ANALYSIS OF SOILBORNE WHEAT MOSAIC VIRUS (SBWMV), WHEAT SPINDLE STREAK MOSAIC VIRUS (WSSMV), AND *POLYMYXA GRAMINIS* IN WHEAT AND BARLEY

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CHAPTER I

INTRODUCTIONS AND LITERATURE REVIEW

Soilborne wheat mosaic virus

Soilborne wheat mosaic (SBWM), is a disease of winter wheat and barley in the central and eastern U.S.A., and is caused by *Soilborne wheat mosaic virus* (SBWMV) (Brakke and Langenberg, 1988). *Soilborne wheat mosaic virus* is transmitted through the soil to wheat or barley roots by the plasmodiophorid vector *Polymyxa graminis* (Brakke et al., 1964). SBWM disease has several descriptions: "green mosaic," "yellow mosaic," "mosaic rosette," and "eastern wheat mosaic disease" (Wiese, 1987). Symptoms of SBWM, in wheat and barley, include light green to yellow mosaic pattern on the leaves. Highly susceptible cultivars (e.g. Vona) can be severely stunted, with rosetting of leaves and excessive tillering (Wiese, 1987). Lower-lying fields may show more extreme symptoms due to the preference of *P. graminis* for wet ground (Wiese, 1987; Ledingham, 1939). The optimum temperature for virus transmission to wheat or barley roots is low (14 to 16°C). Mosaic symptoms and parallel yellow streaks due to SBWM are exhibited on young expanding leaves, as well as leaf sheaths. Later in spring, symptoms decrease and plants seem to outgrow the disease (Wiese, 1987).

SBWM is widely distributed throughout the winter wheat and barley growing areas of the eastern and central U.S.A., Japan, Italy, China, France, Egypt, Argentina, and Brazil (Brakke and Langenberg, 1988; Jianping, 1993). Crop losses due to SBWM disease in winter wheat vary depending on the cereal cultivar, the strain of the virus, and weather conditions. Large areas or entire fields may become so damaged that harvest is

completely abandoned (Wiese, 1987). In the central U.S.A., including wheat-growing areas of Oklahoma, the economic impact of SBWM on annual crop yields can mirror that of barley yellow dwarf disease if SBWM-susceptible varieties are grown (Wiese, 1987). Hence, the best option to control SBWM of wheat lies in the use of resistant or tolerant cultivars. Both hard red winter wheat and soft red wheat cultivars are little damaged by the disease. Other less effective controls include crop rotation and late fall planting dates (Wiese, 1987).

SBWMV, identified in 1923 by McKinney, is one of the earliest known wheat viruses, and the first to be characterized as a soilborne virus (McKinney, 1925). It is a rigid rod-shaped positive-stranded RNA virus consisting of two parts (bipartite) that are encapsidated separately (Shirako and Brakke, 1984). SBWMV virions are found in roots and, occasionally, in leaves (Brakke, 1971; Gumpf, 1971). Crystalline cytoplasmic inclusions and amorphous x-bodies may be found in infected cells (Hibino et al., 1974 a,b). Virions contain 5% nucleic acid, 95% protein, and 0% lipid (Brakke, 1971; Gumpf, 1971). RNA 1 is approximately 7.1 kb in length. Near the 5' end of RNA 1 are two open reading frames (ORFs) encoding two proteins of 150 and 209 kDa, which are the putative replicases. A third ORF, near the 3'end of RNA 1, encodes a 37 kDa protein that is a putative cell-to-cell movement protein (Fig. 1) (Shirako and Wilson, 1993). RNA 2 is approximately 3.6 kb in length, and encodes four proteins. Near the 5' end of RNA 2, is an ORF that encodes a 28 kDa protein. An ORF encoding the 19 kDa coat protein (CP) overlaps the 28 kDa protein ORF. The CP ORF has an opal termination codon at the 3' end and translational readthrough of this codon produces the 84 kDa protein (Shirako and Wilson, 1993). The 84 kDa readthrough domain is required for transmission of the virus

by its vector (*P. graminis*). Near the 3' end is an ORF that encodes a 19 kDa protein. The 28 kDa protein and the latter 19kDa protein have no known functions (Shirako and Wilson, 1993).

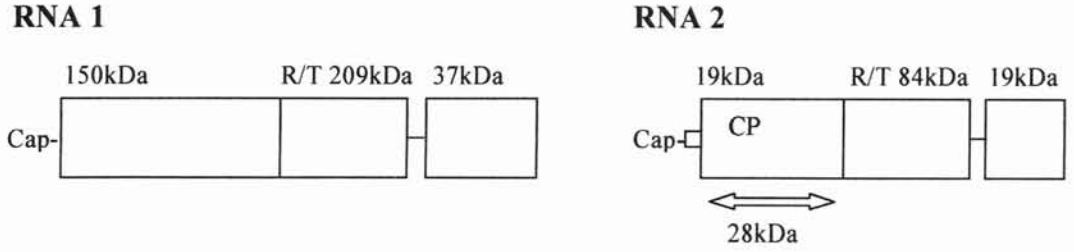


Fig. 1. Soilborne Wheat Mosaic Virus Genome. The boxes represent four ORFs in RNA 1 and RNA 2. The 5' end of each genome segment has a methyl guanosine cap. Molecular weights for each protein are indicated above each box.

SBWMV is the type member of the *Furovirus* (fungus-borne rod-shaped virus) genus of plant viruses (Torrence and Mayo, 1997). Furoviruses are rigid rod-shaped, bipartite, positive strand RNA viruses. Recently the *Furovirus* classification was revised to account for the many differences in segments, nucleotide sequence structures at the 3' terminal end, and movement proteins (Mayo, 1999). The genus *Furovirus* has been divided into four different genera; *Furovirus* (SBWMV), *Pomovirus* (Potato mop-top virus), *Pecluvirus* (Peanut clump virus), and *Benyvirus* (Beet necrotic yellow vein virus). The genus *Furovirus* now consists of SBWMV, *Oat golden stripe virus*, *Sorghum chlorotic spot virus*, *Chinese wheat mosaic virus* and *European wheat mosaic virus* (Shirako 2000). The *Pecluvirus*, *Pomovirus*, and *Benyvirus* genera all contain a triple gene block (Herzog et al., 1994; Koenig et al., 1996) that is also found in the *Potex-*, *Hordei-*, and *Carlavirus*. This triple gene block is necessary for viral cell-to-cell transport

(Huisman et al., 1988; Gilmer et al., 1992). The *Furoviruses* do not have a triple gene block (Mayo, 1999), but contain a 37 kDa putative movement protein encoded by RNA1 (Diao et al., 1999; Shirako et al., 2000). Susceptible host species for SBWMV include *Bromus commutatus, Bromus tectorum*, *Hordeum vulgare*, *Secale cereale*, and *Triticum aestivum* (Brakke, 1971).

There is evidence SBWMV, unlike most plant viruses, uses the xylem for long distance transport from the roots to the leaves (Verchot et al., 2001). Immunolocalization was conducted using segments of roots, stems, and leaves of SBWMV infected wheat plants, and SBWMV antiserum. Paraffin sections were labeled by immunogold silver enhancement, analyzed by light microscopy, and scored for the presence of signal in the xylem vessels, phloem sieve elements, and phloem companion cells (Verchot et al., 2001). In that study, SBWMV accumulated primarily in the xylem vessels of wheat roots, stems, and leaves (Verchot et al., 2001). One hundred percent of the sections contained gold particles in the xylem. Only 11 to 64% of the analyzed sections, contained gold particles in phloem companion cells, and 2 to 9.5% in phloem sieve elements. The fact that SBWMV was detected occasionally in phloem, suggests that movement through the phloem is less likely.

Furthermore, SBWMV was detected in protoxylem in young roots. Viral inclusion bodies also accumulate in xylem and xylem parenchyma (Verchot et al., 2001). Thus we hypothesize that SBWMV may enter the immature protoxylem and replicate in these cells. Then after programmed cell death and xylem maturation, virus is free to move long distance through the vessels.

Wheat spindle streak mosaic virus

Wheat spindle streak mosaic disease, also known as wheat yellow mosaic disease, was identified in the early 1960s, in winter wheat in Japan, in the eastern U.S.A., and in Canada (Slykhuis, 1978; Wiese, 1987). In the U.S.A., the disease was attributed to *Wheat spindle streak mosaic virus* (WSSMV) from the chlorotic spindle-shaped streaks that appeared on the leaves in early spring (Langenberg, 1985). Originally, the disease was thought to be a variant of soilborne wheat mosaic disease, because it is transmitted through the soil by *P. graminis,* and is dependent on cool temperatures. Based on serological analyses, SBWMV and WSSMV were determined to be different viruses (Langenberg ,1985).

Wheat spindle streak mosaic disease tends to be more evenly distributed in fields than SBWM (Wiese, 1987). Symptoms are more prevalent on lower leaves since the plants tend to outgrow the disease as the temperatures warm later in spring. Leaf symptoms can be variable and are more extreme when temperatures are below 20°C. Yellow-green mottling, dashes and streaks develop on the young leaves, are oriented parallel to leaf veins, and taper to form the characteristic chlorotic spindles (Wiese, 1987). If the temperatures stay cool, the centers of the spindles may become necrotic and streaking can continue up to the flag leaf where reddish necrotic streaks develop at the leaf tip (Wiese, 1987). Mild stunting of the infected plants occurs with fewer tillers than normal and decreased numbers of seed heads; however, the seed weight is not significantly affected (Wiese, 1987).

Wheat spindle streak mosaic virus (WSSMV) is widely distributed in France, Germany, India, Italy, China, Japan, Canada, and the U.S.A. In North America, WSSM

disease is endemic in most of southern Ontario and the east central United States including Oklahoma (Wiese, 1987). Annual crop losses to WSSM disease, in southwestern Ontario, may reach 40% in some fields (Wiese, 1987). WSSM occurs on wheat (*Triticum aestivum* and *T. durum*), rye (*Secale cereale*), and barley (*Hordeum vulgare*). WSSM affects both spring and winter grains, but spring cultivars are not often symptomatic (Wiese, 1987).

Low temperatures (<10° C) are more critical for WSSMV symptoms to develop than SBWMV. WSSMV infection does not develop above 20° C, and the disease ceases to be a problem above 18° C (Wiese, 1987). WSSMV has probably the lowest optimal temperature range for a plant virus (between 8 and 12°C), and without the long cool periods in winter and early spring necessary for disease development, the disease is not of any economic importance (Wiese, 1987). Like SBWMV, later planting dates can greatly reduce the rate of infection with WSSMV. Crop rotations can also be of some use in limiting carryover of the virus from year-to-year, however, the liberal use of urea and manure (poultry) also decreases disease incidence (Wiese, 1987).

Wheat spindle streak mosaic virus (WSSMV) is the type member of the genus *Bymovirus*. It is a flexible, filamentous virus (Slykhuis, 1976). Virion particles are 300-2000 nm in length and 16 nm in width. Few particles are seen in host cells, but they can be both scattered and in bundles in epidermal and parenchymal cells, in symptomatic leaves (Slykhuis, 1976).

Bymoviruses are filamentous RNA viruses that are not enveloped (Brunt et al., 1996). Virions are usually slightly flexuous and are transmitted through the soil by a

Plasmodiophorid vector (*P. graminis*) to wheat or barley roots. The definitive species in the group are WSSMV, *Barley mild mosaic virus*, *Barley yellow mosaic virus*, *Oat mosaic virus*, *Rice necrosis mosaic virus*, and *Wheat yellow mosaic virus*.

Thus far the genome sequence of WSSMV has not been analyzed and there has been very little research exploring the biology of the virus. Primarily cytological changes associated with WSSMV have been described, such as prominent pinwheel-shaped inclusion bodies and extensive membrane proliferation in wheat leaf epidermal and parenchymal cells (Slykhuis, 1976).

WSSMV and SBWMV often are detected in the same host. They may cause a synergistic disease that is more severe than the diseases caused by the individual viruses (Kendall and Lommel, 1988). Since they are both transmitted by *P. graminis*, it is likely that the path of SBWMV and WSSMV transmission into, and spread throughout the plant, may be similar (Brakke and Langenberg, 1988).

Polymyxa graminis

Polymyxa graminis Ledingham was first described in 1939 as an obligate parasite of wheat roots (Ledingham, 1939). Ledingham identified this new organism in wheat roots from three different locations in Ontario. Three cultivars of wheat were used as host plants. Later, it was found also in the roots of barley and rye, but not in the roots of oats (Ledingham, 1939). Ledingham found similar resting spores in roots of *Agropyron*, *Scolochloa*, *Rumex*, and *Impatiens*, but zoospores were not always found in conjunction with them. *Polymyxa graminis* is also parasitic on bentgrass (*Agrostis palustris*, L.) (Britton and Rogers, 1963), *Bromus* spp. Leysser, *Avena* spp. L., and *Cynodon* spp.

(Adams et al.,1986; Adams and Jacquier, 1994). Although cereals are the most common experimental host for *P. graminis*, it has also been identified in peanut roots (*Arachis hypogaea* L.) (Thouvenel and Fauquet, 1981).

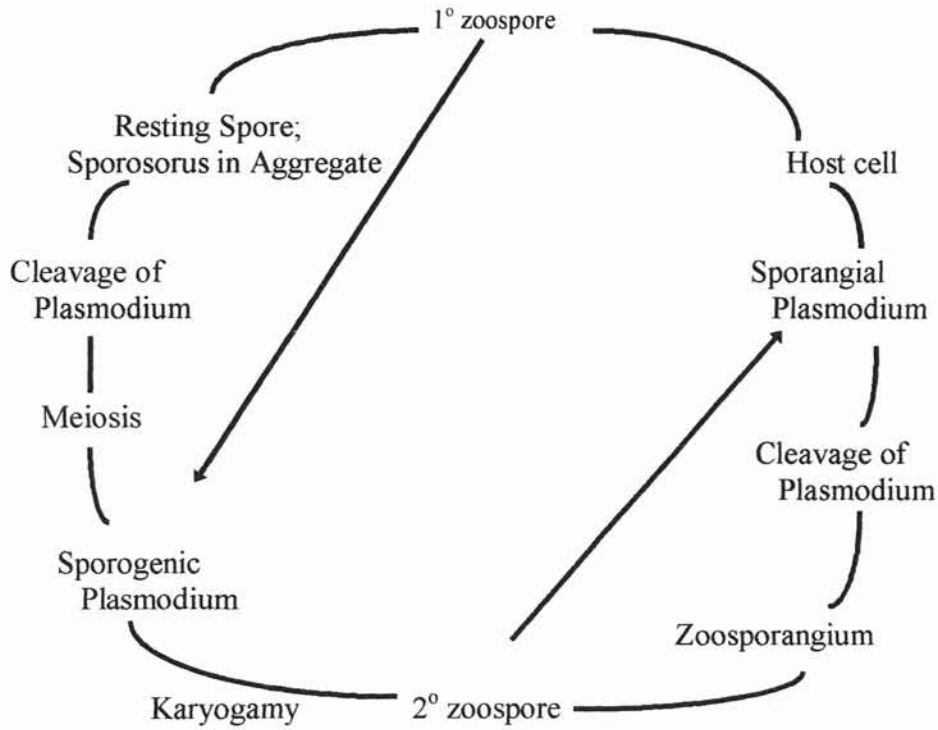


Fig. 2. *Polymyxa graminis* life cycle (Littlefield et al., 1998).

The diagram in Fig. 2. illustrates the life cycle of *P. graminis* with the shunt pathways outlined from secondary zoospores to zoosporangial plasmodia and from primary zoospores to sporogenic plasmodia *P. graminis* primary zoospores directly penetrate the cell wall of the host (Keskin, 1964) and lie uninucleate in the host cytoplasm until they develop further by increasing in size and entering the multinucleate

plasmodial stage (Barr, 1979). A thin membrane surrounds the plasmodium, which elongates, develops many lobes, and can extend through the host cell walls into adjacent cells at maturity, and envelops the sporangial plasmodia (Barr, 1979). Cross walls form and the plasmodia divide into segments that eventually become zoosporangia. Exit tubes form in zoosporangia, which can extend through several cells to reach the host exterior. The protoplasm cleaves to form secondary zoospores, which exit the host through the tubes and swim away. Zoospores may stop swimming after two to three hours and come to rest on a new host (Karling, 1968).

Sporogenic plasmodia start out as a naked amoeba with no universal membrane covering. Many nuclear divisions occur, and a multinucleate myxamoeba is formed, which may take on varied forms and stretch throughout the cell (Barr, 1979). The myxamoebae nuclei undergo meiosis, and cell walls are deposited consisting of an inner hyaline layer, and an outer darker yellow-brown wall, which is fused with the neighboring cells (Barr, 1979). Pressure from being so tightly packaged often causes the cells to be many sided. The individual cells of the sporosori range in size from 5 to 7 μ in diameter. Upon germination, the biflagellate zoospores that emerge from the resting spores are indistinguishable from the secondary zoospores after the short time it takes them to become rounded and begin swimming (Karling, 1968).

There are two economically important *Polymyxa* spp. *P. betae* was identified in sugar beet in Europe (Keskin, 1964), and its hosts include *Atriplex* spp., *Beta* spp., *Chenopodium* spp., *Kochia* spp., *Salsola* spp., *Spinacia* spp., *Amaranthus* spp., and *Portulaca* spp. Only after *P. graminis* and *P. betae* were shown to transmit plant viruses that caused serious economic impact on several agriculturally important crops, was the

genus *Polymyxa* deemed of particular significance to plant pathologists (Karling, 1968). There are now twelve viruses of cereal grains, sugar beet and peanut that have been reportedly transmitted by *Polymyxa* spp. (Adams, 1991).

RESEARCH OBJECTIVES

There is evidence SBWMV accumulates inside *P. graminis* zoospores, and is transmitted by zoospores (Rao, 1968; Rao and Brakke, 1969). There is no evidence concerning WSSMV and *P. graminis* interactions. For both WSSMV and SBWMV, nothing is known about the mechanism by which virus is transmitted by *P. graminis*. For both SBWMV and WSSMV, once virus is deposited in wheat roots, it takes several weeks to observe symptoms in the leaves. Typically, plant viruses move long distance through the phloem; however, we believe that SBWMV and WSSMV may follow an alternative path through the xylem. This is based on previous reports indicating SBWMV uses the xylem for long distance movement (Verchot et al., 2001).

In this study we have conducted experiments to:

1) gather additional evidence that SBWMV may use the xylem for vascular transport. Immunolabeling was conducted to determine if SBWMV movement protein and RNA accumulate in the xylem for viral vascular transport.

2) determine if WSSMV also accumulates and moves through the xylem.

3) establish a laboratory culture of *P. graminis* for studies of virus transmission to plant roots.

CHAPTER II

SOILBORNE WHEAT MOSAIC VIRUS RNA ACCUMULATES IN THE XYLEM WHILE MOVEMENT PROTEIN ACCUMULATES IN THE XYLEM AND PHLOEM IN WHEAT

ABSTRACT

Soilborne wheat mosaic virus (SBWMV) is a member of the genus *Furovirus*. SBWMV is transmitted to wheat roots by the plasmodiophorid vector *Polymyxa graminis*. In a previously published report using SBWMV antiserum and immunogold labeling, we found evidence that SBWMV uses the xylem for vascular movement from the roots to the leaves. Viral coat protein and virion particles were found in the xylem and xylem parenchyma. To further test the idea that SBWMV moves through the xylem, experiments were conducted in this study to determine if viral RNA and movement protein also accumulate in the xylem. In this study we found that viral RNA similarly to coat protein, accumulates in the xylem, while movement protein of SBWMV accumulates in the xylem and phloem.

INTRODUCTION

Five genera of plant RNA viruses, *Furo-, Bymo-, Pomo-, Peclu-*, and *Benyvirus*, include soilborne viruses transmitted by fungal vectors belonging to the order *Plasmodiophorales* (Adams, 1991). Furoviruses, bymoviruses, and pecluviruses are

transmitted by the plasmodiophorid *Polymyxa graminis*; benyviruses are transmitted by *P. betae*; and *Spongospora subterranean* transmits pomoviruses (Adams, 1991). These viruses are associated with fungal zoospores that invade root epidermal cells (Barr, 1988). We do not know if these viruses are internalized or attached to the surface of the zoospores.

The pathway for soilborne virus movement through the vasculature from roots to aerial plant parts has been explored in only a few studies. *Beet necrotic yellow vein virus* (BNYVV), and *Potato mop top virus* (PMTV), were detected in xylem vessels in plant roots (Dubois et al., 1994; Jones, 1975). In immunogold labeling studies with either light or transmission electron microscopy, BNYVV has been found in xylem vessels or xylem parenchyma in infected plant roots (Dubois et al., 1994). Dubois et al. (1994) proposed that BNYVV moves into xylem before differentiation occurs. We propose that SBWMV similarly moves into immature xylem vessels (xylem pole cells) and is translocated upwards in the plant after the xylem vessels mature (Verchot et al., 2001).

There is some evidence that SBWMV uses the xylem for long distance movement (Verchot et al., 2001). In a previous study, SBWMV coat protein was detected in xylem vessels in roots, stems, and leaves by both light and electron microscopy (Verchot et al., 2001). Virion particles in inclusion bodies were found by electron microscopy in xylem parenchyma cells adjacent to xylem vessels.

To further test the hypothesis that SBWMV moves through the xylem, experiments were conducted to determine if SBWMV movement protein and genomic RNA also accumulate in the xylem of infected wheat plants. Cross sections of SBWMV infected wheat roots, stems, and leaves were analyzed using movement protein antibody,

immunogold labeling, and silver enhancement. Cross sections of SBWMV infected wheat roots were also analyzed using *in situ* hybridization to detect viral nucleic acids.

Materials and methods

Plant material

Hard red winter wheat (*Triticum aestivum* L.) plant material infected naturally with SBWMV was obtained from a field nursery located just west of Stillwater, Oklahoma. This nursery has been used for more than twenty years to screen wheat breeder material for reaction to SBWMV. Winter wheat lines and varieties are planted in this area in September and then irrigated heavily for approximately five days just as coleoptiles are emerging through the soil. This protocol facilitates infection of seedling roots by *P. graminis*, and results in consistent and severe infection of wheat that is susceptible to SBWMV. The hard red winter wheat (cultivar Vona) is used as a susceptible check to indicate the consistent occurrence of SBWMV in the nursery, and plant material collected from Vona plants growing in this nursery was used in this study.

Embedding plant material in Paraplast

For light microscopy, root, stem, and leaf segments were taken from six-month-old (Feekes' scale 6-7), symptomatic, field-grown, hard red winter wheat plants (cv. Vona) and embedded in Paraplast (Electron Microscopy Sciences, Fort Washington, Pa., USA) (Berlyn and Miksche, 1976; Verchot et al., 2001). SBWMV infected plants were identified by an enzyme-linked immunosorbent assay of leaf extracts, with SBWMV antiserum (see below). Root segments were collected within ca. four cm of the soil

surface (Verchot et al., 2001). Samples were placed in fixative (10:2:1 dilution of ethanol, formaldehyde, and acetic acid) overnight at 4°C, and then dehydrated in a graded series of 50, 60, 70, 85, 95, and 100% ethanol. Ethanol was replaced with a graded series of 25, 50, 75, and 100% xylene. Paraplast chips were added to the samples at room temperature, and samples were incubated overnight. Samples were infiltrated with Paraplast at 62 °C for three days, and molten Paraplast was changed twice each day. Samples were transferred to plastic molds filled with molten Paraplast and allowed to harden for two h in cool water (Verchot et al., 2001).

Immunogold-silver enhancement for light microscopy

Paraplast embedded wheat root, stem, and leaf segments were sectioned (8.0 μm), mounted on ProbeOn Plus slides (Fisher Biotechnology, Pittsburgh, PA), and used for immunogold labeling studies. The slides were incubated in blocking solution (50 mM Tris-HCl, pH 7.4, 150 NaCl, 2% bovine serum albumin fraction V, 0.1% Tween 20) for one h, and then with SBWMV movement protein polyclonal antiserum, diluted 1:100 (prepared by the Oklahoma State University Hybridoma Center), for one h. The slides were then incubated with secondary polyclonal anti-rabbit antiserum (conjugated to 10 nm diameter gold), diluted 1:50, for one h, and then developed by silver enhancement for 30 min (Electron Microscopy Sciences). The reaction was stopped with sodium thiosulfate, and then the slides were counterstained with 0.5% safranin-O for 10 s. For controls, similar slides were treated with a heterologous antiserum *Wheat spindle streak mosaic virus* (WSSMV), obtained from Agdia (Elkhart, Ind.).

Light microscopy was conducted with a Nikon Eclipse E600 microscope. Images were recorded using Optronics Magnafire digital camera (Goleta, CA) and Image-Pro Plus software (Silver Spring, MD). Figures were arranged using Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA).

Data were collected from sections of three to five blocks of roots, stems and leaves of field grown hard red winter wheat plants (cv. Vona). Fifty to 100 cross sections of each tissue were analyzed. Xylem, phloem, sieve elements and phloem companion cells were scored for the presence or absence of silver staining. Images were collected of cross sections to demonstrate accumulation of silver particles in each tissue.

In situ RNA hybridization and light microscopy

Paraplast embedded wheat roots were sectioned (8.0 μm), and then mounted on ProbeOn Plus slides (Fisher Biotechnology, Pittsburgh, PA). Slides were incubated with an RNA probe that can hybridize to viral RNAs. A digoxigenin labeled antibody that recognizes the RNA probe was used. Then slides were developed with NBT/BCIP. A purple color was produced if viral RNA was present.

The slides were incubated for 15 to 20 min at room temperature in 2X SSC (17.5 g NaCl, 8.82 g sodium citrate/l of water, pH 7.0), and then in proteinase K (1μg/ml of proteinase K dissolved in 100 mM Tris-HCl pH 8.0, 50 mM EDTA) at 37°C for 15 to 20 min. Slides were washed for two min at room temperature in PBS-glycine buffer (0.13 M NaCl, 7.0 mM Na_2HPO_4, 3.0 mM NaH_2PO_4, 2.0 mg/ml glycine, pH 7.0), and then twice with PBS buffer. Then slides were incubated for 10 min at room temperature in 4% paraformaldehyde (pH 7.0) dissolved in PBS. Slides were washed twice with PBS

for five min at room temperature, twice with 0.1 M TEA buffer (1.492 g triethanolamine/100 ml water, 0.5 ml acetic anhydride) for 10 min at room temperature, and then twice in PBS buffer for 5 min at room temperature. Slides were incubated for 30 min at 37°C with prehybridization solution (50% deionized formamide, 5% dextran sulfate, 1% blocking reagent (Boehringer Mannheim), 500 μg/mL poly A, 300 mM NaCl, 10mM Tris-HCl pH 7.5, 1 mM EDTA), and then overnight at 42°C with hybridization solution (prehybridization solution plus digoxigenin-labeled RNA probe).

The plasmid p5114 is a pGEMT plasmid containing a cDNA copy of the 3' 1000 nt of SBWMV genome. Transcripts were prepared using *Apa*I linearized p5114 plasmids, SP6 polymerase, and digoxigenin labeled UTP. Then slides were washed twice with 0.2X SSC for 60 min at room temperature, twice with NTE buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) for 5 min at 37°C, once in 0.2X SSC for 60 min at 55°C, and once in PBS for five min at room temperature.

For antibody labeling, slides were incubated for 45 min with 1 % blocking reagent, and then with 1 % bovine serum albumin fraction V (both in a solution of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Triton X-100). Slides were incubated for two h at room temperature with anti-digoxigenin labeled antibody, (diluted 1:1250 in 100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.3% Triton X-100). Slides were washed twice for 15 min in a solution of 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl, followed by a 10 min wash in a solution of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$.

For developing the reaction, the substrate solution was prepared immediately before use, by diluting 200 μl of NBT/BCIP stock solution (vial 4, Boehringer Mannheim DIG Nucleic Acid Detection Kit) in 10 ml buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM

$MgCl_2$). The slides were incubated in darkness, with the color development solution, for two h to three days at room temperature, and then rinsed in TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA). Slides were dehydrated in a series of 25, 50, 75, and 100% ethanol, for a maximum of five sec in each step, to minimize color loss. The slides were dipped in xylene. A drop of Permount and a coverslip was added to each slide prior to viewing under the light microscope.

Data were collected from root sections obtained from three to five blocks of field-grown wheat roots (cv. Vona). Thirty-one cross sections were analyzed. As in Table 1, xylem, phloem sieve elements, and phloem companion cells were scored for the presence, or absence, of blue staining. Images were collected to demonstrate the pattern of *in situ* labeling.

Experiments were conducted to determine if the pattern of *in situ* labeling resembles the pattern of coat protein accumulation, as previously reported.

Table. 1. Distribution of SBWMV RNA in wheat roots

Tissue	Proportion (%) of positive sections containing SBWMV RNA[a]
Cortex	30/31 (97)
Pericycle	15/31 (48)
Endodermis	15/31 (48)
Phloem	0/31 (0)
Xylem	19/31 (61)

[a] A total of 31 root sections from field-grown wheat plants were paraffin embedded and sectioned. Following *in situ* hybridization, each section was scored positive by light microscopy for the presence of SBWMV RNA in associated tissues. Proportions are the numbers of sections that contained digoxigenin labeled antibody to RNA relative to the total numbers of sections analyzed.

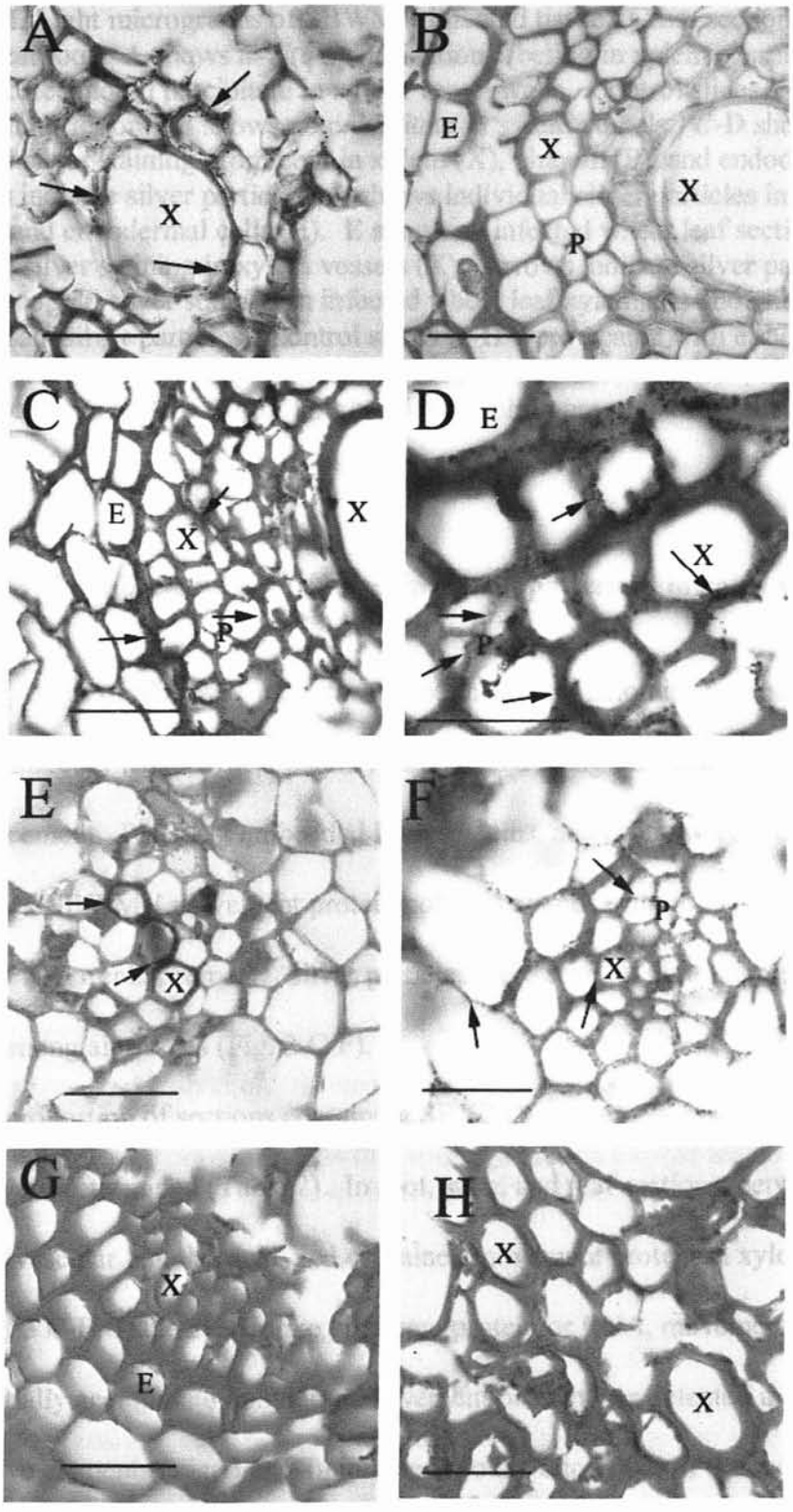
RESULTS

SBWMV RNA accumulation pattern in infected wheat

The pattern of SBWMV RNA accumulation in the vasculature of infected wheat roots was analyzed using *in situ* hybridization. Paraplast embedded roots (cv. Vona) were sectioned and incubated with an RNA probe that hybridizes with SBWMV RNA. The sections were treated with a digoxigenin labeled antibody followed by development with NBT/BCIP, resulting in a purple to black color precipitate where RNA was present (Fig. 3 A, B).

Precipitate was observed in walls of xylem vessels (Fig. 3 A). For a control, infected material was treated with hybridization buffer lacking probe and no precipitate was detected (Fig. 3 B). Viral RNA was detected in all cell types except phloem.

The proportion of sections containing SBWMV RNA in root xylem, phloem, endodermis, pericycle, and cortex was determined. Approximately 61% of sections contained precipitate in walls of xylem vessels and none of the sections contained precipitate in phloem (Table 1). Approximately 48% of sections contained precipitate in both the pericycle and endodermis, and 97% contained precipitate in cortex (Table 1). The fact that phloem cells contained no precipitate supports the notion that SBWMV moves from the roots to leaves through the xylem.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X

Fig. 3. A-H. Light micrographs of SBWMV infected tissue. Cross sections of SBWMV infected wheat roots. **A** shows *in situ* hybridization labeling in xylem vessel walls. (X). Arrows indicate colored precipitate in xylem vessel walls. Control slides treated with hybridization buffer only **B** shows no precipitate in xylem vessels. **C-D** shows immunogold silver staining prominent in xylem (X), phloem (P), and endodermal cells (E). Arrows indicate silver particles. **D** shows individual silver particles in xylem (X), phloem (P), and endodermal cells (E). **E** shows an infected wheat leaf section with immunogold silver staining in xylem vessels (X). Arrows indicate silver particles. **F** shows immunogold silver staining in infected wheat leaf xylem (X) and phloem (P). Arrows indicate silver particles. Control slides **G-H** were treated with either WSSMV antisera or buffer only. Controls show no silver particles in xylem (X), phloem (P), or endodermal cells (E). Bars: 20μm. Light micrographs were obtained with an X100 objective.

SBWMV movement protein accumulation pattern in infected wheat

The pattern of SBWMV movement protein accumulation in the vasculature of field-grown infected winter wheat plants was analyzed using immunogold labeling with silver enhancement. Paraplast embedded leaves, stems, and roots, were sectioned and incubated with SBWMV movement protein polyclonal antiserum followed by immunogold silver enhancement. Silver particles were seen by light microscopy to be distributed among all tissues (Fig. 3 C-F).

The proportion of sections containing SBWMV movement protein in roots, stems, and leaves were determined (Table 2). In root, stem, and leaf sections, between 78 and 100% of the vascular bundles analyzed contained movement protein in xylem and phloem (Fig. 3 C-F; Table 2). Unlike viral coat protein or RNA, movement protein did not preferentially accumulate in xylem. Movement protein was detected in companion cells and sieve elements (Fig. 3 D). In infected roots, 96 to 100% of the sections

contained movement protein in cortex, pericycle, and endodermis. In stem and leaf sections, movement protein was detected in bundle sheath, cortical, and mesophyll cells.

For controls, slides were treated either with WSSMV antiserum or buffer only (Fig. 3 G, H, respectively). No label was observed in any of the control slides.

Table. 2. Distribution of SBWMV movement protein in wheat roots, stems, and leaves

Tissue protein[a]	Proportion (%) of positive sections containing SBWMV movement		
	Leaf	Stem	Root
Cortex			50/50 (100)
Pericycle			48/50 (96)
Endodermis			48/50 (96)
Phloem	85/85 (100)	47/55 (85)	48/50 (96)
Xylem	85/85 (100)	43/55 (78)	50/50 (100)
Bundle sheath		27/55 (49)	
Mesophyll	85/85 (100)		

[a]Following immunogold silver enhancement, each section was scored positive by light microscopy for the presence of SBWMV movement protein in associated tissues. Proportions are the numbers of sections that contained silver labeled antibody to movement protein relative to the total numbers of sections analyzed.

DISCUSSION

SBWMV and WSSMV have been identified in hard red winter wheat growing areas of the U.S.A. and are often detected in the same field (Brakke and Langenberg,1988; Wiese, 1987). In cytological studies of plants co infected with

SBWMV and WSSMV, SBWMV proteins were associated with WSSMV pinwheel-type inclusion bodies (Langenberg 1985; Langenberg 1986). Paraplast-embedded tissues were analyzed with WSSMV antiserum, immunogold silver enhancement, and light microscopy (Fig. 3G) and there was no evidence of WSSMV in the samples.

The strain of SBWMV used in this study does not produce significant symptoms in field-grown hard red winter wheat until approximately 5-6 months after planting. Seed is planted in the autumn and symptoms appear in late February or March. Unlike field-grown hard red winter wheat, the SBWMV symptoms in hard red winter wheat grown in growth chambers are often mild and difficult to identify. In addition, the level of virus accumulation is lower in growth chamber-grown plants than in field-grown plants. For this study, we determined that field-grown plants were more useful because they had stronger symptoms and contained significantly higher levels of SBWMV.

The data presented in a previously published study suggests that SBWMV is likely to move from roots to leaves through the xylem. SBWMV was detected in xylem vessels in root, stem, and leaf sections viewed either by light or electron microscopy (Verchot et al., 2001). In addition SBWMV virion particles were observed in xylem parenchyma cells adjacent to xylem vessels.

The previously published results indicated that virus is present in the xylem and did not preclude the possibility that SBWMV can enter or move through the phloem. The present study was conducted to determine if SBWMV could be phloem associated. We explored the pattern of viral RNA and movement protein accumulation in SBWMV infected wheat plants. We found evidence that viral RNA accumulates primarily in the

xylem. This evidence supports previous studies showing viral coat protein and virion particles in the xylem.

Surprisingly, SBWMV movement protein was detected in phloem as well as the xylem. One explanation is that SBWMV may not move exclusively through the xylem for long distance transport. There may be a subset of virus that can move through the phloem. Viral phloem transport may be important for movement in a source-to-sink direction. Virus may need to use the phloem to move from mature source leaves to young developing sink leaves. Once virus has entered the phloem it may also move from leaves to roots.

Another explanation is that excess movement protein that is not being used by the virus is deposited in the phloem. Evidence of virus accumulating in the xylem and viral movement protein accumulating in both xylem and phloem does not allow us to draw clear conclusions about the mechanism for virus long distance transport. Since we do not have an infectious clone of the virus, we are unable to perform mutational analysis to determine if viral movement protein is essential for virus long-distance transport, or conduct experiments to determine if virus can enter the phloem for source-to-sink movement. The fact that the movement protein accumulated in the phloem is evidence that virus may have been in these vessels; however, it is also possible that the movement protein moved there independently. We do not know, based on these studies, if the fraction of movement protein accumulating in either xylem or phloem tissue functions to mediate virus long distance transport. Further research is needed to test these possibilities.

CHAPTER III

EVIDENCE *WHEAT SPINDLE STREAK MOSAIC VIRUS* ACCUMULATES IN THE XYLEM AND PHLOEM IN WHEAT.

ABSTRACT

Wheat spindle streak mosaic virus (WSSMV) is a member of the genus *Bymovirus*. WSSMV and SBWMV cause a synergistic disease in wheat and are both transmitted to wheat roots by the plasmodiophorid vector *Polymyxa graminis*. In a previously published report using SBWMV antiserum and immunogold labeling, we found evidence that SBWMV likely uses the xylem for vascular movement from the roots to the leaves. Viral coat protein and virion particles were found in the xylem and xylem parenchyma. In this study we conducted experiments to determine if WSSMV also utilizes the xylem for vascular transport. Cross sections of WSSMV infected wheat leaves and roots were analyzed using WSSMV antiserum and immunogold labeling. WSSMV was detected in the xylem and phloem. Unlike SBWMV, WSSMV does not preferentially accumulate in the xylem.

INTRODUCTION

Wheat spindle streak mosaic virus (WSSMV), the cause of wheat spindle streak mosaic disease in winter wheat is the type member of the genus *Bymovirus*. When WSSMV was first identified in the early 1960s in winter wheat, it was thought simply to be a variant of *Soilborne wheat mosaic virus* (SBWMV). That misconception arose

because both WSSMV and SBWMV are transmitted through the soil by the plasmodiophorid vector *P. graminis* to the roots of winter wheat, and both are also dependent on cool temperatures for transmission. However, based on serological analyses, WSSMV and SBWMV were later determined to be two different viruses (Langenberg, 1985).

Mixed infections of both WSSMV and SBWMV commonly occur in winter wheat. The presence of both WSSMV and SBWMV in the same wheat plant may cause a synergistic disease that is more severe than either virus would normally cause alone (Kendall and Lommel, 1988). Since both WSSMV and SBWMV are transmitted by *P. graminis*, it is likely that the path of transmission into, and spread throughout the plant, may be similar (Brakke and Langenberg, 1988).

Since SBWMV moves through the xylem from the roots to the leaves in winter wheat (Verchot et al., 2001), we hypothesize that WSSMV also moves in this manner. Previous studies found SBWMV coat protein accumulating primarily in the xylem of winter wheat (Verchot et al., 2001); therefore, we examined the possibility that WSSMV coat protein also would accumulate mainly in the xylem.

To test the hypothesis that WSSMV moves through the xylem of winter wheat, experiments were conducted to determine if WSSMV coat protein and virion particles accumulate in the xylem of infected wheat plants. Cross sections of WSSMV infected wheat roots and leaves were analyzed using coat protein antiserum, immunogold labeling, and silver enhancement. Light microscopy was used to study the pattern of WSSMV accumulation.

MATERIALS AND METHODS

Plant material

Hard red winter wheat (*Triticum aestivum* L.) plant material infected naturally with WSSMV was obtained from a field nursery located just west of Stillwater, Oklahoma. This nursery has been used for more than twenty years to screen wheat breeder material for reaction to WSSMV. Winter wheat lines and varieties are planted in this area in September and then irrigated heavily for about five days just as coleoptiles are emerging through the soil. This protocol facilitates infection of seedling roots by *P. graminis*, and results in consistent and severe infection of wheat that is susceptible to WSSMV. The hard red winter wheat (cultivar Sierra) is used as a susceptible check to indicate the consistent occurrence of WSSMV in the nursery, and plant material collected from Sierra plants growing in this nursery was used in this study.

The hard red winter wheat cultivar, Sierra, is SBWMV-resistant and WSSMV-susceptible. ELISA analysis was conducted to determine if WSSMV, and/or SBWMV, were present in the leaves.

Embedding plant material in Paraplast

For light microscopy, root and leaf segments were taken from four, 6-month-old (Feekes' scale 6-7) symptomatic field-grown Sierra plants and embedded in Paraplast (Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.) (Berlyn and Miksche, 1976). Paraplast embedding was carried out as described in Chapter 1. WSSMV infected plants were identified by an enzyme-linked immunosorbent assay of leaf extracts, with

WSSMV antiserum (see below). Root segments were collected within ca. 4 cm of the soil surface (Verchot et al., 2001). Samples were placed in fixative (10:2:1 dilution of ethanol, formaldehyde, and acetic acid) overnight at 4 °C, and then dehydrated in a graded series of 50, 60,70,85,95, and 100% ethanol. Ethanol was replaced with a graded series of 25, 50, 75, and 100% xylene. Paraplast chips were added to the samples at room temperature, and samples were incubated overnight. Samples were infiltrated with Paraplast at 62 °C for three days, and molten Paraplast was changed twice each day. Samples were transferred to plastic molds filled with molten Paraplast and allowed to harden for 2 h in cool water.

Immunogold-silver enhancement for light microscopy

Paraplast embedded leaf, and root segments were sectioned (8.0 μm), mounted on ProbeOn Plus slides (Fisher Biotechnology, Pittsburgh, PA), and used for immunogold labeling studies, as described in Chapter 1. The slides were incubated in blocking solution (50 mM Tris-HCl, pH 7.4, 150 NaCl, 2% bovine serum albumin fraction V, 0.1% Tween 20) for 1 h, and then with WSSMV polyclonal antiserum diluted 1:100 (Agdia, Elkhart, Ind.). The slides were then incubated with polyclonal anti-rabbit antiserum (conjugated to 10 nm diameter gold) diluted 1:50, for 1 h, and then developed by silver enhancement for 30 min (Electron Microscopy Sciences). The reaction was stopped with sodium thiosulfate, and then the slides were counterstained with 0.5% safranin-O for 10 s. For control experiments, similar slides were treated with SBWMV antiserum (prepared by the Oklahoma State University Hybridoma Center). For control

experiments, slides were treated with SBWMV antiserum, *Brome mosaic virus* (BMV) antiserum, or buffer only.

Light microscopy was conducted with a Nikon Eclipse E600 microscope. Images were recorded using Optronics Magnafire digital camera (Goleta, California, U.S.A.) and Image-Pro Plus software (Silver Spring, Maryland, U.S.A.). Figures were arranged using Adobe Photoshop software (Adobe Systems, Inc., Mountain View, California, U.S.A.). Data were collected from sections of 4 blocks of roots, and 2 blocks of leaves of field grown Sierra plants. Forty-six cross sections of roots and 40 cross sections of leaves were analyzed. As in Chapter 1, xylem, phloem sieve elements, and phloem companion cells were scored for the presence or absence of silver staining. Images were collected of cross sections to demonstrate accumulation of silver enhanced gold particles in each tissue.

RESULTS

WSSMV coat protein accumulation pattern in infected wheat

The pattern of WSSMV coat protein accumulation in the vasculature of field-grown infected hard red winter wheat plants (cv. Sierra) was analyzed using immunolabeling with silver enhancement. Paraplast embedded leaves and roots were sectioned and incubated with WSSMV coat protein polyclonal antiserum followed by immunogold silver enhancement. Silver enhanced gold particles were seen by light microscopy to be distributed among all tissues. (Fig. 4 A-D).

The proportion of sections containing WSSMV coat protein in roots and leaves was determined (Table 3). In roots, approximately 96% of the vascular bundles analyzed

contained WSSMV coat protein in the phloem, and 100% of the xylem, cortex, pericycle, and endodermis (Fig. 4 C, D; Table 3). In infected leaves, 100% of the xylem, phloem, and bundle sheath cells contained WSSMV coat protein (Fig. 4 A, B).

To verify that WSSMV antiserum binds specifically to WSSMV coat protein present in the vasculature, sections of WSSMV-infected wheat roots and leaves were analyzed either with no primary antiserum (Fig. 4 E) or with SBWMV antiserum (Fig. 4 F), or with BMV antiserum (Fig. 4 G). None of the control antibodies used (gold-conjugated antiserum, SBWMV antiserum, or BMV antiserum) reacted with infected tissue, indicating that under these experimental conditions there was minimal nonspecific labeling.

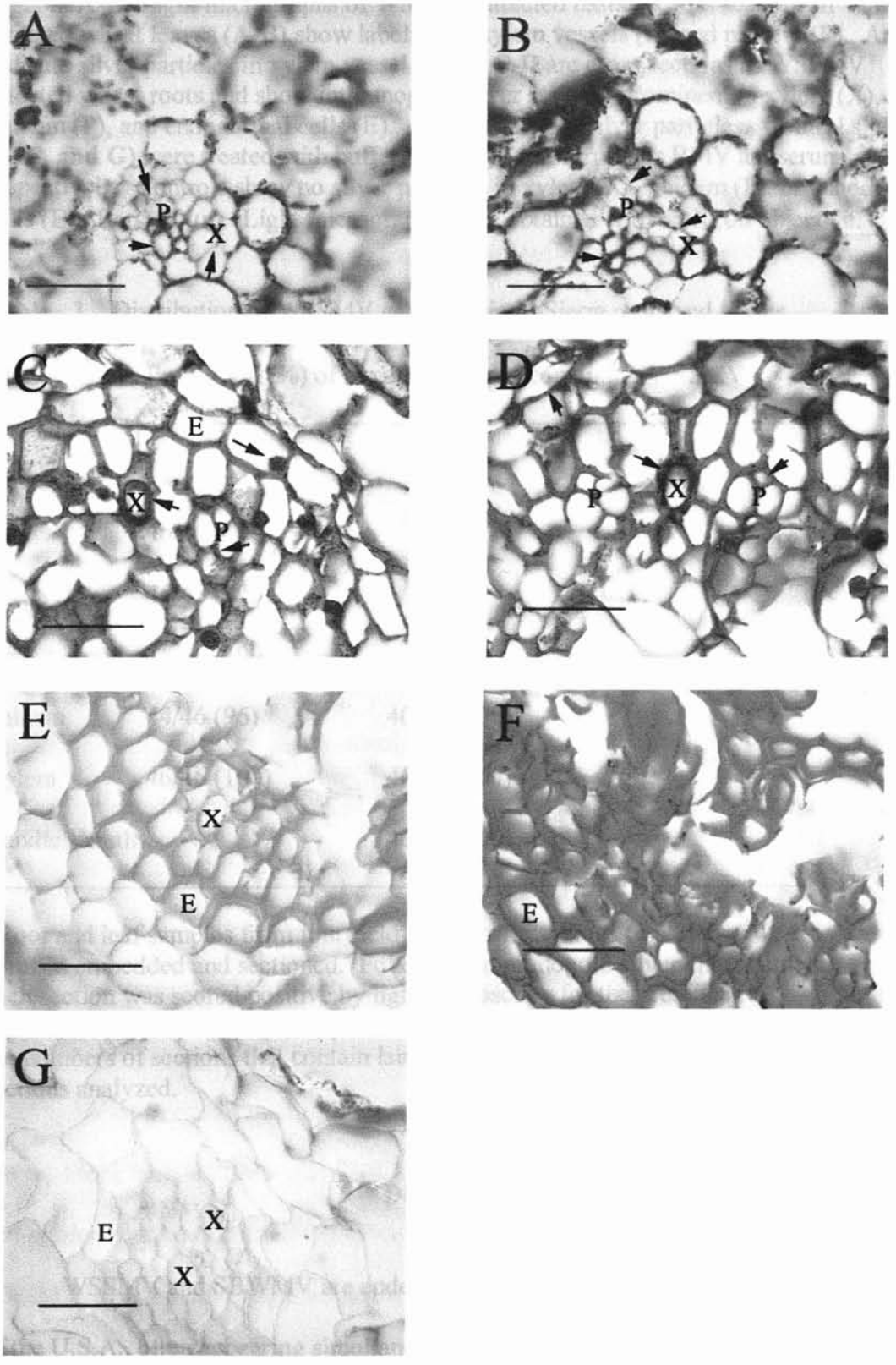
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Fig. 4. A-G. Light micrographs of WSSMV infected tissue. Cross sections of WSSMV infected wheat leaves **(A-B)** show labeling in xylem vessels (X) and phloem (P). Arrows indicate silver particles in xylem vessel walls. **C-D** are cross sections of WSSMV infected wheat roots and show immunogold silver staining prominent in xylem (X), phloem (P), and endodermal cells (E). Arrows indicate silver particles. Control slides **(E, F, and G)** were treated with buffer, SBWMV antiserum, or BMV antiserum, respectively. Controls show no silver particles in xylem (X), phloem (P), or endodermal cells (E). Bars: 20μm. Light micrographs were obtained with an X100 objective.

Table. 3. Distribution of WSSMV coat protein in Sierra roots and leaves.

Tissue	Proportion (%) of positive sections containing WSSMV coat protein[a]	
	Root	Leaf
Cortex	46/46 (100)	
Pericycle	46/46 (100)	
Endodermis	46/46 (100)	
Phloem	44/46 (96)	40/40 (100)
Xylem	46/46 (100)	40/40 (100)
Bundle sheath		40/40 (100)

[a] Root and leaf samples from four field-grown wheat plants (cv. Sierra) were paraffin embedded and sectioned. Following immunogold silver enhancement, each section was scored positive by light microscopy for the presence of WSSMV coat protein in xylem, phloem, and associated tissues. Proportions are the numbers of sections that contain labeled relative to the total numbers of sections analyzed.

DISCUSSION

WSSMV and SBWMV are endemic in the hard red winter wheat-growing areas of the U.S.A., often appearing simultaneously in the same field, and acting synergistically in the same host plant (Brakke and Langenberg, 1988; Wiese, 1987). SBWMV proteins

were shown to be associated with WSSMV pinwheel-type inclusion bodies in cytological studies of plants co infected with WSSMV and SBWMV (Langenberg, 1985, 1986). Because plants used in this study were field-grown for six months prior to embedding in Paraplast, control experiments were conducted to ensure that the samples were only infected with WSSMV. Segments of infected wheat roots (cv. Sierra) were analyzed with SBWMV antiserum, immunogold silver enhancement, and light microscopy (Fig. 4 F), and no evidence of SBWMV was found.

Wheat spindle streak mosaic disease symptoms can be variable, and are more extreme when temperatures are below 10°C. The symptoms are more prevalent on lower leaves since the plants tend to outgrow the disease when ambient temperatures rise later in spring. Due to the difficulty of maintaining growth chamber conditions in the optimal range for WSSMV to flourish (between 8 and 12°C), plants used in this study were obtained from the nursery that routinely exhibits WSSMV symptoms (Wiese, 1987).

In a previous study, SBWMV infected material grown in the same nursery was used to study the pattern of virus accumulation in infected wheat (Verchot et al., 2001). Paraplast and LR-White embedded wheat leaves, stems, and roots were analyzed using SBWMV antiserum light or electron microscopy. SBWMV virus was detected primarily in the xylem suggesting that virus likely uses the xylem for long distance transport from roots to leaves (Verchot et al., 2001).

Since both WSSMV and SBWMV are transmitted to wheat roots by *P. graminis* and produce a systemic infection in wheat plants, we predicted that the pattern of WSSMV accumulation might resemble the pattern of SBWMV accumulation. We also

predicted that since WSSMV and SBWMV cause a synergistic disease, it is likely that they may utilize the same mechanism for vascular transport, via the xylem.

The data presented in this study suggest that WSSMV, unlike SBWMV, does not preferentially move from roots to leaves through the xylem. WSSMV was detected in 100% of the xylem vessels in leaves and roots examined, and also in 100% of the phloem elements in leaves, but only detected in approximately 96% of the phloem cells examined in roots (Table 3). Since WSSMV can be detected in both xylem and phloem tissues, we cannot conclude, based on these observations, whether virus preferentially moves through the xylem or phloem.

CHAPTER IV

PROPAGATION OF AN OKLAHOMA ISOLATE OF *POLYMYXA GRAMINIS* AND *SOILBORNE WHEAT MOSAIC VIRUS* IN HYDROPONICALLY GROWN BARLEY ROOTS

ABSTRACT

Polymyxa graminis is an obligate parasite of winter wheat in the central great plains of the U.S.A. *P. graminis* transmits *Soilborne wheat mosaic virus* (SBWMV) and *Wheat spindle streak mosaic virus*, which can both cause significant yield losses in winter wheat in this region. A viruliferous isolate of *P. graminis* obtained from a field in Oklahoma was propagated in barley roots grown in sand culture. Previous attempts to establish *P. graminis* in wheat roots grown in sand culture were unsuccessful; however in this study we were able to culture the fungus in barley roots. Eleven barley cultivars were tested to identify the most suitable experimental host for *P. graminis.* ELISA analyses were conducted using plant leaves to assess transmission of SBWMV. Two cultivars were identified as useful experimental hosts for studying *P. graminis* and its ability to transmit SBWMV.

INTRODUCTION

Polymyxa graminis Ledingham is a biotrophic parasite of many cereal crops and is a vector for many plant viruses including *Soilborne wheat mosaic virus* (SBWMV), *Wheat spindle streak mosaic virus* (WSSMV) and *Barley yellow mosaic virus* (BaYMV) (Verchot-Lubicz, in press; Adams et al.,1988; Adams, 1991). These three viruses cause

disease in winter wheat and barley causing significant yield loss throughout North America, Europe, and Asia (Brakke and Langenberg, 1988; Chen, 1993). Under field conditions, *P. graminis* infects plant roots in the autumn soon after plants are sown with virus symptoms appearing later in the winter or early spring (Wiese, 1987). Thus the development of control measures to limit *P. graminis* or virus infection is important (Hsu and Brakke, 1985; Himmel et al., 1991; Myers et al., 1993).

Since *P. graminis* is an obligate root parasite, sand-culture techniques are useful to study influences on the fungal life cycle or its ability to transmit plant viruses. A sand-culture method was devised to propagate a U.K. isolate of *P. graminis* in barley roots (Adams et al., 1986; Adams and Swaby, 1988). An intermittent irrigation system, previously used to culture *Olpidium spp.* and other zoosporic fungi, was adapted for the propagation of *P. graminis* in barley (Adams et al., 1986; Adams and Swaby, 1988). That sand-culture system has been valuable for studying the effects of temperature and barley cultivar on *P. graminis* growth and BaYMV transmission (Adams and Swaby, 1988).

Until now, sand-culture techniques have not been successfully used to propagate an Oklahoma *P. graminis* isolate in winter wheat (Verchot-Lubicz and Littlefield, unpublished data). However, an exhaustive search of appropriate experimental wheat hosts has not been conducted. In this study we used a sand-culture method, similar to the one reported by Adams et al. (1986) to propagate an Oklahoma isolate of *P. graminis* in barley roots.

MATERIALS AND METHODS

Plant material and hydroponic system used for propagating barley

In this study, ten cultivars of spring barley were used: B1202, Baronesse, Black Hulless, Bowman, Crystal, Excel, Harrington, Morex, Robust, and Stander. One cultivar of winter barley (Post 90) and one cultivar of winter wheat (Sierra) were used. Seven plants of each cultivar were grown in sterile quartz silica sand (Unimin Corp., Guion, AR) in Conetainers (Ray Leach "Conetainers"^{TM}, Hummert International, Earth City, MO) for each experiment. Thus, a total of 77 plants were planted for each experiment. The conical bottom of each Conetainer was cut off and covered with 100 μm mesh nylon membranes (Spectra/Mesh, Fisher Scientific, Pittsburgh, PA). Conetainers were placed in an ebb and flow hydroponic tank (Foothill Hydroponics, North Hollywood, CA.) (Fig. 5) filled with a nutrient solution (2.5 mM KNO_3, 1.0 mM KH_2PO_4, 1.0 mM $NaNO_3$, 0.75 mM Ca $(NO_3)_2$, 0.75 mM $MgSO_4$, 1.0 mM $FeSO_4$, 0.1 ppm $MnCl_2$, 0.05 ppm H_3BO_3, 0.025 ppm $ZnSO_4$, 0.005 ppm $CuSO_4$, and 0.005 ppm $(NH_4)_2MoO_4$) (Adams et al., 1986). The hydroponic tank was automatically filled for two periods of six hours each day and was maintained in a controlled environmental chamber at 15-to18 °C with a minimum day length of 12 h. The nutrient solution was changed weekly.

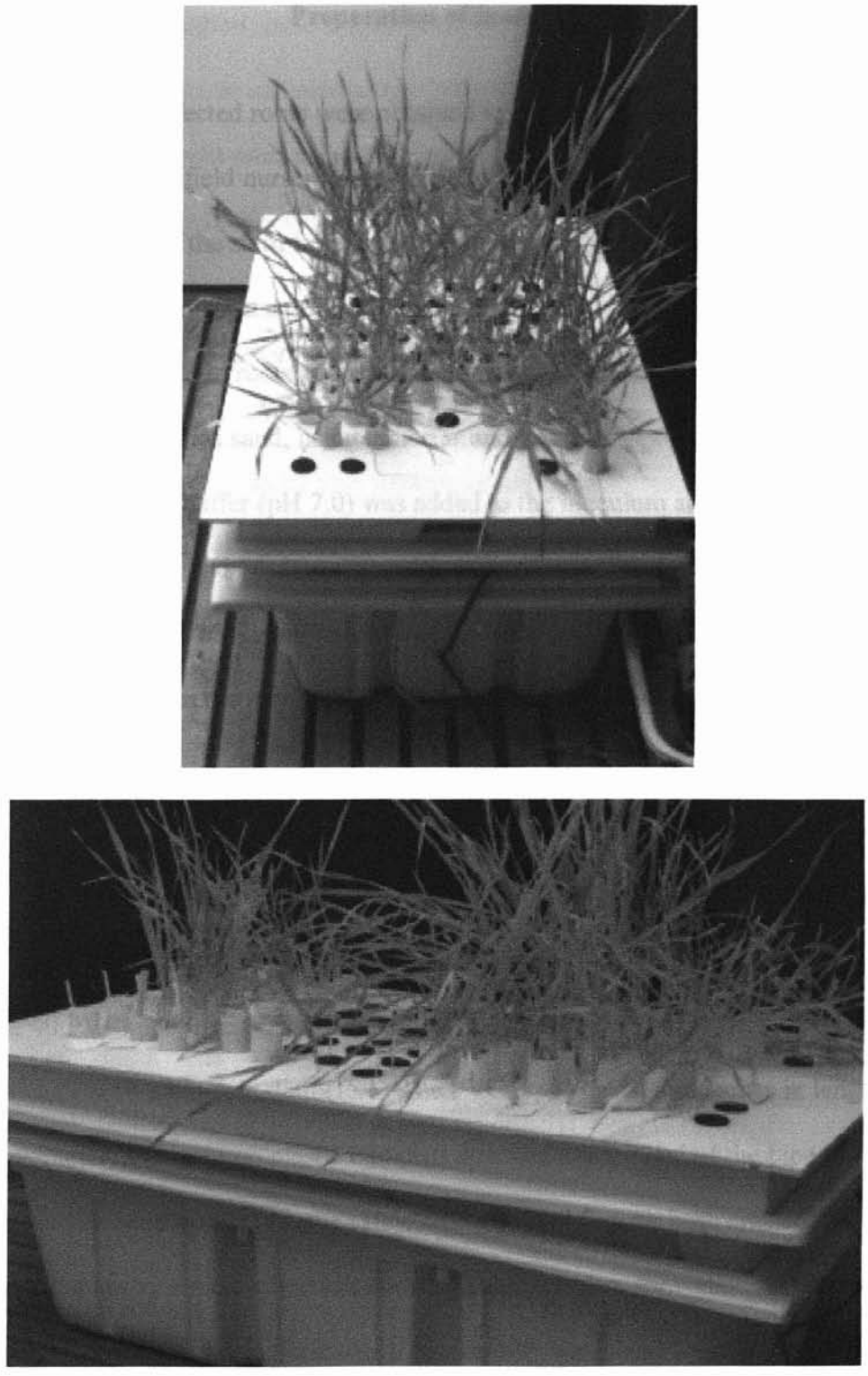


Fig. 5. Sand-culture system used for propagating *P. graminis* in barley roots. The system is an ebb and flow hydroponic system. The lower reservoir contains 40 liters of nutrient solution. A pump fills the top reservoir with solution that bathes the roots continuously for 6 hours, then a timer shuts the pump off for 6 hours.

Preparation of inoculum

P. graminis infected roots were obtained in September 2001 from winter wheat (cv. Vona) grown in a field nursery near the Oklahoma State University campus. Roots were cut at the base of the stem from field grown plants, dried on paper for one week in the greenhouse, and stored in a desiccator at room temperature. To inoculate plants, approximately five g dried roots, were cut finely with a scissors and ground with one tablespoon of sterile silica sand, using a mortar and pestle. Approximately 300 ml cold 10 mM Na-phosphate buffer (pH 7.0) was added to the inoculum and mixed thoroughly.

Three-, seven-, and 14-day-old seedlings were inoculated with approximately three ml of liquid inoculum. Liquid inoculum was pipetted into each Conetainer and remaining solid root material from the mortar was added to the sand in each Conetainer.

Root washing system

With the kind assistance of Dr. Nathan Walker, a large root washing apparatus (Fig. 6), similar to one used for smaller grass roots, was constructed using 1.5 inch schedule 40 PVC pipe, five 0.25 inch brass air hose connections, and five, six-inch pieces of plastic tubing (0.25 inch in diameter). The PVC pipe was twelve inches in length, 1.5 inch in diameter, and had two plastic end caps attached at each end of the pipe as closures. Holes were drilled into the side of the pipe and five air hose connections were attached using epoxy resin to function as one inlet attachment for water entering the system, and four outlet connections which channel the water into four "root-washing containers". Six-inch pieces of plastic tubing (0.25 inch in diameter) were attached to the single inlet brass connection and the four outlet brass connections. The inlet plastic tube

was connected to a distilled water faucet. The four outlet tubes were each connected to 705 ml Rubbermaid plastic containers through holes in the lid. The four Rubbermaid containers served as "root-washing containers".

Water flowed from the faucet through the PVC pipe, through the outlet tube, into the "root-washing container". A square hole was cut into the side of each "root-washing container", which served as a drain, allowing water to flow out of the container. A stainless steel mesh was glued using epoxy resin over the square hole to retain the root mass inside the container. Barley root masses were cut at the base of the stem, most of the sand was shaken off, and the root masses were divided in half longitudinally. Each barley root mass was added to separate "root-washing containers" and using this system, four root masses could be simultaneously washed. Root masses were washed vigorously for one hour to remove sand, and micro fauna.

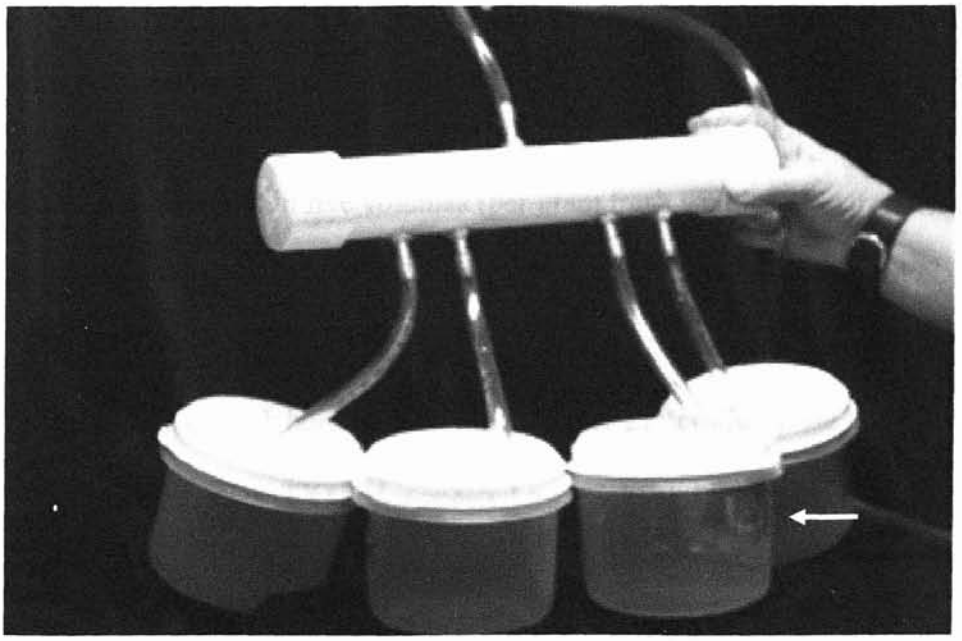


Fig. 6. Home made root-washing system. The stainless steel mesh retained the root mass inside the "root-washing containers" during the washing process.

Microscopy

Individual plants were removed from Conetainers four to six wpi (weeks post inoculation), and washed using the root washing system. Washed roots were placed in distilled water in a 100-ml beaker, and vacuum infiltrated for 20 min to remove air from the interior of the roots. Small branch roots, located within four cm of the stem were examined by light microscopy to detect *P. graminis*. Light microscopy was conducted with a Nikon Eclipse E600 microscope. Images were recorded using Optronics Magnafire digital camera (Goleta, CA) and Image-Pro Plus software (Silver Spring, MD). Figures were arranged using Adobe Photoshop software (Adobe Systems, Inc., Mountain View, CA).

ELISAs

Approximately four to six wpi, plants were scored for presence or absence of virus symptoms. Upper non-inoculated barley leaves (0.5 g), were collected and analyzed by indirect ELISA using monoclonal SBWMV antiserum (Driskel et al., 2002). Leaves were ground in five volumes (per gram fresh weight) of extraction buffer (137 mM NaCl, 8 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 2.7 mM KCl, 10.3 mM Na_2SO_4, 2% Tween 20, 2% polyvinylpyrrolidone [PVP 24 to 40,000], and 0.2% chicken albumin). A 100µl sample of each homogenate was added to a microtiter plate (Nalge Nunc International, Dallas, TX) and incubated overnight at 4 °C. Microtiter plates were washed with buffer containing phosphate-buffered saline plus Tween-20 (PBST) (137 mM NaCl, 8 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 2.7 mM KCl, 10.3 mM Na_2SO_4, and 2% Tween 20) and incubated for 1 h with SBWMV antiserum diluted 1:50 in PBST

containing 0.2% bovine serum albumin (BSA) and 2% PVP 24 to 40,000. Plates were washed with PBST buffer and incubated with horse-peroxidase-labeled secondary mous antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, MD) that was diluted 1:1,000 in PBST containing 0.2% BSA and 2% PVP 24 to 40,000. *o*-Phenylene diamine substrate tablets (Amresco, Solon, OH) were dissolved in 30 ml of sterile H_2O containin 15 µl of 30% H_2O_2, and 100 µl of substrate was added to each microwell. The reaction was stopped with 250 mM H_2SO_4 within 5 min of color development. The absorbance was read at 490 nm on an MRX plate reader (Dynatech Laboratories, Chantilly, VA).

RESULTS

Three barley cultivars are useful experimental hosts for *P. graminis*

Inoculum of *P. graminis* was prepared using roots of winter wheat plants (cv. Vona) grown in a field near the Oklahoma State University campus. Ten spring and one winter barley cultivar were inoculated with *P. graminis* and then scored for the presence or absence of sporosori. Since that isolate of *P. graminis* is known to transmit SBWMV, plants were also tested for the presence of virus in the leaves.

At four to six wpi each plant had a tortuous root system that was extremely difficult to clean of sand particles without damaging the roots, therefore, a homemade root washing system was employed to gently clean plant roots for microscopic analysis (Fig. 6). At four wpi, an average plant root was 30 cm in length and the wet weight averaged four g. The root ball produced was quite massive, considering the diameter of the individual Conetainers was small. The sheer mass of each root system necessitated choosing a fairly small number of root segments for examination.

The greatest proportions of plants that were positive for *P. graminis* sporosori were observed among the plants inoculated three dpp (days post planting) (Table 4). *P. graminis* sporosori were detected in the roots of nine cultivars following inoculation thre dpp. Eight cultivars accumulated greater than 100 sporosori per cm root segment (Table 4) and one cultivar, Stander, accumulated less than 20 sporosori per cm root segments. Between 80 and 100 percent of Harrington, Crystal, and B1202 contained *P. graminis* sporosori (Table 4) indicating that these three are likely the best hosts for *P. graminis*. Although 60 percent of Stander plants were positive for *P. graminis* sporosori, the level of colonization was comparatively low (Table 4). Two cultivars, Baronesse and Robust, did not accumulate *P. graminis* following inoculation three dpp.

Fewer plants were infected with *P. graminis* following inoculation at seven or 14 dpp. Among these plants, *P. graminis* sporosori were detected in the roots of five cultivars. Only one or two plants of each cultivar were positive, indicating that the rate of *P. graminis* infection was low. The greatest *P. graminis* infection occurred in Black Hulless and Post 90, where an average of 20 and 184 *P. graminis* sporosori per cm root were detected, respectively (Table 4). In combination, these data suggest seedlings should be inoculated at three dpp.

Table. 4. Proportion of barley plants infected with *P. graminis* following inoculation 3 7, and 14 dpp.

	Plant inoculated 3 dpp		Plant inoculated 7 –14 dpp	
Barley Cultivar	Proportion infected plants[a]	Avg. no. sporosori/ cm root[b]	Proportion infected plants[a]	Avg. no. sporosori/ cm root[b]
B1202	5/5	>100	1/6	1
Baronesse	0/5	-	0/6	-
Black Hulless	1/5	>100	1/7	20
Bowman	1/5	>100	0/6	-
Crystal	5/5	>100	0/6	-
Excel	1/5	>100	0/7	-
Harrington	4/5	>100	1/6	1
Morex	2/5	>100	1/6	1
Post 90	3/6	>100	2/8	184
Robust	0/5	-	0/6	-
Stander	3/5	<20	0/7	-

[a] Root segments from five to eight plants of each cultivar were observed for the presence of *P.graminis* sporosori. The numbers of plants positive for *P. graminis* relative to the total numbers of plants analyzed for each cultivar are indicated.
[b]The numbers of sporosori were counted in one cm root segment. The average number of sporosori in 6 segments is reported. There was a greater number of *P. graminis* sporosori in roots inoculated 3 dpp (days post planting) than in roots inoculated 7 to 14 dpp.

Microscopy of *P. graminis* in barley roots

In this study emphasis was placed on identifying zoosporangia, sporogenic plasmodia, mature sporosori and resting spores. Fig. 7 shows a diagrammatic representation of the *P. graminis* life cycle taken from Karling (1968) and Ledingham (1939).

In B1202 roots harvested between four and six wpi, most life cycle stages were detected and, often, more than one stage would be observed in a single root segment. Zoosporangia and sporogenic plasmodia were seen in root epidermal and cortical cells (Fig. 8 A-D). Zoosporangia were sometimes tubular and sometimes lobed with cross-walls dividing it into segments (Fig. 8 A, B). Exit tubes, appearing to abut on the cell wall, function as release channels of secondary zoospores to the root exterior (Fig. 8 A).

Plasmodia prior to cleavage and cyst formation were observed (Fig. 8 C-E). Plasmodia (Fig. 8 D) do not have a discernable limiting membrane and were often in motion, changing shape during observation (Fig. 8 D). Ledingham also described plasmodia as being long and slender, and highly motile. Plasmodia (Fig. 8 C) that appeared to be lobed thalli were observed and we were unable to determine if they were plasmodia just prior to cleavage into cysts or developing zoosporangial thalli. Resting spores and mature sporosori were most easy to detect (Fig. 8 E, F). The mature sporosori were spherical or ovoid in shape and contained aggregates of resting spores.

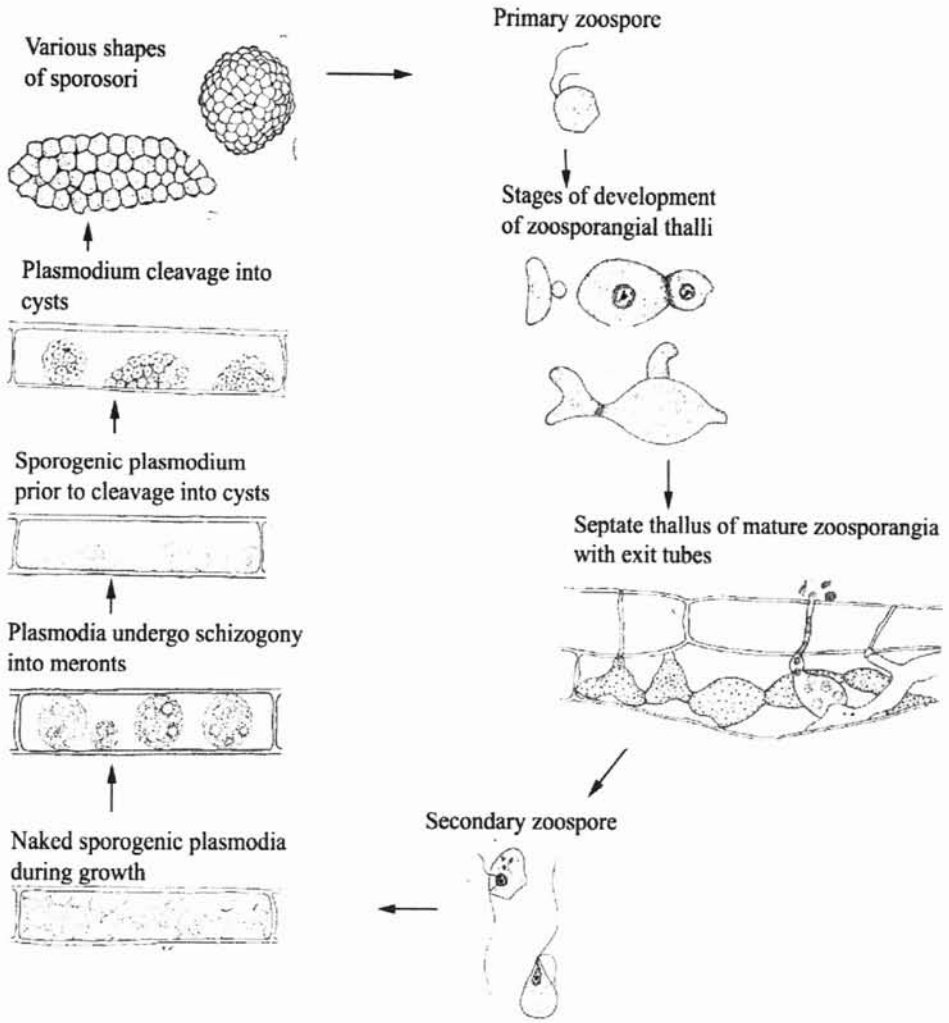


Fig. 7. The *P. graminis* life cycle reported by Karling (1968). Images were taken from Ledingham (1939).

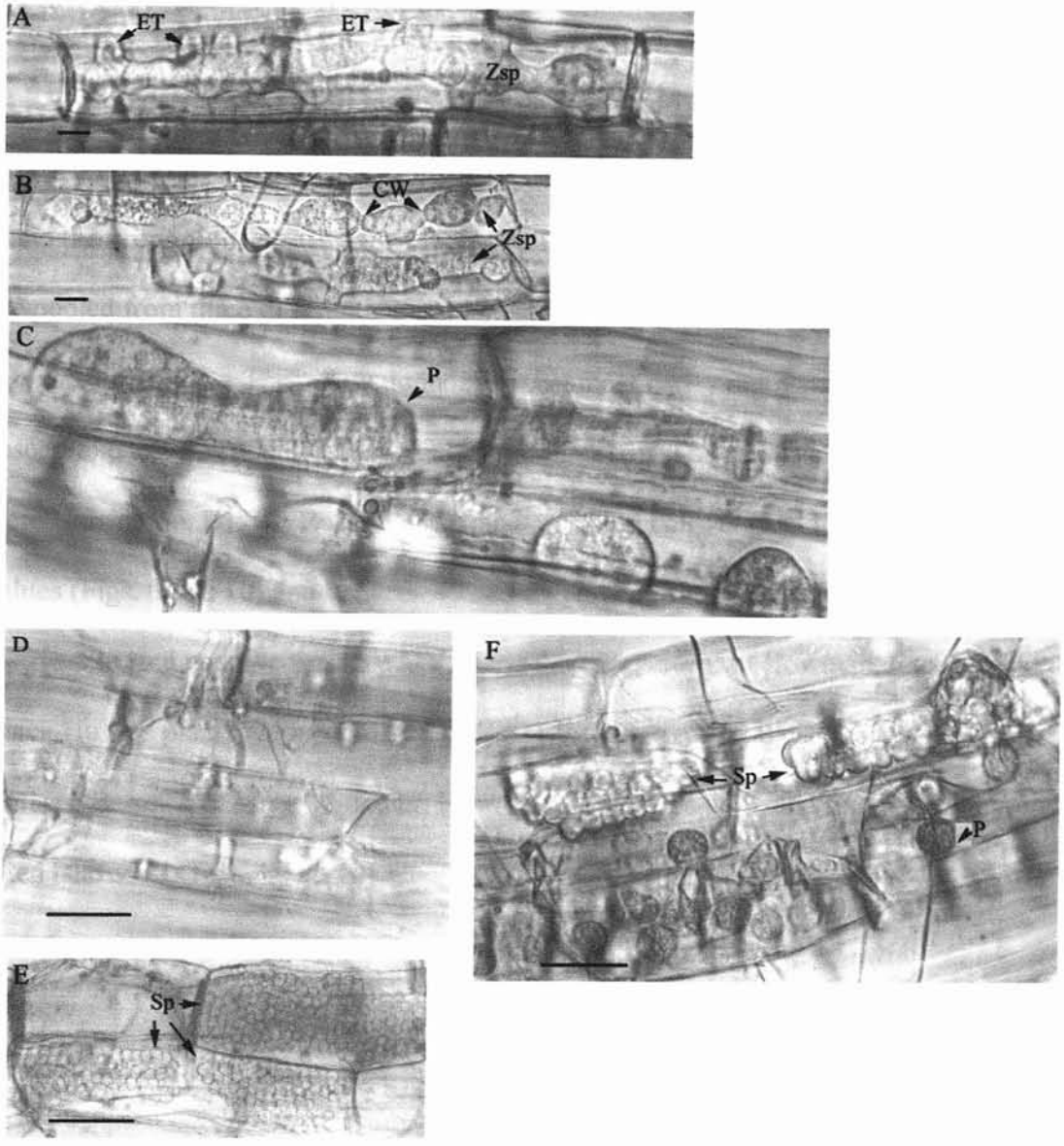


Fig. 8. Images of *P. graminis* in barley roots. **A** Zoosporangium (Zsp) with exit tubes (ET). Exit tubes abut cell wall. **B** Zoosporangium has many barrel-shaped lobes and crosswalls (CW). **C** Plasmodia (P) prior to cleavage into cysts. **D** Plasmodium, often appearing branched, can change shape during observation. **E** Sporosori (Sp) contain numerous resting spores. **F** More than one life cycle stage can be viewed in a root segment. This image contains sporosori and immature plasmodia that likely will cleave into cysts. Bars = 10μm.

SBWMV systemic infection of barley

At most, 55 percent of plants tested for each cultivar were positive for SBWMV (Table 5 and Figs. 9 and 10). The proportions of plants positive for *P. graminis* in Table 4 and the proportions positive for SBWMV, Table 5, do not coincide because these data are pooled from three different experiments that produced extremely different levels of *P. graminis* infection. These data do indicate that most barley cultivars tested were susceptible to SBMWV (Table 5, and Figs. 9 and 10). The most susceptible cultivars were Harrington, B1202, Robust, and Stander. These cultivars had the greatest ELISA values (Figs. 9 and 10) and between 45 and 55 percent of the plants analyzed, from these cultivars, tested positive for SBWMV. Less than 25 percent of Bowman, Excel, Post 90, Black Hulless plants were positive for SBWMV. The ELISA values among Black Hulless, Baronesse, Morex, Stander, Bowman, Crystal, Post 90, and Excel were more often below 0.1 (Figs. 9 and 10).

Table. 5. Proportion of SBWMV infected barley foliage following inoculation 3, 7, and 14 dpp.

Barley cultivar	Proportion infected plants[a]
B1202	6/11
Baronesse	3/9
Black Hulless	2/11
Bowman	2/10
Crystal	3/11
Excel	1/10
Harrington	4/12
Morex	3/11
Post 90	2/10
Robust	5/10
Stander	5/10

[a] Leaves were taken from 2 to 4 plants of each cultivar inoculated at 3, 7, and 14 dpp (days post planting) and analyzed by ELISA to detect SBWMV. The numbers of plants positive, by ELISA, for SBWMV, relative to the numbers of plants analyzed are shown.

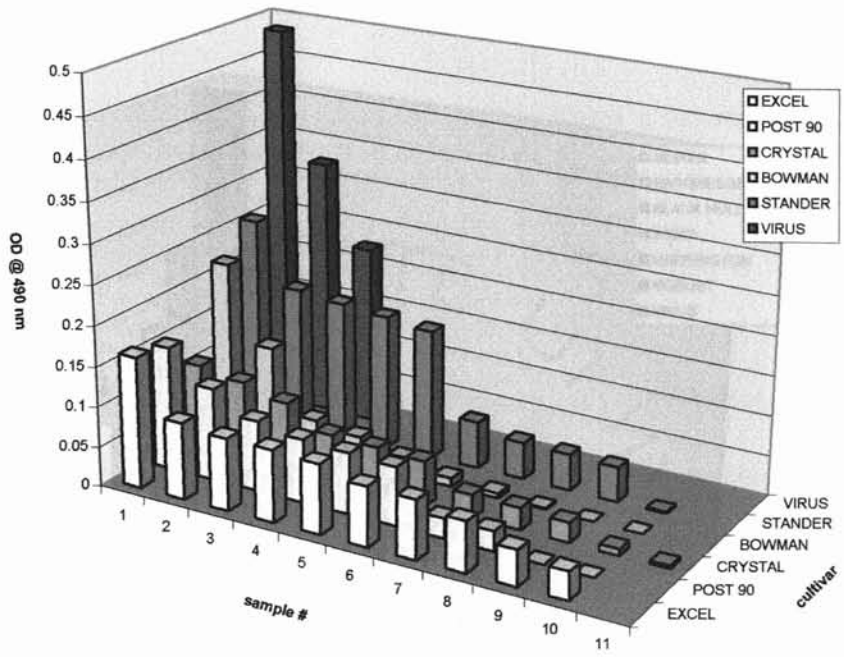


Fig. 9. Five sets of ELISA values obtained from barley grown in sand-culture. ELISAs were conducted using leaf extracts and SBWMV antiserum. ELISA values taken from plants from five cultivars inoculated at 3, 7, and 14 dpp. Values greater than 0.1 were determined to be positive for SBWMV. Between 9 and 12 plants were analyzed for each cultivar. All cultivars were susceptible to SBWMV infection. Each color represents ELISA values for plants of each cultivar.

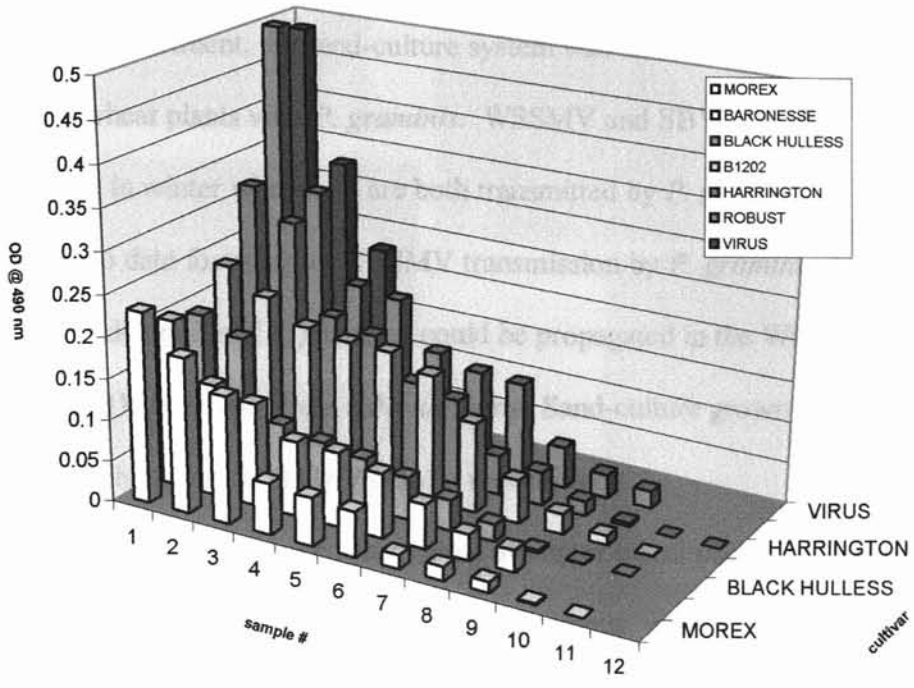


Fig. 10. Six sets of ELISA values obtained from barley grown in sand-culture. ELISAs were conducted using leaf extracts and SBWMV antiserum. ELISA values taken from plants from six cultivars inoculated at 3, 7, and 14 dpp. Values greater than 0.1 were determined to be positive for SBWMV. Between 9 and 12 plants were analyzed for each cultivar. All cultivars were susceptible to SBWMV infection. Each color represents ELISA values for plants of each cultivar.

P. graminis infection of winter wheat using the sand-culture technique

In a single experiment, the sand-culture system was tested to determine if it could be used to infect wheat plants with *P. graminis*. WSSMV and SBWMV often cause a synergistic disease in winter wheat and are both transmitted by *P. graminis*. Since there is no information to date focusing on WSSMV transmission by *P. graminis,* initial tests were conducted to determine if *P. graminis* could be propagated in the WSSMV-susceptible, SBWMV-resistant wheat cultivar Sierra. Sand-culture grown Sierra plants would be useful in the future to study WSSMV transmission.

P. graminis inoculum, derived from roots of winter wheat plants (cv. Vona) grown in a field near the Oklahoma State University campus, was used to inoculate two Sierra plants at three dpp. Root segments were harvested four to six wpi and were studied microscopically to detect *P. graminis*. As in the B1202 barley roots, zoosporangia, sporogenic plasmodia, and sporosori were seen in root epidermal and cortical cells (Fig. 11 A through G).

In these wheat roots zoosporangia at varying developmental stages were observed. The septate zoosporangial thalli were observed with and without exit tubes (Fig. 11 A, B, D and F). Exit tubes develop in mature zoosporangia and pass to the exterior of the cell. Dense regions in the zoosporangia may be secondary zoospores, which will pass through the exit tubes to the exterior of the cell (Fig. 11 B). Young sporogenic plasmodia were observed (Fig. 11 C) that filled the entire cell. Mature sporosori were observed along the length of the root and near the root tip, within the zone of elongation (Fig. 11 E and G).

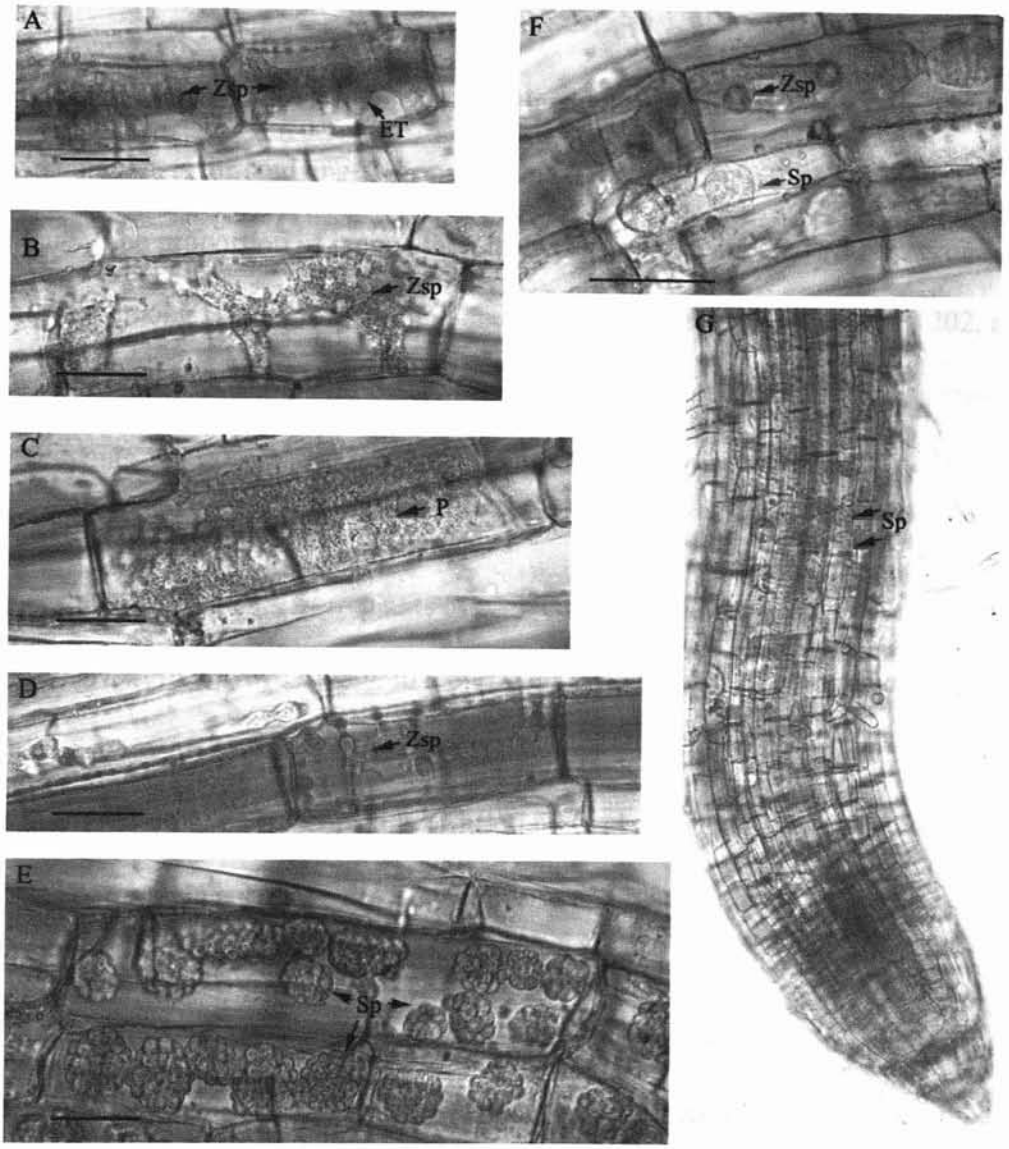


Fig. 11. Images of *P. graminis* in wheat roots. **A, B**, **F** and **D** show different zoosporangial stages (Zsp). **A** shows exit tubes (ET). **C** contains the sporogenic plasmodium (P) that takes up the entire cell. **D** shows young zoosporangia. **E** cells contain numerous sporosori (Sp). **F** shows multiple life cycle stages can occur in a root segment. **G** *P. graminis* infection can occur near the root tip. Bars = 10 μm.

DISCUSSION

Results in these studies indicate that Harrington and B1202 may be useful experimental hosts for studying *P. graminis* and SBWMV using the sand-culture technique. The proportion of inoculated plants that became infected with *P. graminis* was highest among Harrington, Crystal, and B1202 cultivars. Harrington, B1202, and Robust were most susceptible to SBWMV, having the greatest proportion of virus-infected plants and the greatest ELISA values among the infected plants.

To compare host susceptibility to *P. graminis,* we counted the number of sporosori in root segments from plants of each cultivar. Mature sporosori are the most visible *P. graminis* life cycle stage to identify and enumerate. The irregular, grape-like clusters of sporosori scattered in the cortex of barley roots are often pigmented, making them easy to identify microscopically (Littlefield et al., 1998). Often there can be multiple sporosori in a single cell. Zoosporangia and plasmodia are often less refringent than sporosori, and therefore are not as easy to quantify.

In general, plants may be more susceptible to *P. graminis* when inoculated at three dpp than at seven or 14 dpp. *P. graminis* root colonization may depend on both developmental and environmental factors (Adams and Swaby, 1988; Teakle 1988). Previous studies have indicated that *P. graminis* prefers to colonize roots within four cm of the sand surface, where the concentration of oxygen is greater (Gerik, 1992; Verchot et al., 2001). *P. graminis* may also prefer to infect young developing roots. Root cells within the top four cm of the sand culture are developmentally different at three, seven and 14 dpp. At three dpp the roots are barely emerging while at seven to 14 dpp, roots are approximately 10 to 15 cm in length.

The U.K. isolate of *P. graminis* is a vector for BaYMV. In previous studies, *P. graminis*- and BaYMV-susceptibility of cereals and grasses was tested using a viruliferous *P. graminis* isolate from the U.K. as well as European cultivars of barley, wheat, oats, and rye (Adams et al., 1987; Adams and Jacquier, 1994). Studies have not been previously conducted using any U.S. isolates of *P. graminis* or SBWMV. Unfortunately, the barley cultivars tested in the U.K. were not available for our studies, and therefore we could not compare the susceptibility of barley to the different *P. graminis* isolates. In the U.K. studies, 51 accessions of *Hordeum* spp. were tested and all were susceptible to *P. graminis* (Adams and Jacquier, 1994). In contrast, we found variation in the susceptibility of U.S. barley cultivars to the Oklahoma isolate of *P. graminis* and SBWMV. In this study of *P. graminis* infected plants, *P. graminis* sporosori always accumulated to high levels, but the numbers of plants for each cultivar that were positive for *P. graminis* varied.

In previous studies, plants were assessed for the presence of zoosporangia or resting spores and the intensity of *P. graminis* colonization was measured using a scale of zero to three (Adams et al., 1987; Adams and Jacquier, 1994). In this study, we directly quantified the number of sporosori in a random sampling of plant roots. Using this sampling technique we were better able to compare relative differences in infection of different hosts. This method of sampling also allowed us to determine more accurately the best time to inoculate roots, and to maximize *P. graminis* colonization.

In this study we observed that many, if not all, *P. graminis* life cycle stages can be detected simultaneously in one root system. This may be due to the life cycle of the plasmodiophorid itself. Since *P. graminis* regularly produces primary and secondary

zoospores within a short time span, secondary or multiple infections may also occur in one root system. Thus, *Polymyxa graminis* infections in wheat and barley may appear to be asynchronous.

In Oklahoma, *P. graminis* and SBWMV cause disease in winter wheat. In this study, experiments were conducted using one winter barley (Post 90) and ten spring barley cultivars. The infestation rate for *Polymyxa graminis* in Post 90 was less than 50% at three dpp, indicating that it was not a suitable cultivar for sand cultivation of the fungus. Furthermore, Post 90 was not an effective host for SBWMV (Table 5, Fig. 9). Since only one winter barley cultivar was tested, we cannot draw conclusions comparing the differences between spring and winter cultivars. However, these data indicate that while *P. graminis* vectors disease in winter wheat in Oklahoma, this isolate is not exclusively a parasite of winter grown cereals.

CHAPTER V

A COMPARISON OF VIRUS-VECTOR INTERACTIONS BETWEEN SOIL-GROWN WHEAT AND SAND-CULTURE GROWN BARLEY

ABSTRACT

The study of virus-vector interactions between *Polymyxa graminis* and *Soilborne wheat mosaic virus* (SBWMV) and *Wheat spindle streak mosaic virus* (WSSMV) has been complicated by the fact that the plasmodiophorid vectors is an obligate parasite of wheat and other cereal crops. With the advent of the sand-culture system (Adams et al., 1986), further exploration can be made in the field of virus-vector interactions. We previously examined wheat roots grown in infested field soil for the presence of *P. graminis* resting spores, SBWMV viral RNA, coat protein, and movement protein. In this follow-up study, we grew barley in the sand-culture system, embedded root segments containing *P. graminis*, and performed experiments to determine if the accumulation of virus was similar to that of soil-grown wheat roots. SBWMV movement protein and WSSMV coat protein were found in *P. graminis* resting spores. No SBWMV coat protein was found in *P. graminis* resting spores. SBWMV viral RNA was also found in *P. graminis* resting spores.

INTRODUCTION

Known fungal vectors of plant viruses include members of the Chytridiomycetes and Plasmodiophoromycetes. Two different mechanisms are involved in transmission of

plant viruses by these vectors. The spherical tombusviruses and necroviruses are transmitted by zoospores of the chytrid fungus *Olpidium brassicae* or *O. bornavanus* (Matthews, 1991). The viral coat protein is the only known factor to mediate tombusvirus-*Olpidium* interactions. Virions are adsorbed onto the surface of the zoospore and are carried into the plant cell during zoospore penetration. The filamentous furoviruses, benyviruses, bymoviruses, and pomoviruses are transmitted by zoospores of the plasmodiophorid organisms *Polymyxa graminis*, *P. betae*, or *Spongospora subterranean* (Matthews, 1991). These viruses encode a single transmission factor that is produced as a readthrough domain of the viral coat protein. These viruses are carried within the zoospore and are released when the zoospore establishes its own infection in a root cell.

P. graminis is the vector for *Soilborne wheat mosaic virus* and together they cause agronomically important disease in winter wheat grown in the Central Great Plains of the USA (Wiese, 1987). In the previous chapter, we have shown that a sand-culture technique can be used to successfully propagate an Oklahoma *P. graminis* isolate in barley roots (Adams et al., 1986). We also determined that at four weeks post inoculation (wpi), we could detect multiple life cycle stages of *P. graminis* in barley roots suggesting this is the optimum time to study *P. graminis* infection.

The objective of this study was to determine if the sand-culture system is an effective method of exploring virus-vector interactions. We embedded roots from winter wheat grown in fungus-infested soil and from barley grown in sand-culture to determine if the pattern of virus accumulation in culture reflects the pattern of virus accumulation in soil-grown roots.

MATERIALS AND METHODS

Embedding plant material in Paraplast and *in situ* hybridization

Root segments were taken from symptomatic hard red winter wheat plants (cv. Vona) grown in vector-infested soil and embedded in Paraplast (Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.) (Berlyn and Miksche, 1976; Verchot et al., 2001), as described in Chapter II. Root segments were collected within ca. four cm of the soil surface (Littlefield, 1994; Verchot et al., 2001). Samples were placed in fixative (10:2:1 dilution of ethanol, formaldehyde, and acetic acid) overnight at 4 °C then dehydrated in a graded series of 50, 60,70,85,95, and 100% ethanol. Ethanol was replaced with a graded series of 25, 50, 75, and 100% xylene. Paraplast chips were added to the samples at room temperature and samples were incubated overnight. Samples were infiltrated with Paraplast at 62 °C for three days, with molten Paraplast being changed twice each day. Samples were transferred to plastic molds filled with molten Paraplast and allowed to harden for 2 h in cool water (Verchot et al., 2001). For *in situ* RNA hybridization and light microscopy, Paraplast embedded wheat roots were sectioned (8.0 μm), and then mounted on ProbeOn Plus slides (Fisher Biotechnology). Slides were incubated with an RNA probe that can hybridize to viral RNAs, as described in Chapter II. To prepare the RNA probe we used the plasmid p5114, which is a pGEMT plasmid containing a cDNA copy of the 3' 1000 nt of SBWMV genome. Transcripts were prepared using *Apa*I linearized p5114 plasmids, SP6 polymerase, and digoxigenin labeled UTP.

The slides were incubated 15 to 20 min at room temperature in 2X SSC (17.5 g NaCl, 8.82 g sodium citrate/l of water, pH 7.0), then in proteinase K (1μg/ml of proteinase K dissolved in 100 mM Tris-HCl pH 8.0, 50 mM EDTA) at 37°C for15 to 20 min. Slides were washed for two min at room temperature in PBS-glycine buffer (0.13 M NaCl, 7.0 mM Na_2HPO_4, 3.0 mM NaH_2PO_4, 2.0 mg/ml glycine, pH 7.0), twice with PBS buffer and then incubated for 10 min at room temperature in 4% paraformaldehyde (pH 7.0) (dissolved in PBS). Slides were washed twice with PBS for five min at room temperature, twice with 0.1 M TEA buffer (1.492 g triethanolamine/100 ml water, 0.5 ml acetic anhydride) for 10 min at room temperature and then twice in PBS buffer for five min at room temperature. Slides were incubated 30 min at 37°C with prehybridization solution (50% deionized formamide, 5% dextran sulfate, 1% blocking reagent (Boehringer Mannheim), 500 μg/mL poly A, 300 mM NaCl, 10mM Tris-HCl pH 7.5, 1 mM EDTA) and then overnight at 42°C with hybridization solution (prehybridization solution plus digoxigenin-labeled RNA probe). Slides were then washed twice with 0.2X SSC for 60 min at room temperature, twice with NTE buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) for five min at 37°C, once in 0.2X SSC for 60 min at 55°C, and once in PBS for five min at room temperature.

For antibody labeling, slides were incubated 45 min with 1 % blocking reagent then with 1 % bovine serum albumin fraction V (both in a solution of 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Triton X-100). Slides were incubated for two h at room temperature with anti-digoxigenin labeled antibody conjugated with alkaline phosphatase (diluted 1:1250 in 100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.3% Triton X-100). Slides were washed twice for 15 min in a solution of 100 mM Tris-HCl (pH 7.5) and 150

mM NaCl followed by 10 min wash in a solution of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$.

For developing the reaction, the substrate solution was prepared immediately before use, by diluting 200 µl of NBT/BCIP stock solution (vial 4, Boehringer Mannheim DIG Nucleic Acid Detection Kit) in 10 ml buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$). The slides were incubated in darkness, with the color development solution, for two h to three days at room temperature then rinsed in TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA). Slides were dehydrated in a series of 25, 50, 75, and 100% ethanol, for a maximum of five sec in each step, to minimize color loss. The slides were dipped in xylene. A drop of Permount and a coverslip were added to each slide prior to viewing under the light microscope.

Embedding plant material in LR-White and immunofluorescence labeling

Root segments were taken from winter wheat plants (cv. Vona) grown in vector-infested soil, or from sand-culture grown four-week-old barley (cv. B1202) plants. All roots were collected from within ca. four cm of the soil surface. Segments were embedded in LR-White resin (Ted Pella, Inc., Redding, Calif., U.S.A.) as described previously (Littlefield et al., 1998). Root segments were fixed for two h at room temperature under vacuum in a solution containing 0.5% glutaraldehyde, 4.0% paraformaldehyde, and 100 mM sucrose in 50 mM sodium cacodylate buffer (pH 7.2). The samples were rinsed in 50 mM cacodylate buffer without the sucrose, and then post-fixed in aqueous 1% osmium tetroxide for one h at room temperature. Samples were rinsed in water, dehydrated in a graded series of water and ethanol, then infiltrated and

embedded in LR-White resin. Samples were cut with an ultramicrotome (1μ) and affixed on ProbeOn Plus slides (Fisher Biotechnology, Pittsburgh, PA). The slides were incubated in blocking solution (50 mM Tris-HCl, pH 7.4, 150 NaCl, 2% bovine serum albumin fraction V) for one h at 4°C, and then incubated with either undiluted SBWMV movement protein polyclonal antiserum or SBWMV coat protein antiserum (prepared by the Oklahoma State University Hybridoma Center), for one h. Slides were then incubated with secondary polyclonal FITC conjugated rabbit antiserum, diluted 1:50, for one h. Sierra root cross sections were also treated with *Wheat spindle streak mosaic virus* (WSSMV) antiserum obtained from Agdia (Elkhart, Ind.), SBWMV coat protein monoclonal antiserum (prepared by the Oklahoma State University Hybridoma Center), or buffer only. All control slides were treated with undiluted primary antibodies. Slides treated with SBWMV coat protein monoclonal antiserum were incubated with secondary monoclonal FITC-conjugated anti-mouse antiserum, diluted 1:50, for one h. Slides treated with WSSMV antiserum were treated with secondary polyclonal FITC conjugated anti-rabbit antiserum, diluted 1:50, for one h.

Microscopy

Samples were examined for *in situ* labeling of SBWMV RNA in *P. graminis* infected wheat roots using a Nikon Eclipse E600 (Nikon Inc., Dallas, TX) microscope. Images were recorded using the Optronics Magnafire digital camera (Goleta, CA), attached to the Nikon E600 microscope, and Image-Pro plus software (Silver Spring, MD). A Leica TCS SP2 (Leica Microsystems, Bannockburn, IL) confocal imaging system was used to study FITC labeling in sporosori, plasmodia, and zoosporangia found

in *P. graminis* infected wheat and barley roots. The Leica TCS SP2 system was attached to a Leica DMRE microscope. Both microscopes were equipped with water immersion objectives. All images were processed using Adobe Photoshop software (Adobe Systems Inc., Mountain View, CA).

RESULTS

Evidence that SBWMV accumulates in *P. graminis* resting spores in wheat roots

Accumulation of SBWMV in *P. graminis* resting spores in wheat roots was analyzed using immunofluorescence labeling and confocal microscopy. Cross sections of LR-White embedded wheat roots were analyzed using either using coat protein or movement protein antiserum. SBWMV movement protein, but not coat protein was detected in *P. graminis* resting spores (Table 6, Figure 12). In four sections labeled with coat protein antiserum, we viewed 17 sporosori and none were positive (Fig. 12 E). However, in seven sections labeled with movement protein antiserum we viewed 23 sporosori and 100% were positive (Fig. 12 C and D). Fluorescence was concentrated in the root cell walls and also in *P. graminis* resting spores. In root sections treated with a heterologous TMV antiserum, there was no fluorescence detected indicating that nonspecific labeling of the tissue was minimal (Fig. 12 F)

Wheat roots were also embedded in Paraplast and cross sections were analyzed using *in situ* hybridization to detect viral RNA. In three sections analyzed, viral RNA was detected inside nine *P. graminis* resting sporosori (Fig. 13 B). Viral RNA was detected in SBWMV infected wheat roots that were positive controls (Fig. 13 A), as in

Chapter II of this thesis. In sections treated with buffer and antiserum (no RNA probe) there was no label detected, indicating that nonspecific labeling did not occur (Fig. 13 C).

Table. 6. Proportion of *P. graminis* infected root sections of wheat and barley positive for SBWMV or WSSMV

	Wheat roots	***Barley roots***
vRNA	9/9 (s)	ND
CP	0/17 (s)	0/5 (s)
MP	23/23 (s)	16/16 (s, p, z)
WSSMV	ND	4/7 (s, p)
Buffer	0/21	0/6 (s)
TMV	0/22 (s)	ND

Three separate segments of *P.graminis* infested wheat and barley roots were sectioned and examined through *in situ* hybridization (viral RNA) or immunofluorescence confocal microscopy (CP, MP, and WSSMV) for presence of sporosori (s), plasmodia (p), and zoosporangia (z). Approximately 90 sections were examined; since results were similar among all sections, a subset of the data is listed here. Controls consisted of buffer only (no primary antiserum), and TMV antiserum. ND = not determined.

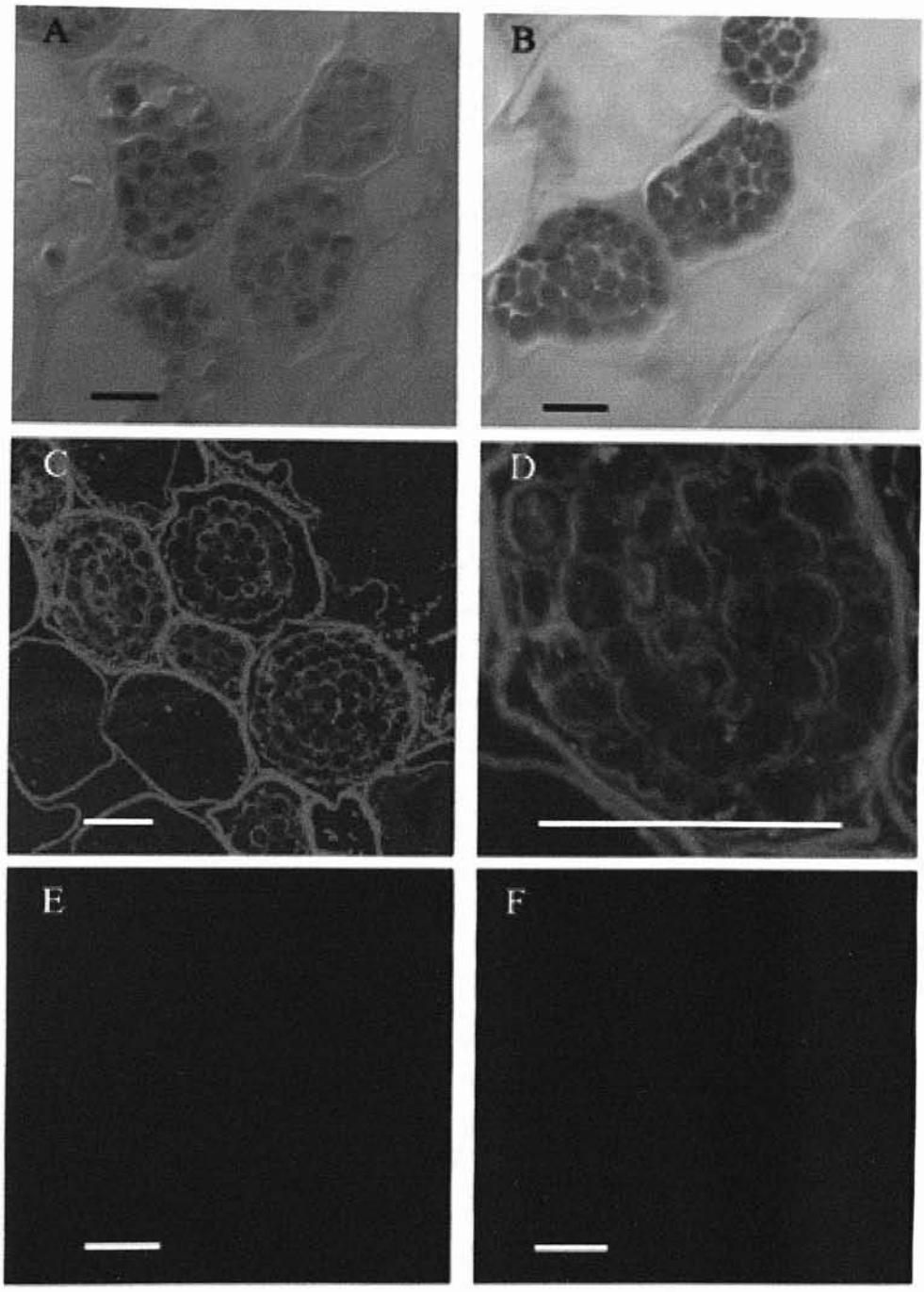
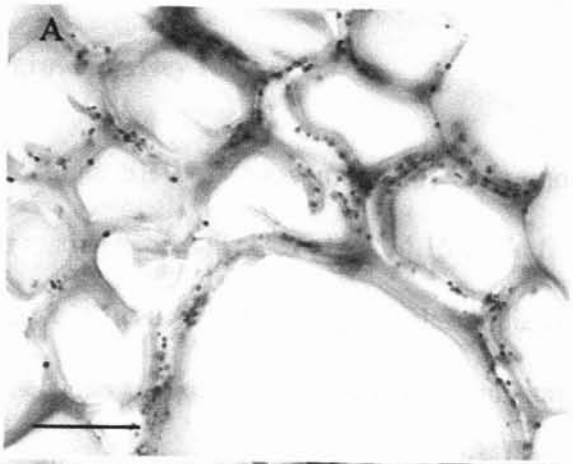
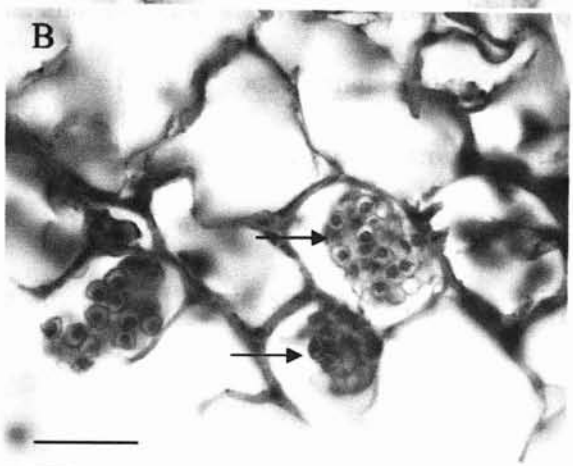


Fig. 12. Immunofluorescence labeling of movement protein in cross sections of wheat roots. **A, B** images taken using the transmitted light detector of the confocal microscope showing cross sections of sporosori. **C, D** images taken of sporosori that were labeled with movement protein antiserum. **E** image of sporosori labeled with coat protein antiserum. **F** images of sporosori treated with TMV antiserum. Bar = 20 μm.

In situ labeling SBWMV-infected wheat roots



In situ labeling SBWMV in *P. graminis* resting spores



SBWMV-infected wheat roots treated with buffer (no probe)

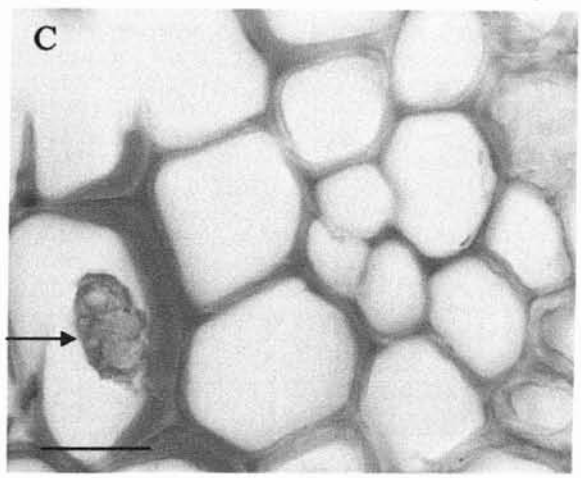


Fig. 13. *In situ* hybridization of SBWM- infected wheat roots, and viruliferous *P. graminis* sporosori. An RNA probe was used to detect SBWMV RNA. **A, B** Purple spots in wheat root cross sections and in sporosori cross sections indicate presence of SBWMV RNA. **C**, No purple spots were detected in samples treated with buffer and no RNA probe. Arrows in **B** and **C** indicate sporosori. Bars = 30 μm.

Evidence that SBWMV accumulates in *P. graminis* resting spores in sand-culture grown barley roots

As in the wheat root cross-sections, SBWMV movement protein, but not coat protein was detected in *P. graminis* resting spores (Table 6, Fig. 14,15). In five sections labeled with coat protein antiserum, we viewed five sporosori and plasmodia and none were positive (Fig. 14 A-F). In 16 sections labeled with movement protein antiserum we viewed 16 zoosporangia, plasmodia, and sporosori and 100% were positive (Fig. 15). Fluorescence was scattered throughout the body of zoosporangia and was concentrated in exit tubes (Fig. 15 A, B, and C). Fluorescence was also scattered throughout the plasmodia except in the vacuoles (Fig. 15 D, E, and F). Movement protein was also detected inside resting spores and along the wall of the spores (Fig. 15G and H) as was observed in wheat roots (Fig. 12 C, D).

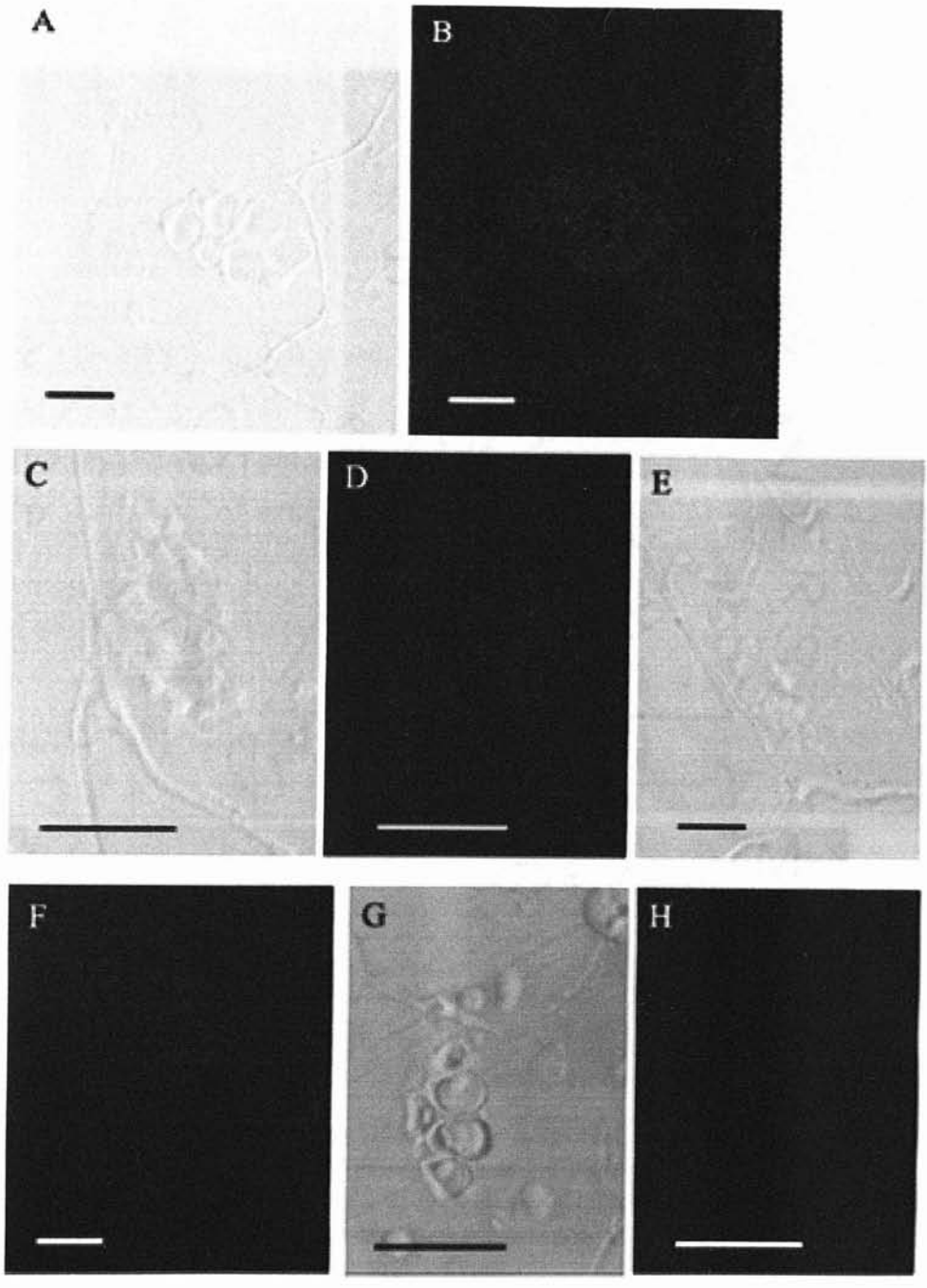


Fig. 14. Immunofluorescence labeling with coat protein antiserum in barley roots. **A, C, E** and **G** are images taken with the transmitted light detector of the confocal microscope. **B, D**, and **F** were treated with coat protein antiserum, and **H** was treated with buffer, no primary antiserum. **A, B** transmitted light and fluorescence images of a sporosorus. **C, D, E**, and **F** transmitted light and fluorescence images of plasmodial stages of *P. graminis*. **G, H**, transmitted light and fluorescence images, respectively, of *P. graminis* resting spores treated with buffer, no primary antiserum. Bar= 20 μm.

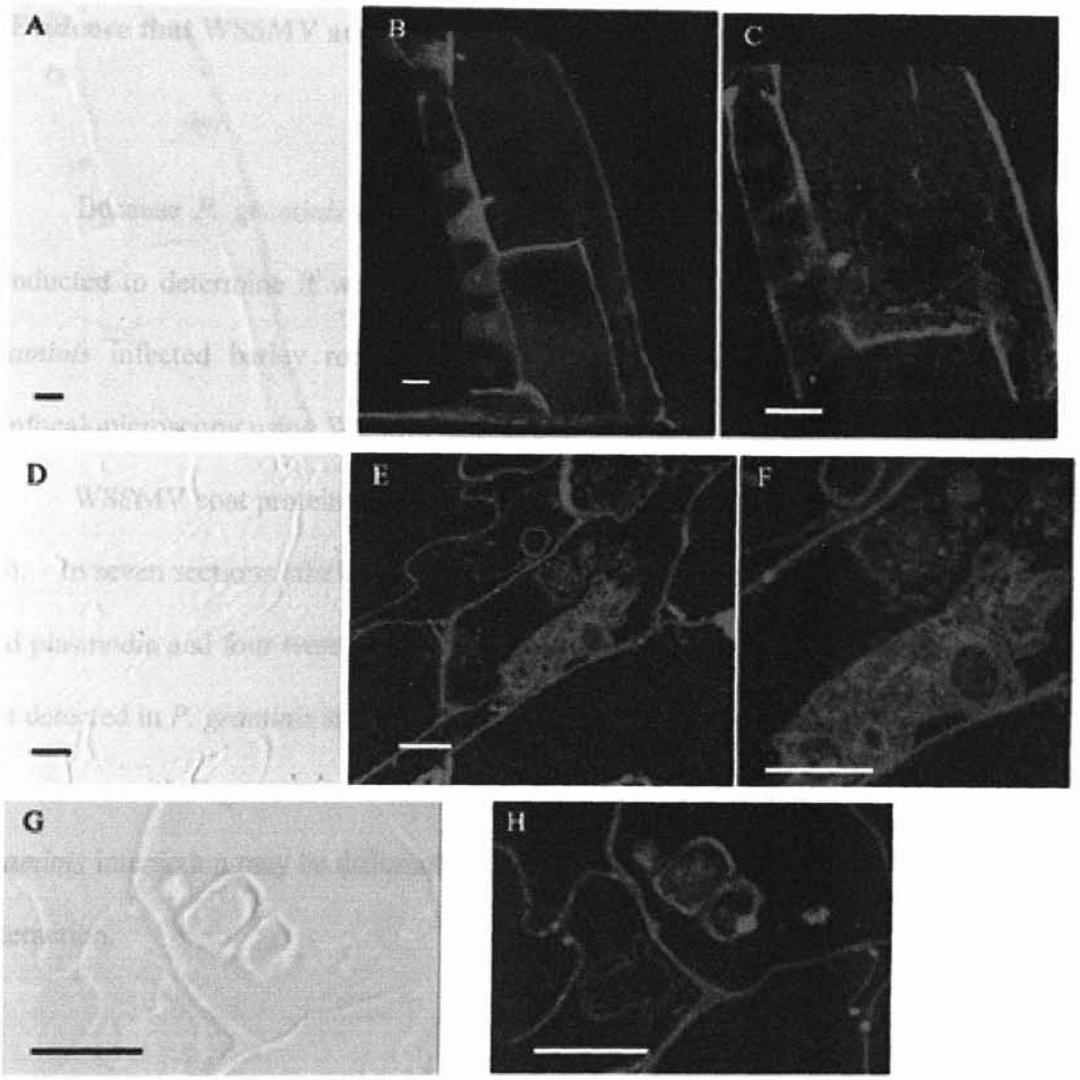


Fig. 15. Laser confocal images of *P. graminis* labeled with SBWMV movement protein antiserum in barley roots. **A P.** *graminis* zoosporangium. **B, C** zoosporangium with exit tubes. **D-F** plasmodial stages. **G, H** sporosori with resting spores. **A, D,** and **G,** transmission images. **B, E,** and **H,** fluorescent images. **C** and **F** fluorescent images enlarged to show internal detail. Bar = 8 μm.

Evidence that WSSMV accumulates in *P. graminis* resting spores in sand-culture grown barley roots

Because *P. graminis* transmits both SBWMV and WSSMV, experiments were conducted to determine if we could detect WSSMV in sand culture grown plants. *P. graminis* infected barley roots were analyzed by immunofluorescence labeling and confocal microscopy using WSSMV antiserum.

WSSMV coat protein was detected in *P. graminis* resting spores (Table 6, Fig. 16). In seven sections labeled with coat protein antiserum, we viewed seven sporosori and plasmodia and four were positive (Table 6). Thus while SBWMV coat protein was not detected in *P. graminis* sporosori or resting spores, WSSMV coat protein was seen in association with *P. graminis*. These data suggest that the mechanism of SBWMV-*P. graminis* interaction may be different from the mechanism for WSSMV-*P. graminis* interaction.

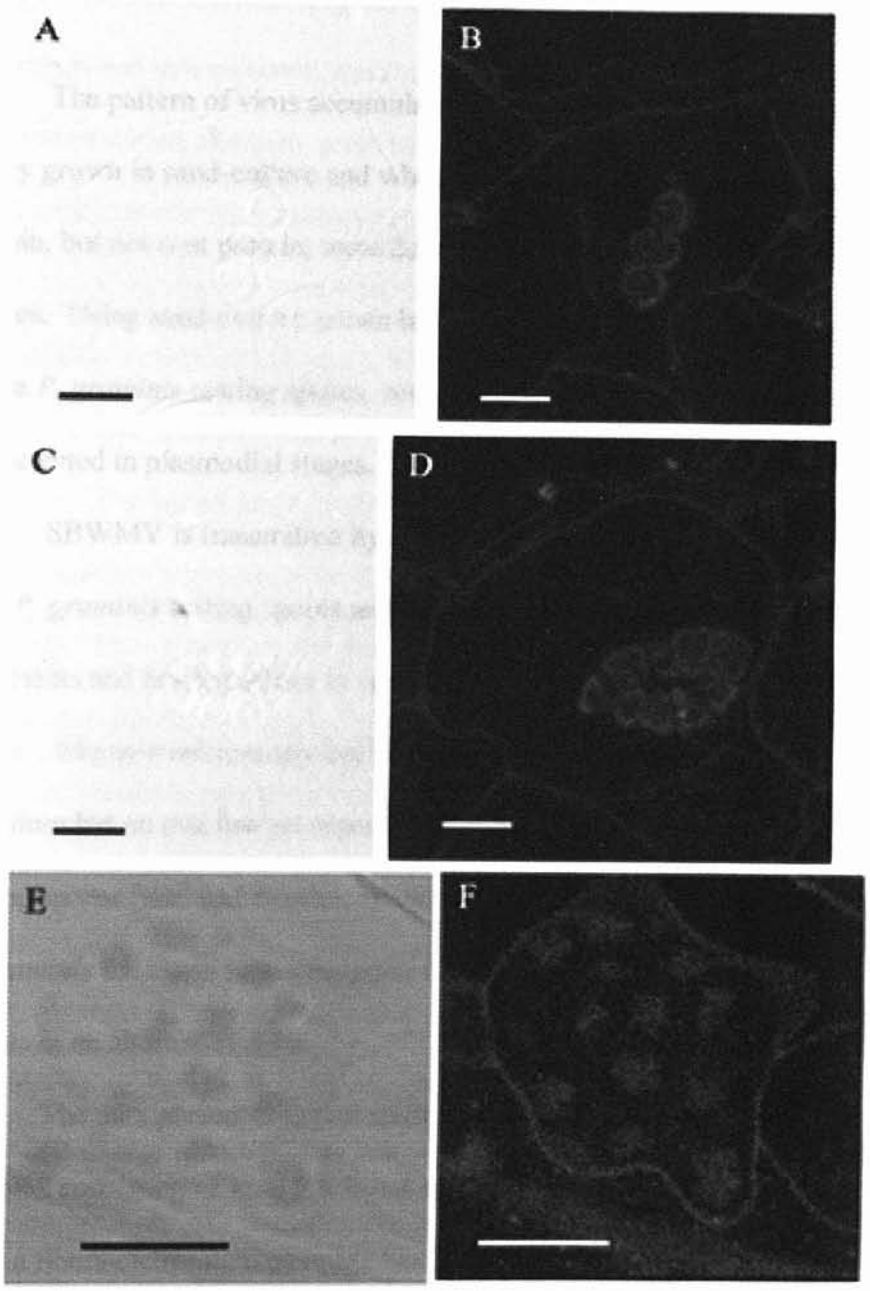


Fig. 16. Transmitted and confocal images of *P. graminis* resting spores (**A, B, C, and D**) labeled with WSSMV coat protein antiserum in wheat roots. Bars= 8 μm.

DISCUSSION

The pattern of virus accumulation in *P. graminis* sporosori was similar in roots of barley grown in sand-culture and wheat grown in soil. SBWMV RNA and movement protein, but not coat protein, were detected in *P. graminis* sporosori in each of the two studies. Using sand-culture grown barley roots we detected SBWMV movement protein inside *P. graminis* resting spores, zoosporangia and plasmodia. Viral coat protein was not detected in plasmodial stages.

SBWMV is transmitted by *P. graminis* zoospores to plant roots. Its association with *P. graminis* resting spores and zoospores is impervious to acid, alkali, or detergent treatments and has led others to suggest the virus is inside the vector (Rao and Brakke, 1969). Electron microscopy has been used to show virus is present in a macerated root inoculum but no one has yet reported virus particles inside *P. graminis* zoospores or resting spores (Rao and Brakke, 1969). The presence of viral movement protein in most *P. graminis* life cycle stages suggests that the virus may be intimately associated with the fungus in an alternative form.

The data presented in this study suggest that there may be a ribonucleoprotein complex consisting of viral RNA and movement protein occurring inside *P. graminis*. Such a ribonucleoprotein complex has been described in relation to virus cell-to-cell and vascular transport. Viral movement proteins, related to the '30K superfamily' of viral movement proteins, mediate viral cell-to-cell and vascular transport by binding viral nucleic acids and carrying them through plasmodesmata and through the vasculature (Melcher, 2000). The SBWMV movement protein is related to the '30K superfamily' and may function in this manner (Melcher, 2000). In relation to *P. graminis* transmission

of SBWMV, the ribonucleoprotein complex may be important for virus movement out of the fungus and into adjacent, uninfected root cells. The ribonucleoprotein complex may be released during zoospore penetration, or through zoosporangial exit tubes (Fig. 17). We do not know if the movement protein mediates movement of the virus out of the fungus, or if the ribonucleoprotein complex is fortuitously released into the cell and then immediately moves into adjacent cells to initiate infection (Fig. 17). SBWMV may require the movement protein to mediate plasmodesmata transport as a first step in virus infection. Further research is needed to investigate these possibilities.

Another explanation for the data is that SBWMV is able to replicate inside *P. graminis*. The occurrence of viral RNA and movement protein in sporosori could indicate replication and translation of viral RNAs. Since SBWMV movement protein is expressed from a subgenomic RNA, movement protein expression would require production of minus strand RNAs and subsequent transcription of subgenomic RNAs. Further *in situ* hybridization experiments to detect minus strand RNAs are needed to test this hypothesis. Since we lack antiserum to other SBWMV proteins (such as the replicase or 19K protein) we could not conduct further experiments to determine if other viral proteins are translated inside *P. graminis*. Such information would help us determine if viral gene expression is occurring inside *P. graminis*.

We present the first evidence that WSSMV accumulates in *P. graminis* sporosori. Unlike SBWMV, WSSMV coat protein was also found in 50% of the *P. graminis* sporosori examined in infected barley roots. WSSMV is a bymovirus and is not related to SBWMV. The mechanism for transmission of WSSMV may be unrelated to

SBWMV, and further research is needed to characterize the mechanism for WSSMV transmission.

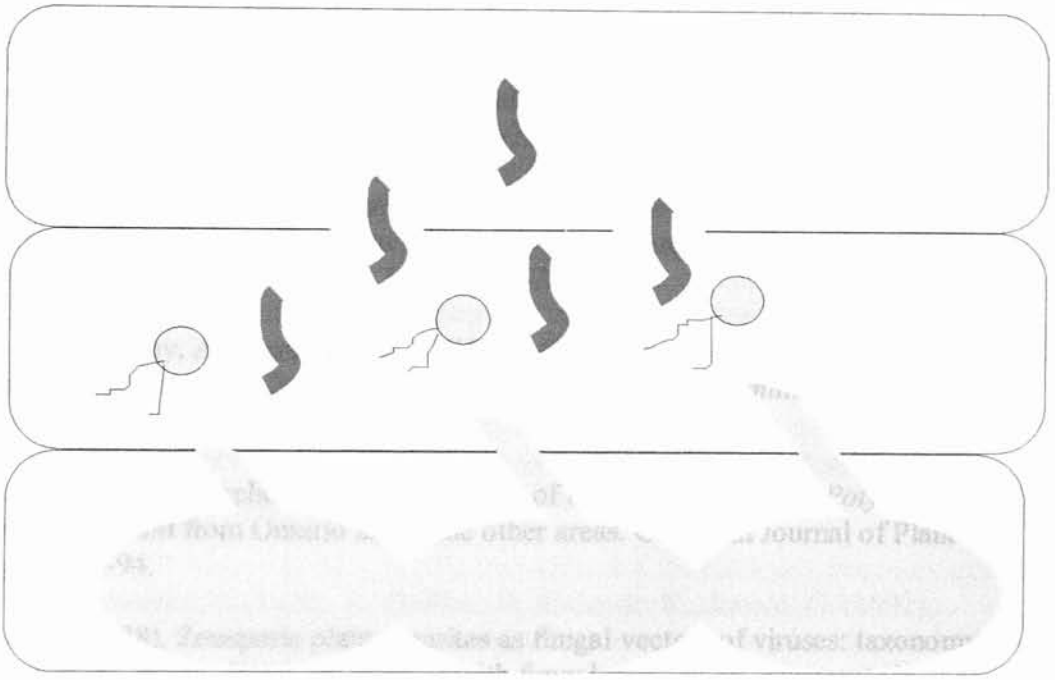


Fig. 17. A model for *P. graminis* release of SBWMV into plant cells. Since movement protein is detected in zoosporangial exit tubes (indicated in pink in the bottom cell), it is possible a ribonucleoprotein complex (indicated in blue) consisting of viral movement protein and RNA are released along with secondary zoospores (indicated in pink in the second cell) into adjacent cells. The complex may first move to adjacent cells that are yet uninfected with *P. graminis* to initiate virus infection there.

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