLO, PV-106, OK964, TAMU, and OSU in one grouping with PV-91 and PV-57 in the other (Figure 20). Banding patterns were similar for isolates OSU, PV-106, COLO, and OK964 following papain digestion and reaction with PAb gave similar banding patterns to the Fast Stain® gels. No bands at all were evident for PV-57, all bands for TAMU were ≤ 31 kDa, and two doublets were visible for PV-91 < 6.9 kDa marker (Figure 21). The V-8 digestion lanes (Figure 22) did not produce bands recognized by the PAb below the 31 kDa doublet for OK964 or PV-106. The COLO and TAMU isolates had a similar pattern of bands as did the PV-91 and PV-57 isolates. The OSU digestion pattern appeared unique. Finally, with N-chlorosuccinimide PV-106 and TAMU produced two doublets between 14-21 kDa, COLO had no bands < 31 kDa, PV-57 and PV-91 were similar in banding pattern, and the OSU isolate had low pH migration problems (data not shown).

Western Blot Analysis of Proteolyzed Capsid with MAb

The non-digested capsid proteins were run in an 4-20 % gradient SDS-PAGE gel and probed with MAbs (Figure 23). The MAb reactive isolates, (OSU, PV-57, and PV-91), cleaved by CNBr produced similar reaction patterns for both PV-57 and PV-91 but the lane for the OSU isolate did not allow a comparison (Figure 24). Trypsin digested proteins, produced identical

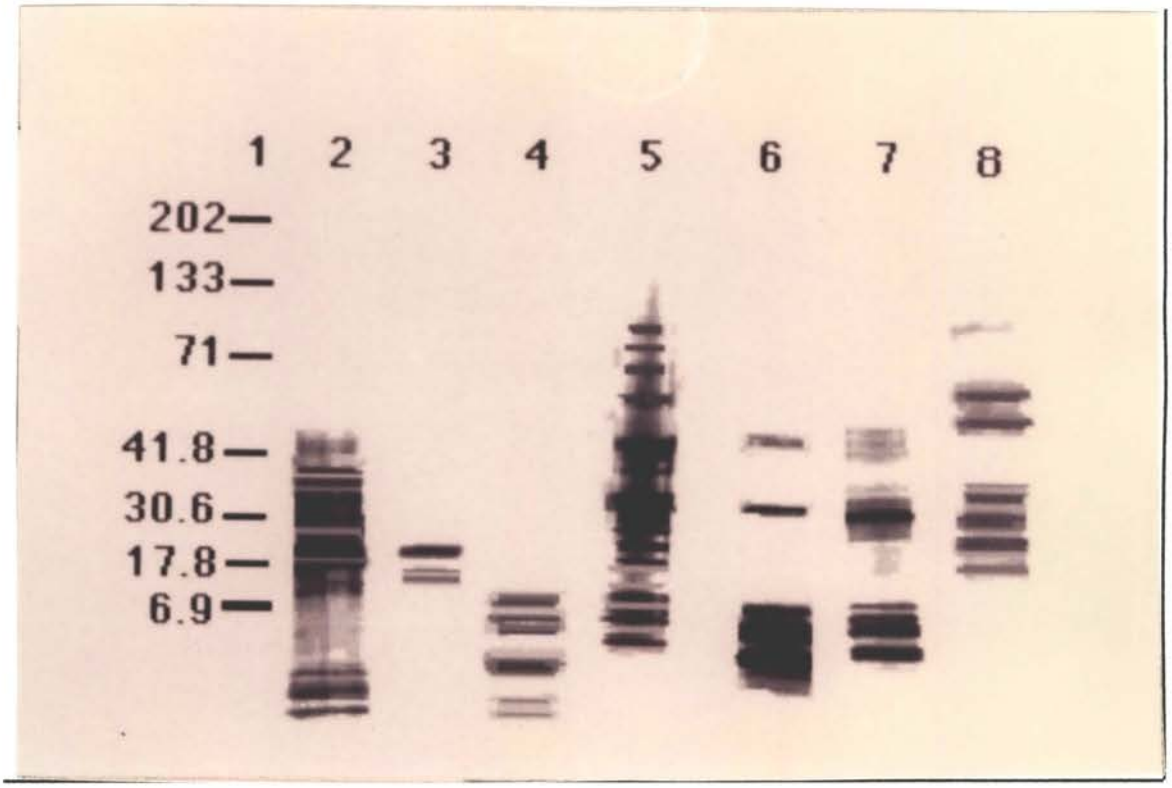

Figure 20. Migration pattern of chymotrysin digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with polyclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91, COLO, TAMU, PV-106, and OK964.

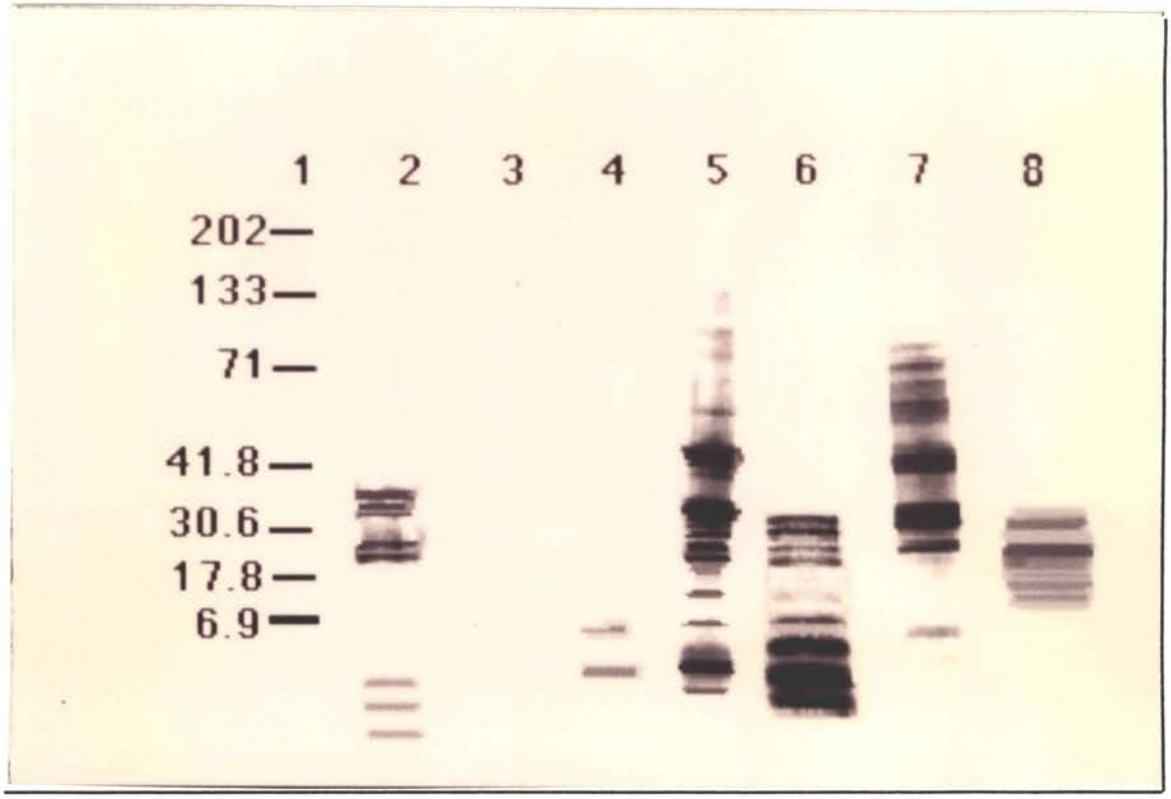

Figure 21. Migration pattern of papain digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with polyclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91, COLO, TAMU, PV-106, and OK964.

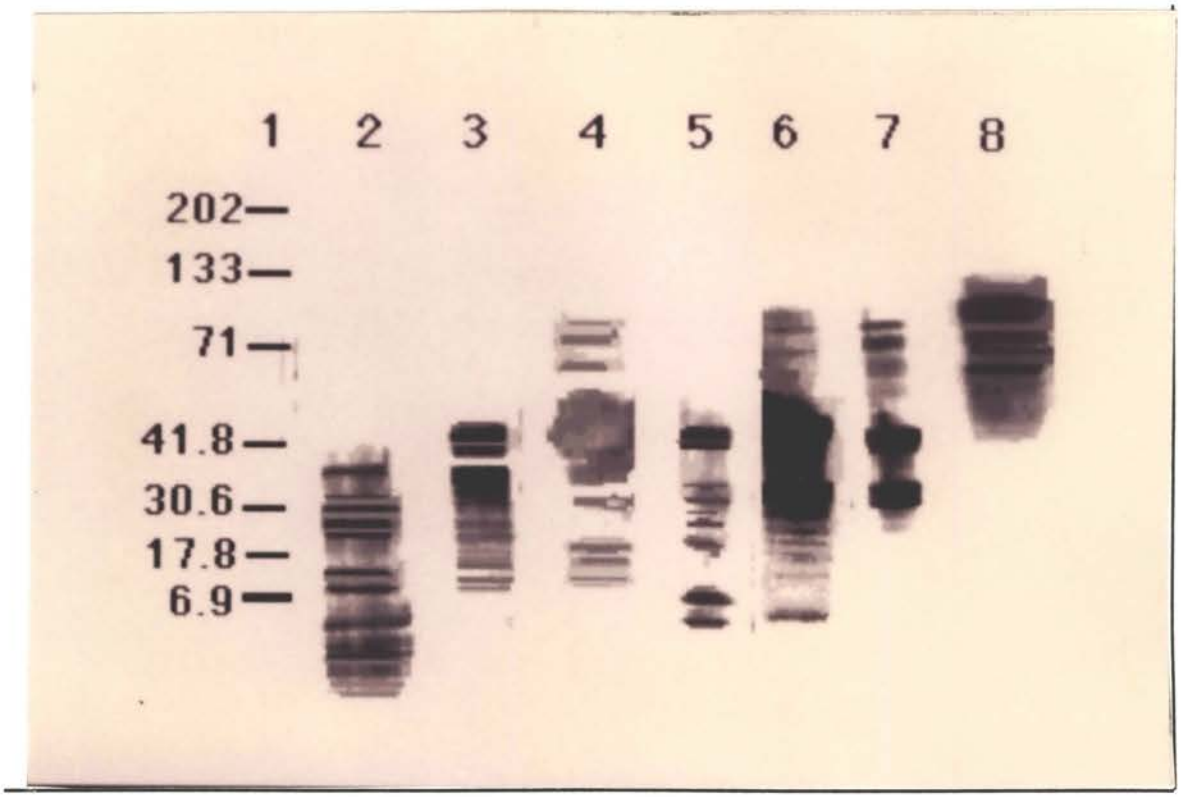

Figure 22. Migration pattern of protease V-8 digested capsid from seven WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with polyclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91, COLO, TAMU, PV-106, and OK964.

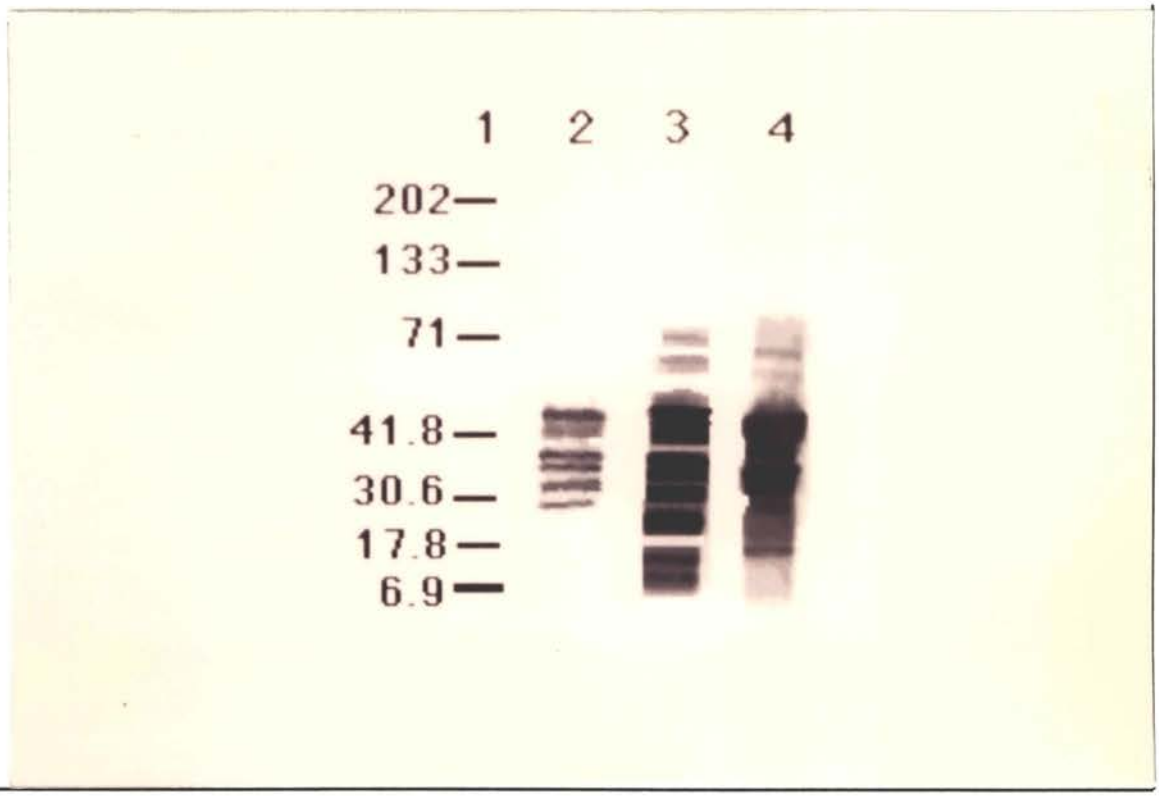

Figure 23. Migration pattern of non-digested capsid from three WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with monoclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91.

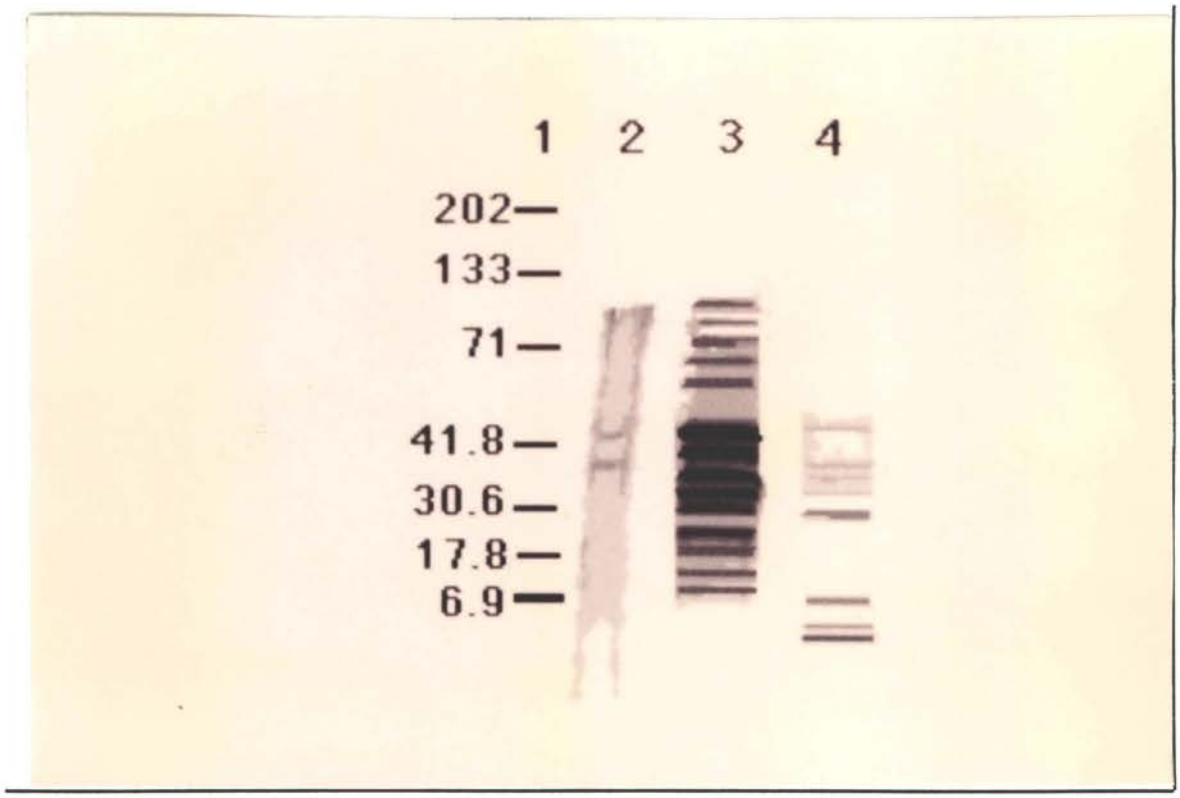

Figure 24. Migration pattern of CNBr digested capsid from three WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with monoclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91.

MAb reaction patterns as previously shown by Sherwood et al., (1990) and the chymotrypsin digested proteins produced similar reaction patterns for all three isolates (Figure 25). No reaction occurred with the papain digestion products for all three isolates (data not shown). The V-8 protease digested proteins of the three isolates again reacted similarly (Figure 26). No reaction occurred for the three isolates with N-chlorosuccinimide and no cleavage occurred with formic acid solution (data not shown).

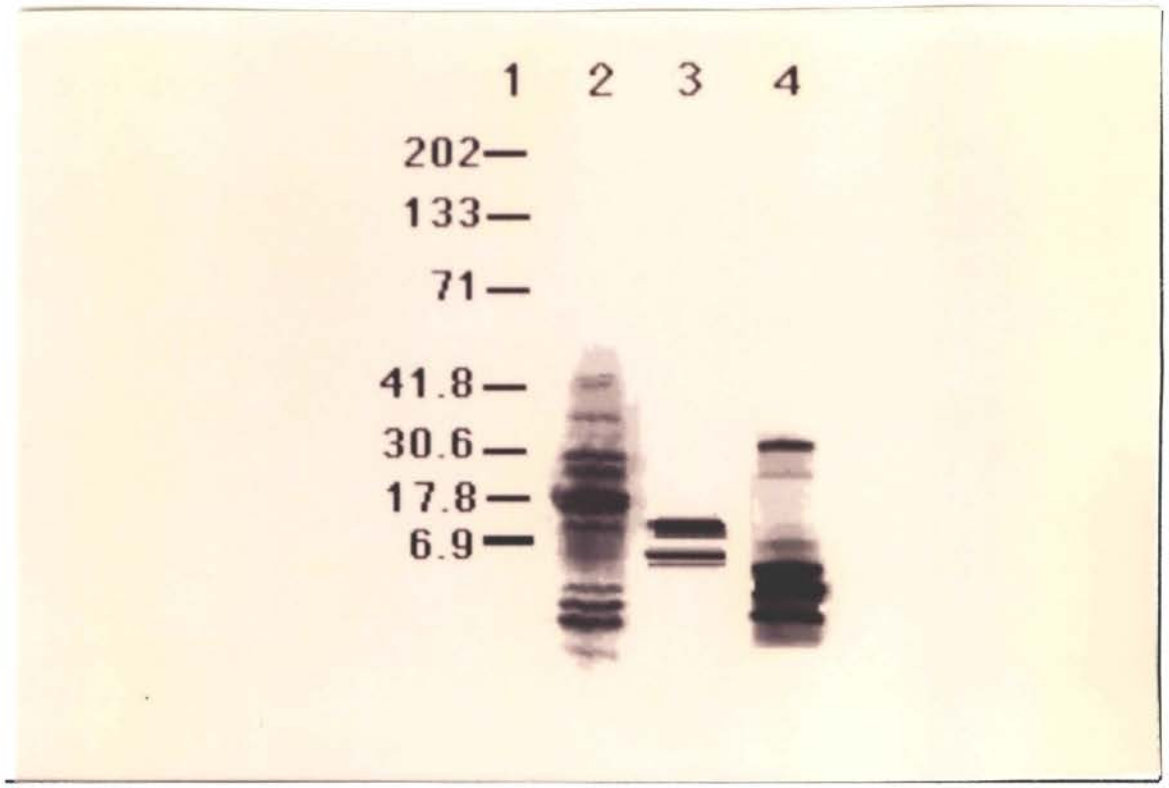

Figure 25. Migration pattern of chymotrypsin digested capsid from three WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with monoclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91.

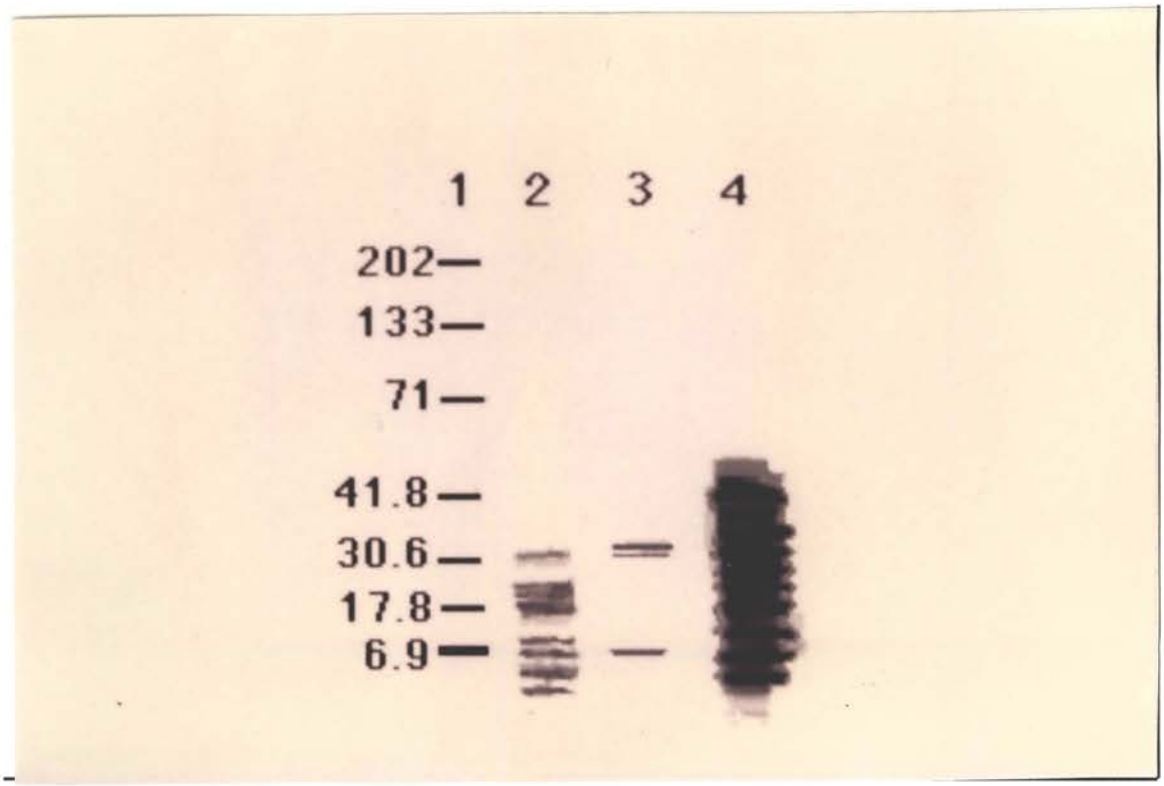

Figure 26. Migration pattern of protease V-8 digested capsid from three WSMV isolates in a 4-20 % precast gradient SDS-PAGE gel probed with monoclonal antibodies. Two μl of BioRad's Kaleidoscope standards in lane 1, followed by: OSU, PV-57, and PV-91.

Discussion

These results indicate the existence of a number of distinct isolates of WSMV that may be separated first by serological reactivity and then grouped based on additional tests. Three isolates (serotypes) reacted with the MAbs indicating distinctive epitopes. This permitted separation of these three isolates from the other isolates used in this study and from isolates since received. These differences have been further characterized by comparison of the reactivity results of the MAbs. The lack of reactivity of MAb 32C-1 under denaturing conditions suggests MAb 32C-1 was produced to a discontinuous antigenic determinant (neotopes) in the intact virion coat protein. Reaction of MAbs 32C-6 and 33A-1 to both native and denatured OSU, PV-57 and PV-91 indicates these MAbs react to continuous antigenic determinants (metatopes).

The lack of spur development in the Ouchterlony double diffusion PAb assay indicated that all isolates contain several common antigens. MAbs did not form a precipitation line at all, which is not uncommon for MAbs in this type of assay, due to a lack of lattice formation. The lack of, (or very weak), Ab/Ag precipitation for PV-91 was most likely not due to a lack of recognition, but due to a low titer in the

infected tissue used or a problem with the migration of the viral particles through the agar due to aggregation of the virions. In any case, obvious confluent precipitation was never achieved for PV-91. Production of precipitations for all other isolates indicates that serologically optimum proportions of the reactants occurred, that these isolates contain identical PAb antigenic determinants (epitopes) resulting in a continuous (confluent) precipitation line, and that there was total absorption of the diffusing Abs by these isolates.

The SSEM was conducted to try to determine the location of antigenic determinants and serotype relatedness of these isolates. In the SSEM micrographs the binding pattern for PAbs and Mabs was similar. By SSEM the binding pattern of PAbs and MAb with the intact and disrupted virions of various WSMV isolates was observed. This allowed the observation that neither the PAb serotypes or MAbs serotypes reacted at only one or the other end of the virion. The Protein-A/gold/Ab complex was seen to lightly bind along the length of the virus and would also bind to disrupted virions. Langenberg (1986) also produced micrographs of WSMV which displayed binding along the virion when using PAbs. The disrupted virions were believed to have been produced due to repeated freeze/thaw cycles. When the purified virions were subdivided into ten 25 μl aliquots with each aliquot to be used only once, disrupted

virions were no longer produced. The observance of Abs bound throughout the formvar grid initially was thought to have been nonspecific binding. But such an array of gold particles was never seen in the various control grids i.e., those lacking Abs, Protein-A\gold\Ab, or virus. Also had the MAbs reacted only to the extremities of the virion then the lack of precipitation in the ODD assay might have been readily explained.

Others have shown that a particular set of Abs would bind only at one end of a virus particle, at both ends, or all along the virion (Dore et al., 1988; Lesemann et al., 1990). Binding to disrupted virion subunits was also shown by Diaco et al., (1986); Dore et al., 1988; Hajimorad and Franki, 1991; and McCullough et al., 1985. Since all MAb clones reacted with the three MAb DAS-ELISA reactive serotypes, SSEM did not help to explain why MAb clone 32C-1 did not react under the denaturing western blot conditions. This would have been possible if I was able to recreate the structural modification that would have led to the loss of neotopes in the virion i.e., the epitope which 32C-1 recognizes. Perhaps comparing the reactivity in SSEM of viral particles taken after mixing in the SDS-PAGE loading buffer, and heating at 95 C for 5 min with non-denatured virions, might indicate the basis for the antigenic modification. Such structural changes have been reported (Dore et al., 1988; Joisson et al., 1992) to occur to

proteins at high pH values or when adsorbed to the solid phase of the microtiter plate used in ELISA e.g., DAP-ELISA technique. Such an occurrence was not observed in either SSEM or DAP-ELISA. MAb clone 32C-1 did react with the intact virion as well as any possibly altered virions of all three MAb DAS-ELISA reactive isolates in DAP-ELISA. The required quartenary antigenic determinant structure of the neotope specific MAb 32C-1 was probably not sufficiently altered by SSEM to prevent its recognition and subsequent MAb/neotope binding with OSU, PV-57, and PV-91.

In the MAb competitive assay 36 comparisons were possible (plate and rep data combined/isolate/reading); however, six of the nine intra-group comparisons were of prime importance. The significant inter-group, (and three intra-group), differences are actually a measure of MAb avidity rather than a determination of competition for, or identification of differing, epitopes. The use of the RCB experimental design inadvertently provided a check on the absorbance level which resulted from the addition of a particular secondary MAb. For example, if absorbance increased after the addition of MAb 32C-6 (A) to MAb 33A-1 (B) [BA], determined relative to the baseline absorbance reading [BB], then the converse should also occur i.e. addition of B to A relative to AA. It is reasonable to assume that if a virion contained, for example, X = 100 epitopes and MAb A recognized 60 of these and B

recognized the remainder, then whether A or B was the primary MAb should not matter. In either case the absorbance level should increase, although at different levels relative to their baseline i.e., 60 % increase over BB or 40 % increase over AA, allowing somewhat for steric hinderance. This would show that the two MAb clones truly recognize different epitopes.

The results of this competitive assay showed that the OSU isolate did not display significant intra-group differences regardless of ELISA method used. This suggests that the MAbs were reacting relatively equally to the antigenic determinant present on the capsid. Those differences observed for PV-57 were limited to the DAP-ELISA using purified virus and even then the significant differences were a reduction in absorbance. Friguet et al., (1983) concluded that a viable conclusion for a reduction in absorbance (a negative additivity test) would be that the MAbs were recognizing the same region of the molecule . Only in one case for PV-57, did absorbance significantly, at less than the 5 % probability level, increase. This was the addition of MAb 33A-1 (B) after MAb 32C-1 (C) or stated another way, the BC combination compared to the CC baseline.

These results suggest that all three MAbs are responding in a similar manner to the antigenic determinants of OSU and PV-57. Thus the results suggest again that these two isolates

belong in the same serogroup.

For isolate PV-91, both the DAS-ELISA (using infected sap) and the DAP-ELISA (using purified virus) showed significant intra-group differences. In the DAS-ELISA MAb 33A-1 (B) increased absorbance above the MAb 32C-1 (CC) baseline. The DAP-ELISA using purified virions, showed that in comparison to the MAb 33A-1 (BB) baseline both MAb 32C-6 (A) and MAb 32C-1 (C) significantly increased absorbance. Also both MAb 33A-1 (B) and MAb 32C-1 (C) increased absorbance above that of the MAb 32C-6 (AA) baseline. These trends were relatively consistent across the four absorbance readings taken. Therefore, whether MAb 32C-6 (A) or MAb 33A-1 (B) was applied first, absorbance increased when either of the other two MAbs were added I.e., AB or AC compared to AA or BA or BC compared to BB. Thus, these two MAbs are recognizing distinct epitopes. The other combinations only worked in one direction i.e., only when MAb 32C-1 (C) was added after either MAb 32C-6 (A) or MAb 33A-1 (B) did absorbance increase, the converse MAb 32C-6 (A) or MAb 33A-1 (B) added after MAb 32C-1 (C) reduced absorbance level and therefore the reciprocal test did not also increase absorbance. This suggests that WSMV isolate PV-91 has a different arrangement of antigenic determinants than the other two MAb reactive serotypes PV-57 and OSU and can be separated from either of them on this basis into a separate serogroup.

The use of agents (especially the proteases) to cleave the coat protein (CP) of the WSMV isolates for the protein fingerprinting procedure, allowed the placement of the isolates into distinctive groups. The Fast Stain® procedure showed that WSMV isolates differed in their banding patterns and could be placed into groups. Although trypsin did not produce any difference in banding pattern among the isolates, it did reaffirm the data of others (Brakke et al., 1990; Sherwood et al., 1990). Only the proteolytic agents were responsible for producing different banding patterns between the isolates using both the protein staining and PAb western blots for overall comparison. The MAbs did not reveal differences between OSU, PV-57, and PV-91. But while their banding patterns were similar, inclusion of the above MAb competitive assay and known differences in virulence, does separate PV-91 from OSU and PV-57. These isolates of WSMV appear to consist of five distinct subgroups. In subgroup-I are OSU and PV-57, PV-91 in subgroup-II, COLO in subgroup-III, TAMU and OK964 (and probably OK994) in subgroup-IV, and PV-106 in subgroup-V. Remembering that these are within the larger grouping i.e., serogroups WSMV-I and WSMV-II and therefore subgroups I and II would be in serogroup WSMV-II. All the other subgroups would be members of serogroup WSMV-I.

The multiple bands seen below the typical CP 31-46 kDa range for the purified viruses not given a cleavage agent,

were probably due to either enzymatic degradation by the host plant, proteases present during the purification procedure, or multiple freeze/thaw cycles, as seen in the SSEM procedure. Others (Brakke et al., 1990; Seifers, 1992; Sherwood et al., 1990) have also observed such capsid degradation and have reported PAb reaction with these degradation products. The protein bands observed at ≈ 66 kDa after applying the Fast Stain® may be, as suggested by Seifers (1992), either inclusion proteins or aggregation of intact and partially degraded protein. Brakke et al., (1987, 1987b) has stated that purified proteins may migrate as dimers and trimers and that these 66 kDa proteins seen in SDS-PAGE were inclusion bodies exclusively if they did not react with PAbs made to the capsid protein. But we observed that these 66 kDa proteins were reacting with both PAbs and, for the OSU, PV-57, and PV-91 isolates, with MAbs as well. Therefore they were probably due to protein aggregation and thus poor gel migration resulting in bands of higher Mr than for the typical capsid bands.

The attempt to protein fingerprint using seven digestion agents, two controls, and seven WSMV isolates provided positive and negative aspects to this study. The major negative aspect was the large number of combinations and determining the optimum results in order to compare isolates. Conversely, the use of only one or two digestion agents such

as trypsin and formic acid would have led to a spurious conclusion that no differences existed between the isolates. Thus once the determination that serotypes of WSMV existed, the incorporation of so many experimental combinations was deemed necessary for a preliminary characterization. From this work chymotrypsin, papain, and protease V-8 were identified as being useful cleavage agents to distinguish between the isolates. CNBr and N-chlorosuccinimide occasionally revealed differences between isolates and with protocol modification, may also provide useful fingerprint information. Therefore, future studies should focus their attention to using the agents identified herein that produce banding patterns useful in characterization and separation of WSMV isolates.

The purpose of a partial cleavage procedure for protein fingerprinting is primarily comparative in order to determine whether two or more proteins are identical or unique. However, if a protein has so many of the cleavage residues, then an overwhelming number of fragments is generated. This is a common problem to all methods of partial digestion (Lischwe and Ochs, 1982). By identification of agents useful for WSMV protein fingerprinting, research can now be intensified with these in slab gels, capillary electrophoresis, or HPLC.

The most probable cause of the migration problems

observed with the chemical cleavage agents CNBr, N-chlorosuccinimide, and formic acid was the extremely low solution pH (pH < 1). An attempt to mitigate this problem was made by the addition of acetone (1:1), centrifugation to precipitate the protein, and subsequent discarding of supernatant. However, this was not completely successful. This technique could be modified further, or conversely for these three chemical reagents, capsid digestion should be performed with intact gel slices (intragel digestion). This would allow multiple washing of the gel slices to significantly reduce the residual CNBr and HCL, formic acid, and N-chlorosuccinimide in solution. Transferral of the gel slices to a solution of 0.125 M Tris/Hcl (pH 6.8), 0.1% SDS, and 10% glycerol containing 0.001% bromophenol blue, would then indicate whether residual acidity remained and the gel slices could be rewashed as required.

Concluding Remarks

The results obtained indicate the existence of serotypes of WSMV. The reactivity of three of the isolates (OSU, PV-57, and PV-91) to the MAbs show that distinct epitopes are present on the CP. The significant differences in competitive inhibition to PV-91 by the three MAbs further demonstrate distinct CP epitopes. While all three MAb clones reacted with PV-91, absorbance increased upon addition of either of the other clones. The isolates used in this study fell into two

serogroups, i.e., WSMV-I, those isolates reacting to only PAbs and WSMV-II, those reacting to PAbs and MAbs.

The N and C termini of the CP of potyviruses are located on the protein surface (Shukla et al., 1987, 1988, 1988b 1989c; Allison et al., 1985). The N terminus is the most immunodominant region in potyvirus particles, and the N terminus constitutes the only large area of the entire potyvirus CP which is variable and virus and strain specific. Epitopes located in the N and C termini of the viral coat protein, are able to differentiate one virus from another (interviral) or even strains of a virus (intraviral) (Jordan and Hammond, 1991,1992; Land and Skopp, 1983; Niblett et al., 1991). Therefore, epitopes located in this area should elicit virus specific antibodies unlike antibodies produced to the core protein [the trypsin resistant core, (TRC)] region which has considerable sequence identity between viruses. Crossreacting Abs are usually produced to the TRC.

Most if not all ELISA procedures are based on the surface properties of the viral protein conformation. This implies the protein or proteins that constitute the viral coat of either the intact (fixed or native) virus or the protein subunits of a disrupted virus. Both the PAbs and MAbs were produced to purified, intact virions of the WSMV OSU isolate by Sherwood et al., (1990) implying the immunodominant and virus specific N and C termini of the coat protein subunits.

Such intact virions would be 45-47 kDa in size made up of the 31-33 kDa doublet proteins, thought to be the basic structural unit (core protein) of the viral helix (Brakke et al., 1990), plus ≈ 14 kDa more protein. Brakke et al., (1990) stated that this extra 14 kDa of protein may serve a biological function as have others (Sako and Ogata, 1981).

Serological testing, with MAbs produced to the N and C termini was a beneficial procedure to determine if WSMV isolates were related or not. The MAbs produced to epitopes located in these N and C termini of the viral coat protein, were able to differentiate WSMV serotypes. This is done to obtain several samples which in turn may harbor various viral serotypes. But with WSMV this was not the case since MAb reactive isolates have not been observed for several years.

As mentioned earlier, both PAbs and MAbs were produced to the OSU isolate which in turn was believed to have been obtained originally from McKinney who collected the type strain of WSMV currently called PV-57 (ATCC). Results presented in this paper suggest that the OSU isolate and PV-57 are very similar, if not the same viral strain. Furthermore, both PV-57 and OSU have been observed to have similar virulence in field tests (data not shown). The other isolate collected by McKinney (1956) and currently labelled PV-91 (ATCC) also reacted to the MAbs. However PV-91 is distinguished by being mild in the degree of symptom

expression produced by the host and thus can be placed in a separate subgroup (subgroup-II). PV-57 and PV-91 are the original isolates of WSMV and have been used in many scientific studies since first collected. Also, Brakke (1971) states that the mite vector was used to transfer PV-57 from infected to healthy wheat prior to donation to ATCC. Therefore, apparently continual transfer by mechanical inoculation for scientific investigations did not affect the ability of the vector to ingest and transfer the virus prior to donation, and there must be another basis for the relative reduction in occurrence of the MAb serotypes in nature.

Records from the Oklahoma State University Diagnostic Lab reveal that MAb based ELISA was once a useful diagnostic procedure for WSMV. It was only relatively recently that diagnostic evaluation (by ELISA) of wheat samples believed to have WSM by visual assessment no longer reacted positively with MAbs. Isolates obtained from two surrounding states, (Texas and Colorado), and from the panhandle of Oklahoma reacted to PAbs but would not react to MAbs. These include not only the isolates used in this paper but others subsequently collected. Geographically distant WSM samples (Oregon) also gave a positive reaction to WSMV with PAbs but not MAbs (data not shown). Recently a MAb positive isolate of WSMV was collected (Dr. Dallas Seifers - personal communication) near the Oklahoma border in south central

Kansas. This suggests that the lack of MAb reactivity to naturally occurring WSMV isolates cannot be attributed simply to the maintenance of the OSU isolate by mechanical inoculation. Viruses constantly transferred by mechanical inoculation instead of using the vector might allow the production of Abs to a mechanically altered form of the virus.

It is imperative that both PAbs and MAbs be produced against a representative member of the MAb non-reactive (ELISA) isolates, to test the MAb (homologous) and PAb (heterologous) antisera, and further study the relationships of these distinct serogroups WSMV-I and WSMV-II. Such antisera would prove that each reactive antisera only reacts with only the unique antigenic determinant of each of the serogroups. Such MAbs should be of high titer to demonstrate a serological relationship or a lack thereof (Matthews, 1991; Jordan and Hammond, 1991).

The discovery that WSMV serotypes exist has practical value i.e., changes in antibody recognition suggests changes in antigenic character which in turn have been attributed to altering vector transmissibility, host range, and virulence levels (Barnett, 1992; Dijkstra, 1992; Hammond, 1992; Matthews, 1991; Shukla et al., 1989). I have found preliminary evidence from field studies that such differences in virulence level occur. Hammond (1992) indicated that characters, such as serological differences, that relate to

biological differences should be given more weight than those unrelated to biology for taxonomic purposes. Thus, the existence of WSMV serotypes by itself is important but in combination with observed differences in virulence level and in their reaction pattern when tested against a variety of wheat cultivars is more important. Often the report of serotypes for various potyviruses is made. But by itself, this is not surprising because of the inherent infidelity of RNA replication leading to diversity without correlation to biological activity. Within the *Potyviridae*, the N-terminal region for the CP gene may be subject to slippage during replicase, or template jumping between positive and negative strands may lead to the evolution of potyviruses (here WSMV) with new CP properties (Hammond, 1992).

The use of N-terminal directed Abs for classification seems to be unjustified because a virus really is more than mere CP (Atreya et al., 1990; Bos, 1992). Rather, classification should be based on a combination of characters. Again, the serological differences of the isolates reported here are also being noted in differences in their reaction to cultivars of HRWW.

The delineation of WSMV serotypes will be a particularly important part of programs attempting to produce resistant wheat cultivars, study WSMV epidemiology, and etiology. The determination of what selection pressure has made the MAb

reactive serotypes much less common in the natural ecosystem, would be an interesting area of research to pursue. Since serotypes and serogroups of WSMV exist this will in turn directly affect wheat growers because identification of serotypes would provide a more accurate assessment of WSM in their fields. Also, knowledge of WSMV serotypes can impact the determination of which serotype exists in particular areas aiding the study of the epidemiology of WSM.

The use of specific Abs is the first step in the determination of a serotype followed by the biochemical characterization of the serotype to elucidate how the original (termed type strain) and the newly discovered serotype differ. By eventually comparing the results of these tests to biological characterizations e.g., virulence on cultivars of wheat, and comparison of serotype/vector transmissibility, placement into differential groups based both on serology and biological characters, will be possible. The early classification of plant viruses failed because they were built around such superficial characteristics as symptomatology and host range (Makkouk and Singh, 1992). Viruses show high rates of mutation and if the mutant is selected by edaphic conditions it may develop into a new variant or strain and possibly into a new virus (Bos, 1992). It has been said that distinction between viruses should be based on the nucleotide sequences of the genome, whereas distinction between strains

should also take biological characteristics into account (Dijkstra, 1992). By the inclusion of as many characteristics as possible certain WSMV isolates may, as happened for Slykhuis and Bell (1966), be found to be sufficiently unique to be considered a new virus.

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APPENDIXES

APPENDIX A

FIRST REPORTS OF AGROPYRON MOSAIC VIRUS IN WHEAT AND MIXED INFECTION WITH WHEAT STREAK MOSAIC VIRUS IN OKLAHOMA.

Montana, J.R., Jacobs, J.L., Hunger, R.M., and Sherwood, J.L. 1994. Plant Dis. 78:432.

In June 1993, a mixed infection of agropyron mosaic virus (AgMV) and wheat streak mosaic virus (WSMV) was identified by DAP-ELISA (AgMV polyclonal antisera provided by Dr. D. Seifers, Kansas State University, Fort Hays Experiment Station, Hays, Kansas) in hard red winter wheat (*Triticum aestivum* L.) in Harper county in northwestern Oklahoma. The estimated yield loss in marketable grain was 80-85% in this field. Symptoms similar to those seen in the field were observed when both viruses were mechanically inoculated to wheat. The viruses were transmitted from infected to healthy wheat by mechanical inoculation. Disease symptoms of infection developed approximately two weeks after inoculation. The viral isolates were separated by mechanical inoculation to *Avena sativa* L. as the exclusive host for WSMV, and *Elytrigia repens* Rydb., exclusive host for AgMV. Isolate separation was verified by DAP-ELISA. In July 1993, possible alternative gramineous hosts were collected at random from the periphery

of the field. Four of 14 grass samples (*Elymus smithii* Rydb., *Bothriochloa laguroides* DC., *Aristida sp.* L., and *Sorghastrum nutans* L.) tested positive for AgMV and *Setaria viridus* L. tested positive for WSMV using DAP-ELISA. This is the first report of AgMV and the first report of a mixed infection by AgMV and WSMV of wheat in Oklahoma.

APPENDIX B

EVALUATION OF WHEAT CULTIVARS AND GERMPLASM TO DETERMINE POSSIBLE SOURCES OF TOLERANCE OR RESISTANCE TO WHEAT STREAK MOSAIC

Fifty-four germplasm lines (Crop Sci., 1993, 33:1120; Phytopathology, 1992, 82:1094), five parents (OK83398, Chisholm, Aurora, Tam 101 and Arkan), plus the checks Rall and Triumph-64 were tested for possible tolerance and/or resistance to WSM under glasshouse conditions during the late winter and early spring of the 1991/1992. Ten seeds of each entry were planted in clumps with five reps in two separate tests (total 10 reps) in non-pasteurized soil in flats. Seeds were treated with Arason Red fungicide to reduce infection from soil-inhabiting pathogenic fungi. When seedlings were eight to ten inches tall (20.32 - 25.4 cm) foliage was clipped using a cordless Black and Decker 'Grass Shear' to stimulate juvenile growth. Seven to ten days after clipping, seedlings were inoculated with the OSU serotype of WSMV using a Devilbiss air gun (as used in the field inoculations) at a low pressure setting [276 KPa (40 psi)], because the plants were more tender than field grown plants. Visual evaluation of symptoms and virus detection using the double antibody

sandwich enzyme-linked immunosorbent assay (DAS-ELISA) were conducted to identify germplasm lines or parents resistant to WSM; however, no resistance was observed. Also, the cultivar Rall, which has displayed field tolerance and/or resistance, did not do so in this study nor has any tolerance since been observed under glasshouse or laboratory conditions. The field resistance is real however and may be related to environmental factors (especially temperature) or another parameter which as of yet has not been identified.

APPENDIX C

PRELIMINARY STUDIES ON THE INTERACTION BETWEEN WHEAT STREAK MOSAIC VIRUS SEROTYPES AND CULTIVARS OF HARD RED WINTER WHEAT UNDER FIELD CONDITIONS

Wheat is affected by a serious viral disease called wheat streak mosaic (WSM) which can significantly reduce total yield, grain weight, and grain quality. Sources of resistance to wheat streak mosaic virus (WSMV) in agronomically acceptable cultivars of hard red winter wheat (HRWW) currently are not available. One cultivar that has shown some promise for tolerance and/or resistance under field conditions to WSMV is Rall. However, while this tolerance or resistance shown against the OSU serotype of WSMV is substantial (Hunger, et al., 1992; Montana unpublished), it is expressed only under field conditions. This cultivar also has severe undesirable agronomic traits such as late maturity and excessive height which can result in lodging of the wheat. Furthermore, as mentioned above, Rall has only been challenged and expressed resistance to the OSU serotype of WSMV. No data were available about the level of resistance in Rall to any other WSMV serotype until this study was conducted in the fall of 1993 and spring of 1994.

The OSU serotype belongs to the proposed WSMV-II serogroup, which is comprised of two other members i.e., PV-57 and PV-91 obtained from the American Type Culture Collection (ATCC, Rockville, MD). These WSMV-II members are identified by their reactivity with both polyclonal and monoclonal antibodies (PAbs and MAbs respectively). Further, Mckinney (1932, 1937, 1949) reported that PV-57 was the virulent and type strain while PV-91 was the mild strain of WSMV. This has since been verified by Carroll et al., (1982) and Montana (unpublished). Other serotypes of WSMV recently have been identified (Montana et.al., 1993) and belong to the WSMV-I serogroup. These isolates react only to the PAbs. Within this serogroup a range of virulence also has been identified, with PV-106 being highly virulent, TAMU, OK964, and OK994 displaying moderate virulence, and COLO displaying relatively mild virulence. Therefore, it was the objective of this preliminary field study to compare the reaction of two HRWW cultivars (Chisholm and Rall) to four serotypes of WSMV (OSU, PV-57, PV-106, and COLO) when inoculated in the fall. A comparison to a non-inoculated control also was included.

The experimental design was a combined randomized complete block / Latin square, designed by Dr. David Weeks of the Department of Statistics (Oklahoma State University) which would place at a premium the statistical effects of the cultivar/serotype parameter. There were 10 blocks per

location with four subplots within each block. Each randomized block consisted of one of the serotypes with two of the randomized subplots planted with Rall and the other two with Chisholm for a total of four reps of the serotype/cultivar combination per location. This resulted in a $a^2b^5c^4$ factorial arrangement.

Field plots were located at the Plant Pathology farm and consisted of two separate locations and soil types termed the "terrace" and the "lower" plots. Data were to be analyzed separately by location and then combined only if no significant effects were observed due to location. A soil fertilization test, (for a 50 bushels per acre yield goal) was conducted on 24 Aug 93. The results showed that for the terrace location, pH was satisfactory (5.6), nitrogen (actual N) was required at 73 lbs, and phosphorus (P_2O_5) at 16 lbs per acre. Potassium was 1.66 times the adequate amount. The results for the lower location showed that pH was again satisfactory (5.9), nitrogen required 54 lbs/acre, while phosphorus and potassium were 1.52 and 2.24 times the adequate amount.

Seeds were planted on 22 Sep 93 at 96 seeds (5 g)/four feet of row. The rows were four feet long with three foot borders between subplots and between blocks. Vona was planted between subplots and blocks as an indicator host for wheat soilborne mosaic virus. Seedings were inoculated on 20 Oct 93

(terrace location) and 22 Oct 93 (lower location). Data collected were a visual assessment of WSM symptom expression, thousand kernel weight (TKW), and total yield. The visual assessment scale was: 0 = no symptoms; 1 = no stunting, leaves light green with a few yellow streaks; 2 = plants slightly stunted, leaves with moderate green and yellow streaks; 3 = plants moderately stunted, leaves with yellow streaks predominating; 4 = plants stunted, leaves with severe yellow and little or no green areas remaining; 5 = plant death imminent or has occurred.

Glean® was applied at 0.333 oz/acre in 20 gallons of water on 26 Oct 93, and Tilt® applied on 08, 22 April, and 6 May 94. The cultivars were visually assessed and tissue sampled (for ELISA) on 24 Mar, 1 Apr, and 20 Apr 94. The tissue samples were stored at −20 C after each sampling until ELISA was conducted. The lower plot was not harvested due to rank growth which led to severe lodging. The terrace plots were not similarly affected because of its sandy loam soil which allowed percolation through the soil profile and also a lower cation exchange capacity than the lower clayey soil type.

Symptom expression was different between serotype/cultivar combinations (Table XI). Both DAP-ELISA and DAS-ELISA also display differences between the various treatment combinations (Table XII and XIII) as did the

Table XI. Wheat streak mosaic symptom rating for the WSMV serotype and HRWW cultivar 1993/1994 field study.

	DATE RATED					
Location:	March 24, 1994		April 8, 1994		April 20, 1994	
"Terrace"	Cultivar		Cultivar		Cultivar	
Serotype	Rall	Chisholm	Rall	Chisholm	Rall	Chisholm
OSU	1.50[1]	2.88	2.00	2.63	2.50	3.50
PV-106	1.88	3.88	1.38	3.56	1.56	3.81
PV-57	1.88	3.65	1.00	3.75	1.25	4.19
COLO	2.00	2.25	1.63	2.56	2.56	3.56
Control	0.63	0.00	0.81	0.00	0.56	0.00
Location:						
"Lower"						
Serotype						
OSU	0.50	2.25	1.69	3.06	1.25	2.63
PV-106	2.00	2.75	2.63	3.63	1.63	3.44
PV-57	1.13	3.13	1.75	3.75	0.69	3.69
COLO	1.00	2.50	2.25	3.31	1.94	2.38
Control	0.00	0.00	0.13	0.31	0.00	0.00

[1]The rating level is the mean of four values averaged over the entire location for each cultivar and wheat streak mosaic virus serotype combination.

Table XII. Wheat streak mosaic virus polyclonal antibody DAP-ELISA values for the WSMV serotype and HRWW cultivar 1993/1994 field study.

	DATE SAMPLED					
Location:	March 24, 1994		April 8, 1994		April 20, 1994	
"Terrace"	Cultivar		Cultivar		Cultivar	
Serotype	Rall	Chisholm	Rall	Chisholm	Rall	Chisholm
OSU	0.257	0.647	0.225	0.907	0.581	1.154
PV-106	0.620	0.911	0.103	0.565	0.243	1.160
PV-57	0.200	0.935	0.077	0.884	0.362	1.354
COLO	0.270	1.023	0.184	0.702	0.933	1.247
Control	-0.005	0.019	0.017	0.041	0.004	0.016
Location:						
"Lower"						
Serotype						
OSU	0.368	0.947	0.329	0.931	0.612	0.912
PV-106	0.497	0.898	0.249	0.874	0.210	0.945
PV-57	0.034	0.946	0.082	0.692	0.237	1.138
COLO	0.521	0.972	0.210	0.583	0.787	0.650
Control	-0.003	-0.002	-0.002	0.036	0.002	-0.001

[1]Each value is the mean absorbance (i.e., optical density) at 405 nm of four replications with three readings/replication. Values ≥ 0.100 are considered positive and values < 0.100 are considered negative.

Table XIII. Wheat streak mosaic virus monoclonal antibody DAS-ELISA values for the WSMV serotype and HRWW cultivar 1993/1994 field study.

	DATE SAMPLED					
Location:	March 24, 1994		April 8, 1994		April 20, 1994	
"Terrace"	Cultivar		Cultivar		Cultivar	
Serotype	Rall	Chisholm	Rall	Chisholm	Rall	Chisholm
OSU	0.195	0.363	0.564	1.245	0.757	1.713
PV-106	-0.001	0.005	0.015	0.007	0.057	0.102
PV-57	0.132	0.835	0.237	1.291	0.663	1.452
COLO	0.005	0.023	0.019	0.011	0.128	0.105
Control	-0.003	0.002	0.013	0.007	0.049	0.097
Location:						
"Lower"						
Serotype						
OSU	0.204	0.890	0.686	1.247	0.635	0.040
PV-106	0.013	0.025	0.040	0.009	0.023	0.040
PV-57	0.008	0.872	0.031	1.119	0.204	0.090
COLO	0.051	0.023	0.000	0.009	0.098	0.033
Control	0.004	0.022	0.005	0.003	0.013	0.040

[1] Each value is the mean absorbance (i.e., optical density) at 405 nm of four replications with three readings/replication. Values ≥ 0.100 are considered positive and values < 0.100 are considered negative.

parameters total yield and thousand kernel weight (TKW) (Table XIV).

The interaction between HRWW cultivars and WSMV serotypes displayed in this study emphasize the importance of continuing these investigations. The importance of the existence of WSMV serotypes and a range of virulence among these serotypes has implications for plant breeders trying to locate sources of resistance to WSM. Wheat growers also may be affected by these findings. If a grower could be told with certainty that his particular field was infected with the COLO serotype, (a relatively mild strain), then concern with the degree of yield loss due to WSM might be lessened. Conversely, if a grower has the highly virulent strain, PV-106, then losses could be expected to be severe.

Table XIV. Reaction of two hard red winter wheat cultivars to wheat streak mosaic virus isolates under field conditions in 1993/1994.

	PRODUCTION			
Location:	Total Yield (g)		Thousand Kernel Weight (g)	
"Terrace"	Cultivar		Cultivar	
Serotype	Rall	Chisholm	Rall	Chisholm
OSU	43.0	25.0	23.4	22.8
PV-106	66.0	20.0	24.7	23.6
PV-57	109.0	17.0	25.1	22.0
COLO	50.0	20.0	24.4	25.0
Control	121.0	148.0	25.0	27.0

[1] The values for Total Yield and Thousand Kernel Weight are the means of four replications, from only the "Terrace" location. The "Lower" location was not harvested

APPENDIX D

POSSIBILITY OF CROSS PROTECTION INDUCED BY MILD SEROTYPES OF WHEAT STREAK MOSAIC VIRUS (WSMV) WHEN SUBSEQUENTLY CHALLENGED BY A VIRULENT WSMV SEROTYPE

Since the viral disease of wheat, termed wheat streak mosaic (WSM), was identified long ago by McKinney (1932 - 1949) much information concerning the virus, its epidemiology, coat protein (CP), and molecular structure has accumulated (Brakke and Ball, 1968, Brakke, 1971; Hunger et al., 1992; Lommel et al., 1982; Montana et al., 1993 and 1994, Montana, 1995; Seifers, 1992; Shulka et al., 1984, Shulka, 1988; Sherwood, 1885, Sherwood et al., 1987 and 1990). The agent responsible for this disease is termed wheat streak mosaic virus (WSMV). Various isolates have been collected and/or field observations have been reported which state that WSMV consists of isolates that differ in virulence. The initial report by McKinney (1950) that both a mild and a virulent (type strain) variant of WSMV existed, was later confirmed by Carroll et al., (1982) and later still the first identification of both serotypes of WSMV (Montana et al., 1993) and a group of isolates that spans the gap between the

two extremes in virulence was reported (Montana, Ph.D. dissertation, 1995). Although these data and observations have been reported, studies have not been conducted to identify the effects of challenging hard red winter wheat (HRWW) with a mild strain of WSMV and then subsequently (within a month or less) providing a secondary challenge by a virulent strain onto the same plant. Purposely infecting plants with a mild strain may act as a protective measure against severe disease by virulent strains which subsequently enter the plant. So far two mild strains have been identified i.e. PV-91 (McKinney, 1937) and COLO (Montana, 1995); two virulent strains, PV-57 (McKinney, 1937) and OSU; and a hyper-virulent strain PV-106 (Montana, 1995). As mentioned above, between these mild and virulent strains a number of moderate strains have also been identified and these are TAMU, OK994, OK964, and Sidney 81 (Montana, unpublished).

Such a challenge on a plant by first a mild and then a virulent strain is termed cross-protection and is defined as the infection of a plant with a strain of virus causing only mild disease symptoms which may protect it from infection by severe strains (Matthews, 1991). For example, if severe reduction in quality and yields can be associated with late infection then growers who regularly suffer such losses should pre-inoculate their plants at an early stage with a mild strain of the virus. While such a technique may be expedient

under very difficult control situations, there are problems associated with this idea and as a general practice it is not recommended. Such pre-inoculations with the mild strain may still reduce yield by 5-10% or more, the infected crop may act as an inoculum reservoir, the mild strain may mutate to a more virulent form, additive or synergistic effects may result from mixed infections, and pre-inoculation with a mild strain is labor intensive. However, it is still an important scientific study because of the information that could be gleaned from the molecular interactions between the two viruses and cytological changes due in the host plant to primary and secondary infection by the two viruses.

Studies should be conducted here at Oklahoma State University with a minimum of two HRWW cultivars and with a minimum of the two required WSMV strains. Since two cultivars in particular, i.e., Chisholm and Triumph 64, have been used for several years in WSMV glasshouse and field tests, they would be well suited for such a cross-protection study. Either the PV-91 (a mild) or COLO (a very mild) strains could be used as initial inoculum and the OSU or PV-57 strains as the virulent secondary inoculum. If this proposed study is conducted as hoped, it should be noted that PV-106, a hyper-virulent strain of WSMV, might not allow the cross-protection effects (if any) to be observed. This is because it may be able to break any protection that the mild strain could

provide. Also, the use of Rall, another HRWW cultivar, should be avoided in the preliminary tests because it too may mask the cross-protective abilities of the mild WSMV types since it has shown a relatively high tolerance to most of the WSMV serotypes tested so far (Montana, unpublished).

APPENDIX E

CORN CULTIVAR AND PURELINES MECHANICALLY INOCULATED WITH SEROTYPES OF WHEAT STREAK MOSAIC VIRUS - A GLASSHOUSE STUDY

Wheat streak mosaic virus (WSMV) is a potyvirus which causes serious yield and quality loss due to the disease, wheat streak mosaic (WSM). Studies concerning the epidemiology of WSM have continued for the last 50 - 60 years. These studies have shown that WSMV is vectored by a eriophyid mite and many monocotyledonous hosts have been identified. These host determinations were important in the epidemiology of WSM because they could provide a host-continuum (commonly called a "green-bridge") between the spring harvested wheat and wheat planted in the fall.

Some researchers have reported that corn is a host of WSMV; however, others have reported corn to be a poor alternative host in the epidemiology of wheat streak mosaic (WSM) under natural field conditions.

Recently, it has been determined that WSMV isolates can be placed into serotypes and serogroups. These serotypes and serogroups (which differ solely by serological assay) have also been characterized further by coat protein fingerprinting

and inclusion of virulence differences on cultivars of hard red winter wheat (HRWW) and are on the verge of being separated into distinct subgroups or strains.

One such WSMV isolate collected in Ohio from corn by L.E. Williams is listed by the American Type Culture Collection (ATCC, Rockville, MD) as PV-106. Only two other WSMV isolates, one collected in Kansas by M.K. Brakke in 1981 called Sidney-81 and the type strain of WSMV called PV-57, have been reported to infect corn. Sidney-81 is able to infect and increase in titer in the corn pure-line N28Ht while the cultivar or line of corn tested was not reported for PV-57. It was reported however, to only be weakly infective on the corn used in the study. No studies have been conducted to test whether other isolates (or serotypes) of WSMV are able to infect and increase in titer in corn.

Therefore, this preliminary study was conducted to evaluate whether differences existed in the ability of the WSMV isolates/serotypes to first infect and then cause WSM in a corn cultivar or four corn pure lines when mechanically inoculated. This was determined visually and by the enzyme-linked immunosorbent assay (ELISA), specifically the direct antigen plating ELISA (DAP-ELISA) assay. These results were compared to a similarly inoculated HRWW cultivar Chisholm, non-inoculated corn and wheat, and to these plants rubbed with abrasive but without inoculum. The four lines were HS-9843,

N-8811, N28Ht, ORO-188, and an unknown cultivar kindly provided by Dr. J.L. Sherwood's lab termed "Myers". These and the Chisholm wheat were planted on 10 Feb 94, inoculated on 25 Feb 94, using infective plant sap in PBS (0.01 M phosphate buffered saline, pH 7.0) and rubbed onto the leaves using an abrasive (1 g 225 μm corundum + 1 g celite) placed in 20 ml of the infective sap. The experimental design consisted of a randomized complete block with seven replications. The WSMV isolates used were: OSU, Sidney-81, PV-57, COLO, and TAMU. PV-106 was not included in this study although as mentioned above it is known to infect corn, because of lack of sufficient inoculum available at the time of this study free from infection by another related virus i.e., agropyron mosaic virus (AgMV), which would have confounded the test results. DAP-ELISA was conducted on 16 Mar 94. The results showed that all of the isolates were able to infect Chisholm but only two were able to infect corn and increase in titer. These were Sidney-81 on the corn pure-line N28Ht and TAMU on "Myers" (Table XV).

These preliminary results give another parameter that can be used in the separation of WSMV isolates i.e., the ability to infect corn. Other pure lines of corn have since been provided by Dr. Wayne Pederson (Dept. of Plant Pathology, University of Illinois at Urbana-Champaign, Urbana, IL) and

Table XV. DAP-ELISA absorbance values of hard red winter wheat cultivar Chisholm, and a cultivar and four pure-lines of *Zea mays* (corn) in comparison to controls. Conducted under glasshouse conditions in the spring of 1994.

Inoculated Plant	Wheat Streak Mosaic Virus Isolates					Controls[1]	
	TAMU	OSU	Sidney-81	COLO	PV-57	non-inoculated	abrasive
Chisholm[2] Wheat	1.503	1.792	> 2.000	1.434	0.664	0.005	0.012
"Myers" Corn	1.000	0.054	0.005	0.030	0.022	0.004	-0.004
N28Ht	0.013	0.024	1.143	0.024	0.011	0.015	0.008
HS9843	0.023	0.031	0.041	0.001	0.005	0.021	0.003
N8811	0.033	0.012	0.025	0.006	-0.003	0.007	-0.007
ORO188	0.005	0.022	0.021	-0.008	0.014	0.026	0.003

[1] Each value is the mean absorbance (i.e., optical density) at 405 nm of seven replications with three readings/replication. Values ≥ 0.100 are considered positive and values < 0.100 are considered negative.

may be useful in further testing of the isolates used in this study. PV-106 should also be included in the second study and tested against these pure lines of corn.

Recently an isolate of WSMV was collected that belongs in the WSMV-II serogroup, (both monoclonal and polyclonal antibody reactive), which also is able to infect two plants considered to be typical WSMV non-hosts e.g., sorghum and pearl millet. These differential hosts and hosts such as corn, which only certain WSMV isolates can infect, provide methods by which isolates can be biologically characterized. Such biological characterizations, as mentioned above, will further aid the placement of WSMV isolates/serotypes into distinct groupings and finally WSMV strains.

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VITA

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