Confirmation of Polymyxa graminis as a Vector of Wheat Spindle Streak Mosaic Virus

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Contribution No. 465 of the Agriculture Canada Summerland Research Station.

The technical assistance of P. L. Sherwood is gratefully appreciated.

Accepted for publication 4 October 1977.

ABSTRACT

SLYKHUIS, J. T., and D. J. S. BARR. 1978. Confirmation of Polymyxa graminis as a vector of wheat spindle streak mosaic virus. Phytopathology 68: 639-643.

Wheat spindle streak mosaic virus was transmitted via water from soil-free roots of wheat plants infected by growing in infective soil, but not from plants grown in sterile soil and infected with the virus by sap inoculation of the leaves. The zoosporic fungi *Lagena radicicola, Olpidium brassicae, Polymyxa graminis, Rhizophydium graminis*, and *Pythium* spp. usually were present in the roots of plants infected from soil, and in the roots of most plants in 10 sets infected successively by root association in sterilized sand. When isolates of each of the zoosporic fungi were cultured on wheat infected with the virus by sap inoculation of leaves, the virus was transmitted by root association in sand only from plants infected with *P. graminis*. The optimum temperature for transmission of the virus in soil, 15 C, is above the optimum for the development of the virus, 10 C, but within the lower range of temperatures favorable for the development of *P. graminis*.

Wheat spindle streak mosaic (WSSM) is widespread in some years in most fields in areas of southern Ontario where winter wheat is grown most intensively (10). It has been reported in several central and northeastern States of the USA, in France, and recently in India (1, 14). It is caused by a soil-borne virus, WSSMV, with very long, filamentous particles, quite distinct from the short rod-like particles of soil-borne wheat mosaic virus (WSBMV) (4), which has not been found in Ontario and was not detected in any of the plants grown in soils used in these experiments.

Because a zoosporic fungus, *Polymyxa graminis* Led. has been associated with the transmission of WSBMV (4, 9), this fungus has been a prime suspect as a vector of WSSMV. Although *P. graminis* usually has been found in the roots of wheat plants infected with WSSMV wherever the disease occurs, other fungi that infect wheat roots in similar ways also generally are present, hence they also were suspect as vectors. One of these, *Olpidium brassicae* (Wor.) Dang., is a vector of several other viruses (7). The other suspects included *Rhizophydium graminis* Led., *Lagena radicicola* Vanterpool and Led., and several *Phythium* species (2, 3, 6, 8).

Attempts to determine a specific vector or vectors from among these were frustrated by slow rates and low levels of transmission (10), specific temperature requirements (9), slow development of symptoms and difficulties in obtaining pure isolates of the zoosporic fungi (2), most of which have not been cultured on artificial media.

The experiments reported here were to indicate some of the specific characteristics and requirements for vector transmission, and to determine if any of these fungi transmitted WSSMV.

00032-949X/78/000 105$03.00/0

MATERIALS AND METHODS

Soil for most of the experiments reported here was a sandy loam collected at the Central Experimental Farm, Ottawa, from a field in which winter wheat had been grown every 3rd yr starting in 1952. By 1967, plants with symptoms of WSSM were observed in this field. Subsequently winter wheat was planted each year to build up and maintain a high level of infectivity. The soil was collected in October, dried on a greenhouse bench, sifted to break down the lumps, and stored dry in a greenhouse where temperatures ranged from about 15 to 30 C.

Winter wheat (*Triticum aestivum* 'Kent') was used as the test host and for multiplying the virus and culturing the zoosporic fungi. Wheat was infected from soil by sowing 10-15 seeds in 7.5-cm diameter pots of the test soil, watering moderately, and growing at 15 C with about 12,000 lux of light for 12 hr per day, genereally for 21 days, but for some tests for 14 days and for others 28 days. To terminate contact with the infective soil, and also to stimulate disease development (13), the young plants were removed from the infective soil, their roots were washed thoroughly with running tap water, then they were re-potted in sterile soil or sand, watered with half-strength Hoagland's nutrient solution, and grown at 10 C. Symptoms usually developed in 60 to 90 days from planting. Roots were examined for the presence of zoosporic fungi after the diseased plants were grown in sand at 15 C or 20 C for an additional 3-4 wk. Generally, by this time resting spores were found easily microscopically (2). A phase-contrast microscope was used particularly when searching for zoosporangia.

The effects of temperature on the development of fungi on wheat were tested by transplanting 2-day-old wheat seedlings in infective soil and growing them at the test temperatures. Periodically, pieces of roots of the plants were mounted in water for examination with a phase-contrast microscope at × 500. The opportune times of observation varied with temperature as follows: after 3 and 5 wk for plants grown at 20 to 24 C, 4 and 7 wk at 13 C, and after 5 and 9 wk at 9 C. Other temperature tests were done with isolates of each fungus grown on the roots of wheat plants in sterile quartz sand.

Thermal inactivation of WSSMV and fungi was tested in infective soil that had been stored dry for several months. The soil was passed through a 2-mm (pore size) sieve then sprinkled with water and mixed until it was in a moist, crumbly condition. It was left in a pile with occasional mixing for 2 days to assure uniform distribution of moisture. For each temperature treatment, a 500-g portion of the soil in a 1-liter flask was heated in a water bath and stirred frequently until it reached the required temperature. It was maintained at the test temperature for 30 min, then spread thinly in pans at room temperature to air for 7 days. Wheat was grown in the soil samples to test for the development of WSSM and the roots were examined for root-invading fungi as described above.

Most tests for vector transmission from the roots of diseased plants were done by root association in sand. The roots of the diseased source plants, whether grown in soil or sand, were washed to remove all soil particles. The plants were transplanted into 7.5-cm diameter pots containing sterile quartz sand with about 10 wheat seedlings from seeds sprouted 2 days at 25 C. The pots were watered with half-strength Hoagland's solution and grown at 15 C for 3 wk. The seedlings then were removed, washed, and planted in pots of sterile soil and grown at 10 C for symptoms to develop.

Several methods were tested for transmission of WSSMV from roots of diseased plants via water. One was an adaptation of the root washings method used successfully for WSBMV (5). Washed roots of diseased plants were soaked in distilled water for about 1 hr, then the roots of 2-day-old wheat seedlings were immersed in the water in a petri dish for 1 day at 15 C. Other methods provided long concurrent periods for release of infective agents from the source roots and infection of the test plants. One method involved immersion of the source and test plant roots in the same beaker or other container, but keeping the roots from contact by separation screens or other barriers. The drip method provided the most convenient test for transmission via water. The source plants were set in sterile quartz sand in 3.8 × 10 cm butyrate centrifuge tubes, each with three small drainage holes in the bottom covered with fine mesh saran screen or Whatman GF/A glass-fiber filter papers. The tubes were supported above 7.5-cm diameter pots of test seedlings in quartz sand. When the source plants in the butyrate tubes were watered with half-strength Hoagland's solution, the drainage water dripped into the pots of test seedlings. Also, samples of the drainage water were collected periodically and examined for zoospores of root-infecting fungi. After the test period for infection, the test plants were transplanted into sterile sand, watered with half-strength Hoagland's solution, and grown at 10 C.

Unifungal cultures of *P. graminis*, *O. brassicae*, *R. graminis*, and *L. radicicola* were obtained from wheat plants grown in infectious Ottawa soil for 3 to 6 wk at 15 C or at 18-20 C. The plants were removed from the soil, the roots were washed free of soil, the plants were set singly in pots of sterile sand, and grown at 15 C for 21-28 days. Pieces of rootlets containing only one fungus were selected microscopically and each placed adjacent to the roots of 2-day-old wheat seedlings planted in sterile quartz sand. After 3-6 wk at 20 C, each plant was checked microscopically to ascertain the presence of the selected fungus and the absence of other zoosporic fungi and nematodes. Although precautions were taken to avoid exposure to dust or contamination with nonsterile soil, the cultures were not free of protozoa, rotifers, bacteria, or saprophytic fungi. Subcultures were propagated by selecting pieces of root with the characteristic spores to inoculate new wheat seedlings. The cultures on roots were stored dry for later use.

Pure cultures of *Pythium* spp. were obtained by selecting pieces of roots from plants grown in infectious soil and planting them on nutrient agar containing 200 ng/ml neomycin sulfate to suppress bacterial growth. Selected outgrowths from the pieces were transferred to tubes of nutrient agar. For infection of wheat plants, the *Pythium* isolates were grown for 1 wk on water agar or on autoclaved hemp seeds in water, then mixed in sterile soil or sand and 3-day-old seedlings were planted in the mixture. In one experiment, the hemp seed cultures were poured over the roots of the seedlings before planting.

To test for transmission of WSSMV by unifungal isolates, wheat seedlings were inoculated with the fungi in pots of sterile soil or sand and grown at 15 C for 2 wk, inoculated with WSSMV by the artist's airbrush method (12), and grown at 10 C for 6 wk. The plants that developed symptoms of WSSMV were removed, planted in 7.5-cm diameter pots of sterile sand with about ten 2-day-old test seedlings and grown at 15 C for 3 wk. Then the test seedlings were removed, planted in pots of sterile soil, and grown at 10 C for 12 wk for symptom development. Controls included plants infected with the fungi but not inoculated with WSSMV, and plants inoculated with WSSMV by the airbrush method but grown in sterile soil without any of the suspect fungi.

RESULTS

Temperature in relation to the development of zoosporic fungi in the roots of seedlings infected from soil.—To determine if the temperature range for the development of any of the fungi corresponded with the temperature range of 7 to 17 C for transmission of WSSMV in soil (11), wheat roots were examined periodically while growing in infective soil at different temperatures. The fungi, *L. radicicola*, *O. brassicae*, and *R. graminis*, were observed on or in the roots after 3 wk at 20 and 22 C, 4 wk at 14 C, and 5 wk at 9 C. When the roots were observed again 2-3 wk later, *L. radicicola* was more abundant, and *P. graminis* was observed at each temperature except at 9 C. *Olpidium brassicae* and *R. graminis* had formed resting spores at all temperatures

with little evidence of having invaded additional tissues. The development of *L. radicicola* and *P. graminis* appeared to be reduced in rootlets invaded by *Pythium* spp. In such roots, they were observed only as resting spores. *Pythium* spp. were frequently associated with root necrosis.

Experiments in which wheat seedlings were inoculated with unifungal cultures incubated at different temperatures and examined after 3, 4, and 6 wk indicated different temperature preferences for the different fungi (Table 1). Although *L. radicicola* developed abundantly at temperatures of 13 to 24 C, it developed slowly at 9 C. Both *O. brassicae* and *R. graminis* developed abundantly at 9 to 22 C, but were not found at 24 C. Although *P. graminis* grew optimally at 15 to 22 C, its development was slight at 24 C and 13 C, and only a trace at 9 C. These results show that each of these fungi could develop in wheat roots at temperatures favorable for transmission of WSSMV from soil.

Comparison of thermal inactivation of wheat spindle streak mosaic virus and zoosporic fungi in soil.—The effects of heating infective soil to different temperatures before planting wheat were compared in two experiments. The symptoms of WSSM developed on plants grown in soils heated to 50 C but not 52.5 C. As indicated by the presence or absence of the respective fungi in the roots of plants grown in the samples of soil after the different heat treatments, *L. radicicola* remained viable after heating to 45 C but not 50 C, *Pythium* spp. to 50 C but not 52.5 C, and *P. graminis* and *R. graminis* to 52.5 C but not 55 C. *Olpidium brassicae* remained viable in soil after heating to 60 C. These results indicate that *L. radicicola* was inactivated at a temperature lower than that required to prevent the development of WSSM, therefore it must not be the principal vector of the virus. Any of the other fungi, which were inactivated at a temperature equal to or higher than required to prevent the development of WSSM, could be vectors.

Root-to-root transmission of WSSMV via water with and without root contact.—Attempts to transmit WSSMV to 2-day-old wheat seedlings by immersing them in water in which the washed roots of naturally diseased plants had soaked for 1 hr (5) did not succeed. Root-to-root transmission was demonstrated in tests in which the roots of test seedlings were immersed with the roots of naturally diseased plants in water or half-strength Hoagland's solution in a petri dish or beaker for 2 days at 15 C with or without root contact. Root-to-root transmission of WSSMV was demonstrated most conveniently and reliably by growing source and test plants in small pots of sterile sand for 2 to 4 wk at 15 C. When the sources were diseased plants that became infected with the virus while growing in infective soil, the numbers of test plants that became infected while grown in the same pots as different source plants varied from 0% to 100%, but the average was about 40% (Table 2). Test seedlings also became infected when grown in separate pots of sterile sand which received the leachate that

TABLE 1. Degree of development of isolates of zoosporic fungi on wheat at different temperatures

Temp[b] (C)	Amount of infection[a]			
	Polymyxa graminis	*Lagena radicicola*	*Olpidium brassicae*	*Rhizophydium graminis*
24 ± 1	++	++++	0	0
22 ± 1	++++		+++	+++
20 ± 1	++++	++++	++++	++++
15 ± 1	++++	++++		
13 ± 1	++	++++	++++	++++
9 ± 1	+	++	+++	+++

[a]Results summarized from five separate experiments. The symbols represent: 0 = no infection, + = trace, ++ = slight, +++ = moderate, and ++++ = abundant infection.
[b]Temperatures were tested in different experiments.

TABLE 2. Transmission of wheat spindle streak mosaic virus from roots of wheat plants infected from soil or by sap inoculation to test seedlings growing in quartz sand in the same pots or in separate pots watered with the leachate from the pots containing the diseased plants

Source plants		Plants which become infected when grown:		
Means of infection	Soil in which grown	In same pots (roots associated with source plants)	In separate pots (watered with leachate from pots containing source plants)	
			Not filtered	Filtered[a]
Soil	Infectious field	104/236[b]	43/245	24/56
Sap inoculation	Non-infectious field	1/230		
Sap inoculation	Steam-sterilized	0/98		
Not infected	Non-infectious field	0/87		
Not infected	Steam-sterilized	0/75		

[a]Whatman GF/A glass-fiber filter paper.
[b]The fraction represents: (number of infected plants)/(total number of test plants).

dripped from pots of sand containing the source plants suspended above them. Even when the leachate was filtered through Whatman GF/A glass-fiber filter paper before entering the pots of test seedlings, some of the seedlings became infected. Biflagellate zoospores were observed in the leachate from time to time.

The virus was not transmitted by root association in sand from source plants that were infected with the virus by sap inoculation while growing in sterile soil. Only one of 230 (1/230) test plants developed WSSM after root association in sand with plants that were infected with the virus by sap inoculation while growing in soil from a field of noninfective soil at Ottawa that had not grown wheat for more than 20 yr.

Zoosporic fungi in relation to successive transmission of WSSMV by root association in sand.—The initial set of plants for this experiment was grown from seed in infectious soil at 15 C for 3 wk, then in sterile soil at 10 C until symptoms developed. Their roots were washed free of soil. They were planted singly with about 10 test seedlings in 7.5-cm diameter pots containing quartz sand and incubated at 15 C for 3 wk. The test seedlings then were removed, washed, planted in sterile soil, and grown at 10 C. The first 10 to 15 plants to develop symptoms were used as source plants to inoculate a third set of seedlings and so on to the 10th set of seedlings 33 months after the experiment was started.

All of the 14 plants in the initial set grown for 3 wk in infective soil were infected and developed WSSM symptoms. The numbers of test plants that developed symptoms in the successive sets, which were inoculated by root association with the roots of plants that became diseased in the preceding set, varied from 21 to 94%. Infection was high even in the ninth and tenth sets. It appeared that transmission by this procedure could continue indefinitely.

When the roots of the first set of plants infected directly from infective field soil were examined microscopically, *L. radicicola*, *O. brassicae*, *P. graminis*, *Pythium* spp., and *R. graminis* were found. It was hoped that by selecting the first plants to develop symptoms in each set of plants as sources to infect each successive set, there might be a selection toward a vector fungus and freedom from fungi that were not vectors. The same fungi were found in the roots of plants examined from successive sets, including the tenth set when the procedure was terminated. It appears that the conditions favorable for successive transmission of the virus also were favorable for transmission of all of the fungi.

Tests for transmission of WSSMV with selected root pieces from diseased plants.—A series of tests was conducted to transmit WSSMV to seedlings in sterile soil or sand by placing on their roots selected pieces of roots from naturally diseased plants bearing specific zoosporic fungi. Although infection sometimes occurred when the major portion of the root system of naturally diseased plants was planted with test seedlings, none of 178 wheat seedlings developed symptoms of WSSM from growing in association with any of the selected pieces of root.

Transmission of wheat spindle streak mosaic virus by isolates of zoosporic fungi.—Unifungal cultures of each of the fungi were tested for transmission of WSSMV by root association in sand from wheat plants infected with the virus by sap inoculation of the leaves. Variable percentages of test plants became infected with WSSMV in all experiments in which the roots of the virus-infected

TABLE 3. Root-to-root transmission of wheat spindle streak mosaic virus (WSSMV) from manually inoculated wheat plants infected with isolates of root-infecting fungi

	Plants which became infected with WSSMV in:					
Wheat source plants infected with:	Expt. 109	Expts. 134, 195, 198	Expts. 235, 260, 333	Expt. 328	Expt. 358	Expt. 361
No fungi + WSSMV	0/15[a]	0/290	0/98	0/61	0/63	0/42
No fungi, no virus	0/17					0/16
Lagena radicicola + WSSMV	0/15		0/103	0/90		
Lagena radicicola, no virus	0/14					
Olpidium brassicae + WSSMV		0/195	0/94	0/150		
Olpidium brassicae, no virus		0/38				
Polymyxa graminis + WSSMV	7/15	47/209	13/97	133/392	35/126	10/102
Polymyxa graminis, no virus	0/14	0/21			0/62	0/34
Rhizophydium graminis + WSSMV		0/157	1/99[b]			
Pythium aphanidermatum + WSSMV			0/79			
Pythium aristosporum + WSSMV			0/81			
Pythium arrhenomanes + WSSMV			0/239			
Pythium rostratum + WSSMV			0/83			
Pythium tardicrescens + WSSMV			0/330			
Pythium torulosum + WSSMV			0/8			
Pythium ultimum + WSSMV			0/23			
Pythium volutum + WSSMV			0/158			

[a]The fraction represents: (number of infected plants)/(total number of test plants).
[b]When the roots were examined, *P. graminis* as well as *R. graminis* was found.

source plants were infected with isolates of *P. graminis* (Table 3). None of the test plants became infected with WSSMV in tests with any of the other fungi unless *P. graminis* also was present. For example, in one experiment, one test plant became infected from a source plant infected with an isolate of *R. graminis*, but when the roots of the source and test plants were re-examined, *P. graminis* as well as *R. graminis* was found. None of the test plants became infected by root association with source plants infected with the virus but not infected with any of the fungi, or with plants infected with *P. graminis* but not with the virus.

DISCUSSION

The long persistence of WSSMV in dried, finely sifted soil, and its transmissibility via water from the roots of naturally infected plants supported the hypothesis that the virus was harbored in and transmitted by a root-infecting, zoosporic fungus. The conditions suitable for transmission of WSSMV from soil or from roots of diseased plants also were suitable for infection by and development of each of the suspect fungi. For this reason, most of the experiments intended to determine if there was a correlation between the presence of a specific fungus and transmission of the virus failed because several of the fungi always were present.

The only experiments which demonstrated an association between the presence of a specific fungus and transmission of the virus were those involving unifungal isolates established on the roots of plants grown from seed in sterile sand or soil and inoculated with the virus by sap inoculation of the leaves. It was necessary to examine roots of culture plants repeatedly and to examine the roots of test plants that developed mosaic symptoms, to determine if fungi other than the desired one were present. These tests, repeated with different isolates of each fungus, confirmed to our eventual satisfaction that of those tested *P. graminis* was the only one that transmitted WSSMV.

Some of the methods used successfully for transmitting soil-borne wheat mosaic virus by *P. graminis* (4, 9) were not successful in our experiments with WSSMV. One of these was the immersion of the roots of test seedlings in water in which the washed roots of naturally diseased plants had soaked. Transmission of WSSMV occurred if the roots of the diseased plants and test seedlings were immersed together in a small volume of water for several days, or if the water that leached from sand supporting the roots of diseased plants was allowed to drip for several days into pots containing test seedlings. We also failed to achieve transmission of WSSMV by placing pieces of roots containing *P. graminis* from diseased plants adjacent to roots of test seedlings. Transmission was generally successful if diseased test plants were potted in sand with test seedlings and grown together for several days. These results and experiments on the effects of temperature (11) indicate that vector transmission of WSSMV is slow and occurs only at a limited range of temperatures.

Earlier results showed that the optimum temperature for the development of WSSMV in wheat is about 10 C, with little development at 15 C, but the optimum temperature for transmission of the virus is about 15 C, with little occurring at 10 C (11). *P. graminis* developed most readily at 15 to 22 C, but slowly at 10 C. It appears that the optimum temperature for transmission of the virus, 15 C, is a compromise between the optimum temperatures for development of the virus and for the development of the fungus vector.

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