SOME EXPERIMENTS ON THE SEED TRANSMISSION OF BARLEY STRIPE MOSAIC VIRUS IN BARLEY WITH ELECTRON MICROSCOPY*

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In 1962, the author reported the results of some experiments on the seed transmission of barley stripe mosaic virus (BSMV), and summarized his observations on the mechanism of seed transmission of BSMV as follows: 1) The seeds can be infected either through pollen and ovule. 2) The rate of embryo infection or the rate of seed transmission is determined already in the early stage of seed development. 3) Marked increase and decrease of seed infection does not occur at the later stage of seed development and at the germination period. In other words, it is not likely that new invasion to or inactivation of the virus in the seed takes place at these stages. 4) The virus in endosperm does not seem responsible for the seed transmission. These observations were brought about by the embryo culture test and also dissection and inoculation test, using barley seeds and their embryos at various stages of maturation. The author made further experiments by using electron microscope together with embryo culture and inoculation technique. The results obtained were not conclusive, but it was conspicuous that the rate of embryo infection determined by electron microscopic observation seemed to be higher than that of seed transmission or embryo culture infection. This was somewhat different from the author's former supposition that the virus inactivation in the germinating seeds was not likely to take place. Under these circumstances, several experiments were carried out to ascertain the relationship between virus particle association with seed and seed transmission.

MATERIALS AND METHODS

The seeds taken from BSMV infected Kenyoshi No. 3 barley were used throughout the experiments. For the prepatations of electron microscopy to detect BSMV particles, small cut piece of whole embryo, scutellum or

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endosperm were dissected from the moistened seeds. The cut piece was dipped for a moment into a droplet on a slide glass of distilled water of about 0.005 ml to which a small amount of serum albumen had been added. Then, a roopful of suspension taken from the above mentioned droplet was mounted on a carbon coated colodium film on a copper grid. The specimens were evaporated to dryness at room temperature and shadow-casted with cromium. Observations were made at 6～7,000 magnification under the Hitachi HS-6 electron microscope. Additional two or three specimens prepared from the same embryo or endosperm were used to confirm the observation when no virus particle was found. For the embryo culture, solidified White's media was used. Dissected embryos were placed on the media in the petri dish and incubated at 24°C. Germinated embryos were transfered into the test tubes containing the same media and cultured under the glasshouse conditions. Seedling infection was examined at the first leaf stage in most of the cases, as the seed-borne symptoms of 6-rowed variety such as Kenyoshi No. 3 had been found to appear at that stage of seedling without exception (Inouye, 1962). Other details of the experimental methods are to be described later in the results.

RESULTS

1. *Comparisons between the Rate of Seed Transmission and Embryo Infection*

BSMV particle association with embryo was examined under the electron microscope on 100 seeds of Kenyoshi No. 3 to detect the virus infection. Observations were made 5 replications by the use of each 20 embryos. The other 100 seeds, each 20 seeds taken seperately 5 times,

TABLE 1

Comparisons between the rate of seed transmssion and virus particle association with embryo

	Seed transmission		virus particle associated embryo	
	%	θ	%	θ
1	70.0	56.79	90.0	71.56
2	79.0	62.72	85.0	67.21
3	75.0	60.00	70.0	56.79
4	68.7	55.98	85.0	67.21
5	63.2	52.65	75.0	60.00
Total	71.3		81.0	
Av.	71.2	57.628	81.0	64.554

$t=2.17$ $0.1>p>0.05$ $\theta=\arcsin\sqrt{p}$

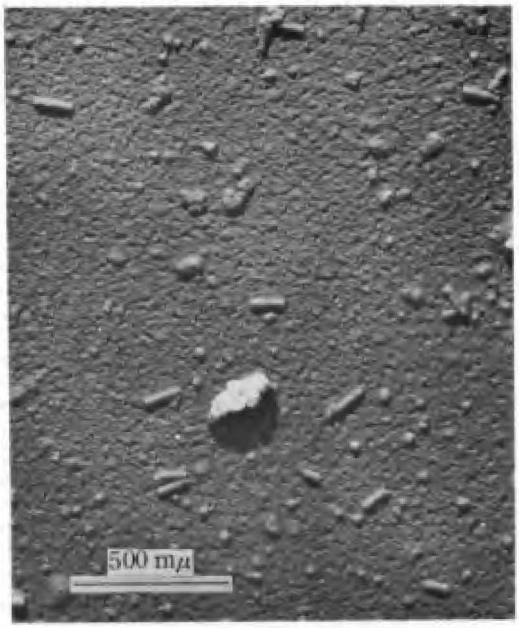

Fig. 1. Electron micrograph of a typical area from a preparation of BSMV infected barley embryo.

were sown in the glasshouse and the seed transmission was examined. Although the rate of seed transmission was likely about 10% lower than that of infected embryo, as seen in Table 1, the difference observed here was not significant statistically. This means that there are some difficulty to trace the evidence accurately only by the comparison of the rate of seed transmission and embryo infection. Accordingly, it was found necessary to take a more direct method that the seed-borne symptoms be examined individually on the seed and embryo which have already been checked for the presence of virus particles with electron microscopy. A typical area from an infected embryo is shown in Fig. 1.

2. *Accuracy of Particle Observation under the Electron Microscope to detect BSMV Infection*

Each of twenty dissected embryos of Kenyoshi No. 3 barley which had been used for electron microscopic observation was separately subjected to an inoculation test. Each of the embryo was ground in about 0.8-1.0 ml of M/10 phosphate buffer at pH 7.0 and inoculated to each 20 seedlings of healthy Hosogara No. 1 barley using a small amount of carborundum as an abrasive. Experiments were repeated 3 times. Results are seen in Table 2. Most of the embryos in which BSMV particles were observed, gave positive results of inoculation test. However, two of those embryo gave

TABLE 2 A

Accuracy of particle observation with electron microscope to detect the BSMV infection of seeds

Experiment	Seed No.	1	2	3	4	5	6	7	8	9	10
A	Embryo	+*	+	−	+	+	−	+	+	+	+
	Endosperm	+	−	−	+	−	−	+	+	+	+
	Infectivity of embryo extract	3/19	1/21	0/19	4/20	8/20	0/20	4/20	2/20	4/20	3/20
B	Embryo	+	+	+	+	+	+	−	+	+	+
	Endosperm	+	+	−	+	−	+	−	+	+	+
	Infectivity of embryo extract	1/20	5/20	7/17	3/20	10/18	8/19	0/20	12/18	4/19	3/20
C	Embryo	+	+	+	+	+	−	+	+	+	+
	Endosperm	+	+	+	+	+	−	+	+	+	+
	Infectivity of embryo extract	3/20	0/21	5/20	9/18	5/20	0/19	6/20	17/20	3/21	4/21

Experiment	Seed No.	11	12	13	14	15	16	17	18	19	20
A	Embryo	+	+	+	+	+	+	+	+	+	+
	Endosperm	+	−	+	+	+	+	+	+	+	−
	Infectivity of embryo extract	6/20	10/20	5/18	5/20	0/20	14/21	5/20	13/20	6/19	5/20
B	Embryo	+	+	+	+	+	+	+	−	−	−?
	Endosperm	+	−	+	+	+	+	−	−	−	−
	Infectivity of embryo extract	9/19	13/19	13/20	6/19	10/20	9/19	6/20	0/19	1/19	5/19
C	Embryo	+	+	+	+	−	+	+	+	+	+
	Endosperm	+	+	+	+	−	+	+	+	+	−
	Infectivity of embryo extract	8/22	2/19	4/20	4/20	3/24	5/20	9/20	7/18	3/20	5/19

*+ and − sign indicate positive and negative result of EM observation for virus particles.

TABLE 2 B

Summarized result of the experiment in Table 2A

Virus particles in Embryo		Virus particles in Endosperm	Infectivity of embryo extract	
+	52*	+	+	41
		+	−	2
		−	+	9
		−	−	0
−	8	+	+	0
		+	−	0
		−	+	3**
		−	−	5

* Number of the seeds.

** One of the negative results is not conclusive because of the unsuitable conditions of the preparation for electron microscopy.

negative results. Three of the embryos, on the contrary, in which no virus particle was found, showed positive results, though one of them was thought to be caused probably by the unsuitable conditions of specimens for electron microscopy. These results obtained are not yet conclusive because of the nature of the techniques of the both tests, but it seemed likely that both electron microscopy and inoculation test might have almost the equal chance of detecting embryo infection. Observation of BSMV particles in embryo was rather easy in most cases, but not in the case of endosperm. Accordingly, some of the negative results obtained in endosperm does not seem highly reliable. As seen in the table, the infectivities of the extracts of embryo were variable and weak in some cases. It was, therefore, noticed that, under the experiment conditions, the electron microscopic observation seemed to have more advantages than the inoculation test, as long as specimens for electron microscopy be prepared carefully.

3. *Distribution of BSMV Particles in Germinating Embryo*

Specimens for electron microscopy were prepared with sctuellum and pulmule dissected from each 20 seeds of Kenyoshi No. 3 barley moistened in refregerator for 1 and 3 days. Similary, after 11 days when the seeds germinated and their first leaves developed to about 1/2 length of the elongated

TABLE 3

Distribution of BSMV particles in germinating embryo

Experiment	Seed No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	Scutellum	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	Pulmule	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+(+)*		+	+
B	Scutellum	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	−	−	+	+	+
	Pulmule	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	−	−	+	+	+
C	Scutellum	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−
	Coleoptile, primary leaf	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	+	−
	Young roots	+	+	+	+	+	+	+	+	−	+	+	+	+	+(−)**		+	+	−	+	−

Results of EM observation in germinating seeds after the incubation for 1 (A), 3 (B), and 11 days (C) in refregerator.

* Not conclusive. A few particles, probably due to the contamination.

** Not conclusive. Preparations for EM were not suitable for the observation.

coleoptile, the specimens were also prepared from scutellum, primary leaf and coloeptile and young primary roots. Table 3 shows the results. There was almost no difference in virus particle association among organs and stages in germinating embryo, with two exceptions probably due to the contamination and the unsuitable conditions of the preparation. Consequently, it was found that electron microscopic observation about the

scutellum could be an appropriate method at least to detect embryo infection reasonably well at the early stage of germination.

4. *Relationship between Embryo Infection and Seed Transmission*

The results of the above mentioned experiments indicate that electron microscopic observation of dissected scutellum is a reliable method to detect embryo infection. To ascertain whether all of the embryos, in which BSMV particles were detected, should develop the seed transmission or not, the following experiments were carried out. In the Experiments 1 and 2, each 50 seeds of Kenyoshi No. 3 which had been used for the electron microscopy

TABLE 4 A

Seed transmission of BSMV in relation to the virus particle association with embryo

Seed No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Virus particles Scutellum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+
Virus particles Endosperm	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+
Seed transmission	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+

Seed No.	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Virus particles Scutellum	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+
Virus particles Endosperm	+	+	−	+	−	−	+	−	−	+	+	−	−	+	+	−	+	+	−	−
Seed transmission	+	+	−	+	−	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+

Seed No.	41	42	43	44	45	46	47	48	49	50
Virus particles Scutellum	+	+	+	+	+	+	+	+	+	+
Virus particles Endosperm	+	+	+	+	+	+	+	−	+	+
Seed transmission	−	+	+	+	−	+	+	+	+	+

TABLE 4 B

Summarized result of the experiment in TABLE 4A

Seed transmission		Virus particles		
		Scutellum	Endosperm	
+	41	+	+	32
		+	−	9
		−	+	0
		−	−	0
−	9	+	+	3
		+	−	0
		−	+	2
		−	−	4

were tested further for seed transmission, and also the other each 50 seeds were used for embryo culture test in the additional Experiments 3 and 4. After the electron microscopy, in the Experiment 1 and 2, each of the

TABLE 5

Summarized result of the Experiment 2, relationship between virus particle association with embryo and seed transmission

Seed transmission		Virus particles		
		Scutellum	Endosperm	
+	40	+	+	34
		+	−	6
		−	+	0
		−	−	0
−	10	+	+	4
		+	−	1
		−	+	2
		−	−	3

seeds was sown seperately to avoid the contact transmission of the virus in sterilized sand in petri dish and was observed for seed-borne symptoms, whereas the dissected embryos were cultured in test tubes in the Experiment 3 and 4. When no virus particle was found in the embryo, the additional two or more specimens for electron microscopy prepared from the same embryo were observed.

Experiment 1. Table 4 shows the results. BSMV particles in embryo and endosperm were detected in 44 and 37 out of 50 seeds, respectively, whereas the seed transmission occurred in 41 seeds. Three out of 44 seeds, in which embryo infection had been confirmed, developed healthy seedlings, but the others produced mottled seedlings. None of the embryo in which BSMV particles had not been detected developed seed-borne symptoms.

Experiment 2. The experiment was carried out in a similar way to the above. An additional electron microscopy was made after the observation of seed transmission to ascertain whether an albino and the symptomless seedlings were virus-free, and for this purpose the preparations from the scutellum, which had mostly been digested and shrivelled at that time, primary leaf and roots were also tested. As seen in Table 5, quite similar results to the Experiment 1 were obtained. Five out of 45 seedlings which had been grown from the BSMV particle associated embryo showed no mottling. An albino seedling grown from the infected embryo found to be diseased. No virus particle was found in primary leaf and roots of these symptomless seedlings.

TABLE 6 A

Experiment 3—Relationship between embryo infection and seed transmission (Embryo culture test)

Seed No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Virus particles in scutellum	+	+	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+
Seedling infection	+	−	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+
Virus particles in Scutellum	+	−						−			−									
Virus particles in Primary leaf	+	−						−			−									
Virus particles in Roots	+	−						−			−									

Seed No.	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Virus particles in scutellum	−	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Seedling infection	−	+	+	D	+	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+
Virus particles in Scutellum	−					−									−	−				
Virus particles in Primary leaf	−					−									−	−				
Virus particles in Roots	−					−									−	−				

Seed No.	41	42	43	44	45	46	47	48	49	50
Virus particles in scutellum	+	+	+	+	+	+	+	+	+	+
Seedling infection	+	−	+	+	+	+	−	+	+	+
Virus particles in Scutellum		−		+			−			
Virus particles in Primary leaf		−		+			−			
Virus particles in Roots		−		+			−			

D: Not germinated

TABLE 6 B

Summarized result of the Experiment 3 in TABLE 6A

Seedling infection		Embryo infection	
+	40	+	40
		−	0
−	9	+	5
		−	4

However, a small amount of virus particles, which may possibly be due to the contamination from endosperm, was found in the specimens prepared from the scutellum of some of these seedlings, though the basal portion of coleoptile, including the scutellum adjacent to the softened and almost digested endosperm, had been thoroughly washed with running water.

Therefore, the embryo culture technique seemed to be preferable in order to prevent the contamination with endosperm.

TABLE 7

Summarized result of the Experiment 4, relationship between embryo infection and seed transmission (Embryo culture test)

Seedling infection		Embryo infection	
+	41	+	41
		−	0
−	8	+	2
		−	6

Experiment 3 *and* 4. Similar to the Experiment 1 and 2, BSMV particles in scutellum was investigated, but in order to examine seed transmission, embryo culture technique was applied in the Experiment 3 and 4. After the observation of the symptoms of seedling in embryo culture test, scutellum, primary leaf and roots of all of the symptomless seedlings together with some of the mottled seedlings, were examined further to detect virus particle association. The results obtained in these two experiments were very similar to those of the Experiment 1 and 2. No virus particles could be detected in scutellum, primary leaf and roots of the symptomless seedlings. These seedlings were found to be apparently virus-free.

TABLE 8

Alteration of BSMV particle association with germinating embryo

Seed No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Virus particles in scutellum A	++	++	++	++	++	+	+	++	++	+	−	−	++	++	+	++	+	+	++	+
Virus particles in scutellum B	++	+	++	+	+	+	+	+	+	±	−	−	+	++	+	++	+	+	+	++
Seed transmission	+	+	+	+	+	−	+	+	+	+	−	−	+	+	−	+	−	+	+	+

Seed No.	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
Virus particles in scutellum A	+	+	+	++	+	++	+	+	+	++	++	−	++	++	+	+	+	++	+	+
Virus particles in scutellum B	++	+	+	±	+	++	+	+	++	++	±	−	+	+	+	++	+	+	++	+
Seed transmission	+	−	+	+	−	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+

Seed No.	41	42	43	44	45	46	47	48	49
Virus particles in scutellum A	+	+	++	+	−	+	++	+	+
Virus particles in scutellum B	++	±	++	++	−	+	+	++	+
Seed transmission	+	−	+	+	−	+	+	+	+

Results of EM observation in germinating embryo after the incubation for 1 (A) and 5 days (B) at room temperatures.

Throughout those four experiments, the followings were observed: the seedling infection did occur only through embryos carrying BSMV particles, but not through the embryos with no virus particles. However, some limited individuals of the infected embryos developed virus-free seedlings. Virus particles found in endosperm were not responsible for seed transmission.

5. *Alteration of BSMV Particle Association with Germinating Embryo*

The results obtained in the foregoing observations showed the unidentity of seed transmission and embryo infection. Hence, virus particles association with germinating embryo had to be examined further to know in what stage of germination some of the embryo or the young seedling became virus-free. Virus particles in germinating embryo were traced by the similar way to the former experiment on distribution of virus particle in germinating embryo. Only the scutellum was used for virus particle observation in this experiment. Specimens for electron microscopic observation was prepared using the seeds which had been moistened and incubated 1 and 5 days at room temperatures. After 5 days of incubation, all the seeds were germinated, when coleoptile developed about 10 mm in length but folded primary leaf was still in coleoptile. The amount of virus particles observed under the electron microscope was roughly recorded, but it was difficult to give a reliable estimation. As seen in Table 8, the data obtained are quite similar to those of the result shown in Table 3. These results suggest that virus inactivation may have not yet occurred until the early stages of embryo germination, nor may have not yet completed if the inactivation should advance gradually.

DISCUSSION

In many cases of seed-transmitted viruses thereis some evidence to suppose that the virus is associated with embryo. Although the infection of immatured embryo is confirmed in some of the viruses that are not seed-transmitted such as southern bean mosaic virus in bean (Cheo, 1955; Crowley, 1959), the results reported recently by some workers suggested that it is generally essential for seed transmission that the virus can enter embryo before the critical period (3, 4 5, 6, 7, 8, 9). However, the results and the opinion about the relationship among embryo infection and seed transmission and embryo maturation which have been presented by several workers are not similar, and the question is yet to be fully answered. Schippers (1963) reported that bean common mosaic virus was not inactivated in the embryo during maturation, nor did inactivation occur during storage and germination. Tsuchizaki (1965) found that both cowpea mosaic virus in cowpea and azuki-bean mosaic virus in azuku-bean were transmitted only through the embryos carrying the virus at sufficient concentrations, and virus inactivation did

occur in embryos carrying the virus at low concentrations during germination. Iizuka (1965) reported that the rate of soybean mosaic virus infection in almost matured soybean embryo was found to be higher than the rate of seed transmission but in immatured and also matured and dried embryo the rate was nearly equal to that of seed transmission. On the other hand, the rate of soybean stunt virus infection in immatured soybean embryo was lower than that of seed transmission, whereas the rate of infection in matured embryo was almost equal to that of seed transmission. The author (Inouye, 1962) reported the results of embryo culture test that he could not detect any apparent difference among the rate of BSMV infection of barley embryo at various stages of seed maturation and the rate of seed transmission. In the present paper, however, the author's former supposition that it was not likely that inactivation occurs at the stage of seed development nor in germination must be corrected, in view of the finding in this paper that the rate of seedling infection in embryo culture did not mean the rate of embryo infection but seed transmission.

According to the results obtained in this paper, about 4-11% of embryo carrying BSMV particles developed virus-free seedlings. In other words, all of BSMV infected embryo did not always produce seed-transmitted seedlings. This looks very similar to Tsuchizaki's observation on cowpea mosaic and adzuki-bean mosaic, though the relationship between virus concentration in embryo and seed transmission of BSMV is yet to be clarified.

Although some part of the observation in this paper is not as yet conclusive, the period when inactivation of BSMV in some of the germinating embryos does occur is supposed to be about the period when primary leaf emerges from coleoptile.

Based upon the findings of this paper, the author presents in the following his rectified supposition about the seed transmission of BSMV:

1) Seed transmission of BSMV in barley is caused by the embryo infection either through pollen or ovule prior to a certain critical period. The virus associated with endosperm is not responsible for seed transmission.

2) It is not likely that virus inactivation in embryo occurs during the maturation and storage.

3) A small number of the infected embryos produce healthy seedling, which must be due to the virus inactivation in germinating embryo.

It remains, however, obscure whether the virus inactivation takes place suddenly at some particular period or gradually during the germination. More detailed and closer experiments should be made further to clarify the question whether the disappearance of virus particles in some of the virus infected embryo at germination is really due to the virus inactivation, and also whether all the virus particles in germinating embryo could be inactivated, and again what factor should be responsible for partial inactiva-

tion of virus in germinating embryos. Further, it must also be clarified whether the inactivation is attributed to the existence of some high energy phosphate compounds in embryo, as Cadwell (1962) has suggested.

SUMMARY

Several experiments to clarify the relationship between infected embryo and seed transmission of BSMV in barley were carried out by the use of electron microscopy. Both virus particle observation and inoculation test appeared to have almost an equal chance to detect the infected embryo. BSMV particles were detected in scutellum, pulmule and primary roots of all of the infected embryos at the early stages of germination. However, about 4-11% of BSMV infected embryos developed virus-free seedlings. A supposition about the seed transmission of BSMV is presented as follows, correcting the author's former supposition in 1962;

1) Seed transmission of BSMV in barley is caused by the embryo infection through either pollen and ovule prior to a certain critical period. The virus associated with endosperm is not responsible to seed transmission.

2) It is not likely that virus inactivation in embryo occurs during the maturation and storage.

3) A limited number of the infected embryos produce healthy seedlings, which must brobably be due to the virus inactivation in germinating embryo.

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