Concentration and Infectivity of Barley Stripe Mosaic Virus in Barley

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ABSTRACT

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Barley stripe mosaic virus (BSMV) was extracted by grinding infected barley *(Hordeum vulgare)* leaves in 0.5 M sodium orthoborate, pH 9.0, and purified by differential and density-gradient centrifugation. The concentration of extractable virions in the youngest two leaves of infected plants grown at 13, 17, 21, and 25 C was approximately the same for plants from the two-leaf to the flag-leaf stage, suggesting that the infected plant has no long-lasting acquired immunity from the virus. In plants pretreated for 3 days at 25 C, symptoms were more severe at a lower temperature than at a higher temperature, but this difference was not correlated with virion concentration. Polyacrylamide gel electrophoresis of BSMV RNA showed two RNA components for the Type strain, three for the ND18 strain, and four for the AM strain. Component I had the largest molecular weight RNA and component IV, the smallest. The ratio of component I to II in AM strain increased to about 1:1 when infected barley plants were maintained at 13 C, but reverted to the normal 1:3 ratio when plants were returned to 25 C. Component IV could not be recovered from flag leaves of plants infected with AM strain. No changes in proportion of components I to II or loss of any components were observed with plants infected with ND18 or Type strain kept at 13 C until maturity.

The course of a viral disease in a plant is reflected by both concentration and specific infectivity of the virions. The concentration of virions often decreases after the acute phase of the disease, possibly because of host immunity. The specific infectivity may decrease through virion inactivation or through increased synthesis of noninfectious particles, such as short rods in some multicomponent viruses (12).

Several reports have appeared on the symptoms of barley plants infected with barley stripe mosaic virus (BSMV) and grown at different temperatures, and on the virion concentration in the early phases of the disease. Kankam and Arny (3) found that the BSMV concentration was higher at 1 week (acute phase) than at 2 weeks (early chronic phase) after inoculation in both resistant and susceptible cultivars. They suggested that virion concentration in both cultivars paralleled symptom severity. Singh et al. (11) concluded that disease severity increased with increasing soil and air temperatures. The tolerant cultivars (C.I. 3212-1 and C.I. 4219) showed severe infection at 24 C and 28 C, but few symptoms at 16 C. Rao and Brakke (10) reported that the virus induced a pronounced mosaic at 13 C, but the virus moved slowly at this temperature. Only 10% of inoculated plants showed systemic symptoms at 13 C without special treatments, such as keeping plants in the dark for several days. The effects of light and temperature on the intensity of the mild chlorotic symptoms in Glacier barley seedlings were established by McKinney in 1954 (5). In 1965, McKinney and Greeley (6) made an extensive study of development of symptoms in barley seedlings with time, but did not measure virion concentration. Pring (8) measured BSMV virion and RNA concentration in the four youngest leaves of barley for about 2 weeks after inoculation, and in corn over a longer period (9).

We have examined infected barley plants from the two-leaf stage to the flag-leaf stage for major changes in concentration of extractable virions, infectivity of purified virions, symptoms, and proportion of RNA components. Barley plants grown at four different temperatures infected with three strains of barley stripe mosaic virus were surveyed.

MATERIALS AND METHODS

Type (American Type Culture Collection PV #43) (1) and Argentine Mild (AM) strains of BSMV were originally obtained from H. H. McKinney. The North Dakota 18 (ND18) strain was obtained from R. G. Timian. The Type strain has two RNA components and ND18 has three (2, 4). Strain AM usually has four RNA components, but some cultures have only two or three and are called 2-RNA AM or 3-RNA AM. The RNA components are called I, II, III, and IV; with I having the highest molecular weight, and IV the lowest. The virus was maintained in barley (*Hordeum vulgare* L. 'Moore' and 'Black Hulless'), by mechanical inoculation. The plants were grown either in a greenhouse (25 ± 1 C) or in growth chambers (21 C, 10 Klux, 14-hour days).

Virus purification and assay.—The two top leaves of BSMV-infected barley were harvested and ground in 0.5 M sodium orthoborate, pH 9 (10 ml/g). The crude extract was centrifuged for 20 minutes at 8,000 rpm in a Servall SS34 rotor and the supernatant then was centrifuged at 28,000 rpm for 2 hours in a Spinco #30 rotor. The resulting pellet was homogenized in a small volume of 0.1 M PO_4 buffer, pH 6, containing 0.1% Igepon T-73. The homogenate was centrifuged at low speed and the supernatant solution was placed on 15 ml of a 20% sucrose pad containing 0.2% Igepon T-73. After centrifugation at 28,000 rpm for 3 hours in a Spinco #30 rotor, the pellet was resuspended in 0.1 M PO_4 buffer, pH 6. The preparation was centrifuged at low speed and, if still greenish, was centrifuged through another 20% sucrose pad containing 0.1% Igepon. The final step consisted of density-gradient centrifugation on preformed sucrose gradients containing 0.02% Igepon T-73 at 23,000 rpm for 1.75 hours (Spinco SW 25.1 rotor) if the samples were to be fractionated, or at 25,000 for 4.0 hours (Spinco SW 27 rotor) if the virus was to be concentrated in a pellet for RNA gel electrophoresis. The amount of purified virions was determined by measuring with a planimeter the areas under the peaks on the scanning patterns from density gradients and then converting the areas to micrograms using an extinction coefficient of 2.5 ($E^{0.1\%}_{254\ nm}$).

The virion zones were collected as the gradient columns were scanned. These solutions were diluted to give 10 ml per gm of leaf tissue used as a source. Celite (1%) was added and the solutions were rubbed on the leaves of 30-40 barley plants growing in the greenhouse.

Gel electrophoresis.—Electrophoresis of virus RNA was performed in 2.5% polyacrylamide that contained 0.5% agarose in TPE (0.02 M Tris, 0.02 M NaH_2PO_4, 0.001 M EDTA, pH 7.4). The gels (9 cm long, 6 mm diameter) were pre-electrophoresed for 2 hours prior to the addition of 0.05- to 0.10-ml samples of purified virion preparation disrupted by heating at 60 C for 10 minutes in a dissociation buffer (0.5% mercaptoethanol, 1% SDS, 1 mM EDTA, 0.05 M phosphate, pH 7.2, 10% sucrose). The samples were electrophoresed at 5 mA/gel for 4 hours at room temperature. After electrophoresis, the gels were soaked in distilled water and scanned at 260 nm in a Gilford model 2410 linear transport system.

Effect of temperature on concentration and infectivity.—Barley plants (cultivar Moore) in the two-leaf stage were inoculated with Type strain of BSMV and held at 25 C for 3 days to assure initial infection and systemic spread of the virus. The inoculated plants then were separated into four sets and moved to growth chambers with temperatures of 13, 17, 21, and 25 C (10 Klux, cool-white fluorescent bulbs, 14-hour days). Four days after the plants were placed in growth chambers, samples of the two top leaves in each temperature set were harvested and analyzed for virion concentration by purification, infectivity assay, and electron microscopy. The next analysis of the two top leaves from other infected plants in each set was made 3 days later, and weekly thereafter until the flag-leaf stage. Healthy

TABLE 1. Effect of extracting medium on the concentration, infectivity, and length of virion particles of barley stripe mosaic virus[a]

Extracting medium		Infectivity[b]			Concentration (μg virions/gm leaf tissue)	Rod length[f] (nm)
		Crude sap[c]	Low speed pellet[d]	Purified virus[e]		
Distilled water		50	22	7	115	131
Potassium phosphate, 0.01 M pH 7		53	29	0	59	190
Boric acid 0.5 M adjusted with NaOH to:	pH 5	41		0	58	
	pH 7	39		16	151	
	pH 9	42	2	47	227	211

[a]Average of two or three trials. Samples from a given batch of infected leaf tissue were ground in a high-speed blender for 2 minutes with 10 ml of extractant per gram of tissue. The virions were purified as described in the Materials and Methods section, and the concentration measured by density gradient centrifugation. Samples were removed for infectivity assay at various stages of purification.

[b]Percentage of 30-40 inoculated plants showing symptoms in 14 days.

[c]Extracted sap (1/10) diluted tenfold with phosphate buffer, 0.01 M, pH 7 to give a final dilution of 1/100 (gm leaves/ml).

[d]Pellet from second low-speed centrifugation (after the first high-speed cycle) resuspended in phosphate buffer equal to volume of supernatant (1/10) and diluted tenfold (to 1/100) for inoculation.

[e]Virus zones collected from centrifuged density-gradient columns and diluted to 1/10. The concentration of virions when inoculated was thus one-tenth that of column 5.

[f]Average of 100 virus particles measured at ×8,200 magnification using as standard a carbon replica of a 54,664 diffraction grating on a 74-μm (200-mesh) copper grid. Actual spacing of the grating is 462.9 nm.

noninoculated plants were provided as checks.

RESULTS

Extraction buffers.—Tests were conducted to determine whether the buffer in which the leaves were ground would affect the amount of extractable virus. Preliminary experiments indicated that high-pH, high-salt buffers extracted more virions than did neutral, low-salt buffers or neutral, high-salt buffers (0.5 M sodium acetate, 0.5 M sodium citrate, and 0.4 M NaCl in 0.1 M potassium phosphate). These tests were made on the two youngest, systemically infected leaves harvested from greenhouse-grown plants 10-12 days after inoculation. A maximum concentration of about 400 μg of virus per gm of leaf tissue was obtained. Approximately the same amounts of virions were extracted by grinding infected leaves in the two high-pH, high-salt buffers that were tested, that is, 0.5 M sodium borate, pH 9.0, and GPS (0.1 M glycine, 0.3 M NaCl, 0.05 M K_2HPO_4, pH 9.4). However, the virions that were extracted in borate were less aggregated and easier to purify than were those extracted in GPS. Additional tests were done to compare 0.5 M sodium borate at pH 5, 7, and 9 with distilled water and 0.01 M potassium phosphate, pH 7.0 (Table 1). The latter two extractants were used in previous purification procedures in this laboratory. Of the extractants tested, sodium borate, 0.5 M, pH 9.0, extracted the most virions but also resulted in more highly aggregated virions as shown by their longer average length. Since high yield was the goal, the borate was used in all subsequent experiments. Unfortunately, it also extracted many other plant components as well as virions and preliminary purification, as outlined in the Materials and Methods, was needed before the extracted virions could be assayed by density-gradient centrifugation.

Concentration and infectivity of virions in plants.—This experiment was designed to detect relatively large differences; e.g., order of magnitude differences, in virion concentration that might exist in infected leaves from plants of different ages. Such differences had been observed in corn in which flag leaves from BSMV-infected plants may have a pronounced mosaic but contain little or no detectable virus (9). The bioassay was designed to detect large differences among a large number of samples, rather than to detect accurately small differences among a small number of samples. Virion concentration was measured by density-gradient centrifugation of purified virions and by infectivity of purified virions inoculated at a constant dilution relative to the amount of tissue; i.e., 1 gm of leaf tissue per 10 ml of solution.

Large differences in concentration of virions were not found in leaves harvested from plants of different ages grown at different temperatures (Fig. 1). The results suggest that there might be a periodicity in concentration of a smaller order of magnitude; e.g., over a 3- to 4-fold range. However, the results in Fig. 1 are averages of three experiments. Inspection of the data of the individual experiments does not support the idea of a regular, repeatable periodicity. Variation in size of the youngest harvested leaf could account for some of the variability (7).

All preparations of purified virus were infectious. Changes in specific infectivity, if present, were less than 10-fold and were not detected by the assay used. The specific infectivity was rather low throughout, perhaps a reflection of the time required to purify the virus and of the inoculation medium (0.02% Igepon T-73 plus sucrose).

The data in Fig. 1 are for the virions in the two youngest leaves of the plant. A few spot checks showed fewer extractable virions in older leaves of the same plants than in the two youngest leaves.

Symptoms.—Plants kept for 3 days after inoculation at either 13 C or 25 C and then maintained at 25 C until maturity, showed striping symptoms on the third leaf that were confined to the middle portion of the leaf at 10 days after inoculation. The second leaf or the inoculated leaf was symptomless. The following week, the whitish stripes were more prominent on the fourth leaf, but the symptoms did not extend to the tips. At the third week, long, yellowish stripes had developed on both sides of the fifth leaf. Symptoms had disappeared on most of the third and fourth leaves. By the fourth week and until the tenth, the symptoms had become long, wide, diffuse stripes that made the plants appear generally chlorotic. Symptoms on older leaves always disappeared before the leaves dried out.

Plants maintained continuously at 13 C after inoculation remained symptomless. No virus was recovered from these plants. However, if plants were kept

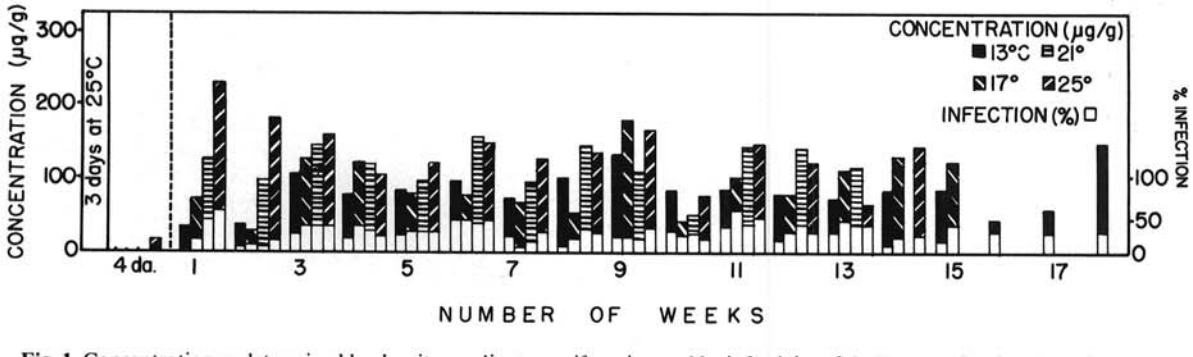

Fig. 1. Concentration as determined by density-gradient centrifugation and by infectivity of the Type strain of barley stripe mosaic virions in barley plants grown at different temperatures. Virions were purified before assay and were inoculated at a concentration of 1/10 (gm of source leaves per milliliter) to 30-40 Moore barley plants for the infectivity assay. Average of three trials.

for 3 days after inoculation at 25 C, then maintained at 13 C until maturity, the symptoms on the third leaf were severe and the striping patterns were more prominent than those on plants held continuously at 25 C. At the second week, broken stripes covered the third leaf, in contrast to plants kept continuously at 25 C, which showed no symptoms at the tips of the leaves during the same period. By the fourth week and thereafter, streaks were quite prominent but were confined only to certain areas of the leaf. Older leaves of plants maintained at 13 C also tended to retain symptoms longer than did those kept at 25 C. Newly developing leaves commonly remained symptomless for some time at this lower temperature (13 C).

Plants kept initially at either 13 C or 25 C and then transferred weekly, biweekly, or monthly from 13 C to 25 C and vice versa, developed symptoms resembling those of parallel plants held at 13 C or 25 C without transfer. Symptoms generally were more severe at 13 C than at 25 C. The cyclical temperature pattern did not give rise to a cyclical pattern of symptoms, such as might have formed if symptom severity were determined at a certain stage of leaf development and were dependent on temperature. The virion concentration per gram of leaf tissue usually was higher when plants were harvested after a 1, 2, or 4 week period at 25 C (depending on the experiment) than if harvested after a period at 13 C.

Proportion of ribonucleic acid components.—A change in proportion of BSMV AM strain RNA components, especially in the ratio of component I to II, was observed in barley plants kept at 13 C from 2 weeks after inoculation until the flag-leaf stage (Fig. 2). At 13 C RNA's I and II were present in almost equal amounts in all assays except for the RNA analysis 1 week after inoculation. Component II remained three times greater than component I throughout the growth of the plants at 25 C. Ratios of component I to II were the same in plants infected with 3-RNA AM as in plants infected with 4-RNA AM at both 13 C and 25 C. Component IV could not be recovered from the flag leaves of a 4-RNA AM-infected barley at 13 C but traces occasionally were observed at 25 C. Infected plants that showed equal amounts of component I and II at 13 C reverted to production of these components in the typical 1:3 ratio when returned to the 25 C growth chamber. Type and ND18 strains had the same ratio of components in plants of all ages and in plants at 13 C and at 25 C. Attempts to follow the ratio of components in "free" (i.e.,

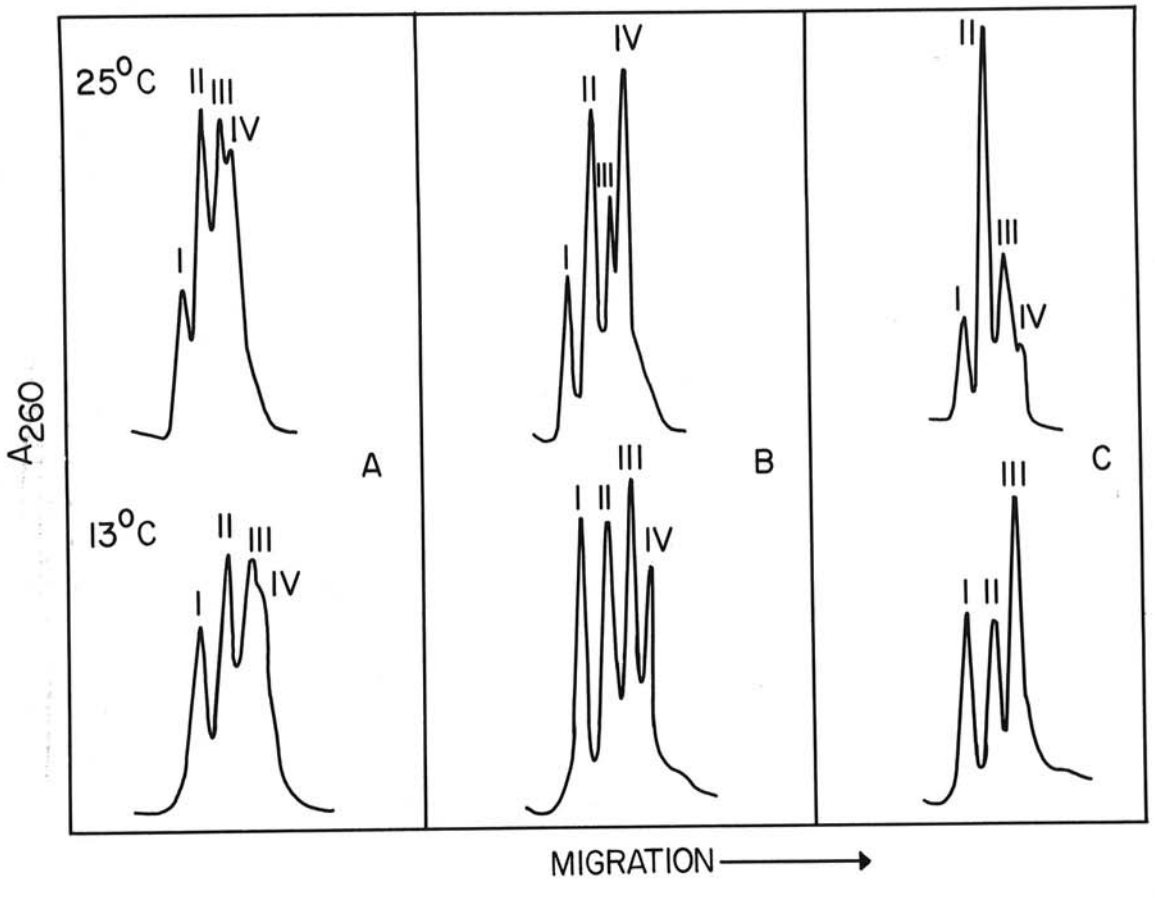

Fig. 2-(A to C). Gel electrophoresis of the Argentine Mild strain RNA of barley stripe mosaic virus. The plants were grown at 25 C (upper curves) and 13 C (lower curves). The RNA was extracted from purified virions at various time intervals: **A)** 1 week after inoculation, **B)** 1 month after inoculation, and **C)** at the flag-leaf stage. Note the difference between the level of RNA I compared to RNA II at the two temperatures. Relative mobilities are not comparable.

nonencapsidated) RNA (8) were unsuccessful.

No AM virions could be isolated 3 days after inoculation from plants at 25 C, but all four components of AM virions could be detected 4 days after inoculation. The proportion of RNA components was similar in virions from the second, third, fourth, or fifth leaves, or in the whole infected plant.

DISCUSSION

The purification procedure in which 0.5 M sodium orthoborate buffer at pH 9 was used gave a consistently high yield of virions. The virion concentration, which varied from about 50 to 400 μg/g of tissue, also depended on the growth conditions of the host plants. Plants in the growth chambers generally yielded fewer virions than plants in the greenhouse, probably because of differences in light intensity.

The concentration of virions extractable from the two youngest leaves remained nearly constant until the flag-leaf stage of the host; i.e., throughout the chronic phase of the disease. Earlier research had indicated a peak concentration in the acute phase of the disease (3, 7, 9). If cyclic changes occur in virion concentration during the chronic phase, they must be of a shorter period and lower amplitude than would have been detected by the present experimental design. Since the two youngest leaves usually were used in this study, the high concentration of virions throughout the life of the plant implies continued synthesis in young tissue, with little or no host immunity. This may explain why BSMV is the only cereal virus known to be seed-transmissible.

The temperature shift experiment shows that the symptoms are different at 13 C and 25 C; that symptoms once formed remain relatively constant at 13 C but decrease in intensity at 25 C; and that the virus moves slowly at 13 C, but rapidly at 25 C. Since the virus moves slowly at 13 C, the concentration in infected cells may be higher at 13 C than at 25 C. The amount of virus was not always correlated to the severity of symptom expression.

Infected plants that were transferred weekly or biweekly from one temperature to another had more severe and longer-lasting symptoms than did plants kept at one temperature. The amount of virions was always higher in plants that were held at 25 C prior to analysis. The longer retention of symptoms at 13 C probably was primarily related to the slower growth of the plant at that temperature. Other workers (3) suggested that the infectivity titer of BSMV in entire plants was higher at 24 C than at 16 C 2 weeks after inoculation. These inoculated plants, however, were not given a pretreatment period before moving them to the growth chambers. The low concentration at 16 C could reflect slow movement, rather than slow multiplication.

When a 4-RNA AM strain is inoculated to barley seedlings, the smallest component (RNA IV) cannot be recovered from the flag leaves of infected plants. The 3-RNA AM recovered from the flag leaves retained its three-component character during subculture; it was difficult to get RNA IV back once it was lost. Ribonucleic acids III and IV of AM BSMV also can be lost by transferring the virus at high dilutions. Details of these experiments will be published elsewhere.

Both the ratio of component I to II and symptom expression are affected by temperature. Symptoms are more severe and longer lasting at 13 C. When plants with a high proportion of RNA I are placed back at 25 C, a temperature at which the symptoms eventually become less pronounced, the RNA ratio returns to its usual value. Among the properties surveyed here, the one most promising for additional investigation appears to be this change in the ratios of components of the AM strain.

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