10. A SEED BORNE VIRUS OF *PHASEOLUS AUREUS* (ROXB.)

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Introduction

A virus causing mosaic and ringspot symptons on *Phaseolus aureus* (Roxb.) has been studied with regard to symptomatology, host range, physical properties and serological relationships. The virus was mechanically transmitted and has a host range similar to tobacco ringspot virus (TRSV). The causal virus was found to have a thermal inactivation point between 65° and 70°C., a dilution end point between 1: 1,000 and 1: 10,000 and the *in vitro* stability was 4–5 days. Infected leaves of *Phaseolus aureus* dried over calcium chloride retained infectivity for 20 days. In antigenic analysis by Ouchterlony double diffusion technique in agar medium, a distinct precipitation band was obtained when antigenic sap was reacted against antiserum to TRSV strain NC58 of U.S.A. The virus was found to be extensively seed borne in *Phaseolus aureus* cultivars Golden Glory and MI. 1, and was recovered from seed coats, cotyledons and embryoes of dried seeds collected from infected plants. In cross pollination studies the infected pollen and infected ovule have been determined to be equaly efficient in the transmission of this virus to the progeny. The possibility of the use of gametes from *Phaseolus mungo* to obtain cultivars of *Phaseolus aureus* that would show no virus seed passage has been demonstrated in a limited interspecies cross pollination studies.

Phaseolus aureus (Roxb.) commonly known as "green gram" is widely cultivated as a grain legume crop in Sri Lanka. In the past several years a mosaic disease of viral origin has been known to affect yields drastically. A total of seven viruses has been recorded elsewhere on this plant (17). In Sri Lanka at least three viruses have been isolated from mosaic infected plants. This paper presents the results of investigations conducted on one of the virus causing mosaic disease of green gram.

Materials and methods

Experimental material was obtained from plants found naturally infected in the field and from infected seed samples sent to the laboratory for tests by the extension workers. The seedlings were raised in an insect proof green house maintained between 20° and 35°C. The preparation of leaves of homogenization consisted of washing the leaves in distilled water, blotting off the excess water and slicing the lamina tissue into small strips. The diluent medium was neutral potassium phosphate buffer consisting of 0.0061 molar dipotassium hydrogen phosphate and 0.0039 molar potassium dihydrogen phosphate. The homogenate was strained through four layers of cheese cloth and inoculated to test plants by rubbing the abaxial surface of the leaves with sterile cotton gauze saturated with the infective extract. Celite or carborandum at one per cent level was added to facilitate inoculation. The dilution of the sap extract was calculated by dividing the original weight of leaf tissue by the resultant volume of extract.

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In cross pollination studies the female parent was emasculated 18–24 hours prior to pollination. Freshly collected pollen was used and these were dusted on stigmas with a camel hair brush. The detection of seed transmission was by germination tests and infectivity assays. The possible presence of the virus in apparently healthy seedlings was detected by inoculation of sap extracts to 8–10 days old seedlings of *Luffa acutangula*. The presence of viral antigen was detected serologically by Ouchterlony double diffusion test in agar medium (Ouchterlony 1956 & 1962). The diffusion medium consisted of 1 per cent Noble* agar disolved in 0.01 molar neutral phosphate buffered saline and 0.02% sodium azide. The tests were made with antigen wells deployed in a ring around the antiserum well. The antigen and antiserum wells being 1.0 cms apart. The antiserum was used undiluted and appropriate controls were instituted to assure that positive reactions were between antigen and antibody and did not involve extraneous constituents. The diluent medium for antigenic sap was 0.01 molar neutral potassium phosphate containing 0.14 molar sodium chloride.

Results

1. Disease symptoms The symtoms differ widely depending on the cultivar and the season. Virus like symptoms were observed in all green gram fields inspected, the most common in the Maha** season on the cultivars Golden Glory and MI–1 consisted of reddish brown necrotic ringspots 4–20 mm in diameter on the underside of leaves, on petioles, stems and pods. These ringspots often coalised to produce a mosaic effect and the leaves become brittle. The virus induces a high percentage of sterility due to flower abortion, non fertile pods and partial filling of grains. Other variable symptoms in the Yala*** season were chlorotic or light green small or large irregularly shaped patches on leaves. These symptoms tend to increase in intensity as the crop matured. The infected plants were stunted, the nodes exhibited a slight swelling, the root system was poorly formed.

2. Hosts in nature The virus was isolated from several weed species found infected in and around green gram fields. The most frequently observed infected weeds were, *Crotalaria intermedia, Phaseolus lathyroides, Cleome pentaphylla, Impatiens balsamia, Heriotropium corymbosum* and wild *Cucurbit* sp. of plants.

Among the cultivated plants the virus was detected by infectivity and by serodiagnosis on *Glycine max, Hibiscus esculantus, luffa acutangula, Lycopersicum esculentum, Nicotiana tabacum, Passiflora edulis* cultivar *flavicarpa, phaseolus mungo,* and *Trichosanthes anguina.*

3. Transmission The virus was found to be easily transmitted by mechanical inoculation with sap extracts from infected plants. The aphids, *Aphis gossypii* and *Myzus presicae* failed to transmit this virus after acquisition feeding periods of 15 minutes, 1 hour, 3 hours and 25 hours.

In two of seven trials mites of *Tetranychus* sp. successfully transfered this virus from diseased to healthy test plants. The plant parasitic nematodes tested failed to transmit this virus from diseased to healthy test seedlings.

4. Host range The virus was mechanically inoculated to a range of host plants. A diversity of symptoms were obtained depending upon the conditions of the test. Many of the indicator hosts exhibited an initial severe reaction to the virus infection

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* a product of General Biochemicals, Inc., Ohio, USA

** Maha season refers to the period from October to March when the North East monsoon is experienced. (Wet season)

*** Yala season refers to the period from April to September when South West monsoon is experienced. The dry zone of Sri Lanka does not receive much precipitation due to South West monsoon. (Dry season)

followed by a recovery phase where the emergent leaves were found to be symptomless. The results of host range studies are in Table 1.

5. Physical properties Seedlings of *Phaseolus aureus* two to three weeks after infection were used in tests for physical properties.

Therma linactivation point: Infectivity of sap extracts was abolished when infectious sap was maintained in serological tubes at 70°C for 10 minutes. Infectivity was however, retained when sap extracts were maintained at 65°C for 10 minutes.

Table 1. Host range, incubation periods and symptoms produced on indicator hosts in insect proof green house

Host	Incubation period	Symptoms	
Amaranthus sp.	6-8[a]	L[b]	Mottle R[c]
Canavalia ensiformis	6-12	L	Mild mottle
Cleome pentaphylla	6-8		Mottle & mosaic
Glycine max	6-10		Mosaic & dark angular patches on pods
Hibiscus esculentus	8-12	L	Mosaic & leaf deformation
Impatiens balsamina	12-18		Mild mosaic
Luffa actangula	4-6		Mosaic, severe systemic necrosis of leaves & stem
Lycopersicum esculentum	8-10		Mosaic & leaf deformation
Mirabilis jalapa	6-8		Mosaic & leaf distoration
Nicotiana tabacum	6-8		Mosaic, & leaf deformation
Passiflora edulis f. *flavicarpa*	—[d]		Symptomless[e]
Petunia hybrida	10-12		Faint mosaic (R)
Phaseolus lathyroides	14-24		Pronounced mosaic as plant matures
Phaseolus mungo	8-12	L	Mosaic as plant matures
Phlox drummondii	10-12		Mosaic & ringspots
Physalis peruviana	6-8		Severe mosaic & leaf deformation
Spinacia oleracea	6-8		Mosaic & leaf deformation
Trichosanthes auguina	4-6	L	Severe mosaic & systemic necrosis
Vigna sinensis	8-12	L	Mild mosaic (R)
Zinnia elegans	14-18		Mild mosaic (R)

a) Incubation period in days.
b) L represents the appearance of local lesions on inoculated leaf.
c) R indicates recovery from infection.
d) Indicates incubation period not determined.
e) Tested by infectivity.

Dilution end point: The virus was found to tolerate a dilution between 1: 1,000 and 1: 10,000. No infectivity was obtained at dilutions at and above 1: 10,000.

Longivity *in vitro*: Repeated ageing tests indicated that the virus lost infectivity after storage at room temperature for 3–4 days.

Ageing *in vivo*: When infected leaf samples were dried over calcium chloride infectivity was retained for 10–20 days.

6. Serological relationships In serological tests partially clarified sap extracts were reacted with antiserum to tobacco ringspot virus (TRSV. N. C. 58) of U.S.A. A positive precipitation reaction was obtained with antigenic sap up to a dilution of

1:8. The precipitin line was closer to the antiserum well indicating that the antigen fraction of the Sri Lanka strain of the virus had a faster mobility compared with the globulin fraction.

7. Seed transmission Seeds collected from infected plants had wrinkled seed coats with a slight swelling at the hilum. Seedlings emerging from such seeds remained symptomless till the cotyledonary stage. Symptoms of chlorotic and necrotic ringspots appear in the first few leaves and these become more marked as the plant matures and if the environmental conditions favour disease development.

Location of the virus in the seeds: Composite samples of portions of disected seed coats, cotyledons and embryos from seeds showing signs of infection were separately triturated with equal part weight for volume of 0.01 molar neutral potassium phosphate buffer and assayed by infectivity and serology for the presence of infectious virus or its antigen. The virus and its antigen were detected in the seed coats, cotyledons and the embryos indicating that the passage of this virus into the seeds of *Phaseolus aureus* is one of true embryonic transmission.

Relationship between age of plant at infection and seed transmission: Healthy plants of two cultivars of green gram were inoculated at intervals of about 10 and 20 days before and after pollination. The extent of seed transmission obtained in the different treatments is in Table 2.

Table 2. Relationship between time of infection and seed transmission in two cultivars of *Phaseolus aureus*

Treatment	Time of inoculation and extent seed transmission				
Cultivars	P −20	P −10	P[a)]	P +10[a)]	P +20
MI-1	9 (27)[b)]	2 (28)	0 (31)	0 (44)	0 (18)
Golden Glory	11 (29)	2 (26)	0 (21)	1 (17)	0 (22)
Controls	Non inoculated and extent seed transmission				
MI-1	0 (25)	0 (18)	0 (14)	0 (16)	0 (19)
Golden Glory	0 (21)	0 (28)	0 (42)	0 (31)	0 (17)

a) P = Time of pollination (+) or (−) followed by number indicates approximate number of days (−) preceeding (+) following pollination.

b) The number in parenthesis refers to the number of seeds examined by germination test.

The results in Table 2 indicate that for effective seed transmission the mother plant must become infected at least 10 days prior to pollination.

Genetic differences to seed passage of the virus: A total of five cultivars of green gram was examined for gentic differences to seed passage. The seedlings were inoculataed at the cotyledonary stage and the rate of seed transmission was traced through four consecutive generations. The infected seeds from the early generations were used in the successive generations. The results are in Table 3. In the cultivars Golden Glory and S/B — 16 an increase in the rates of seed transmission from 68 to 91 percent and 28 to 61 percent respectively was achieved as a result of passage through four consecutive generations. In the other three cultivars the rates of seed transmission of this virus were not significantly altered as a result of passage through four successive infected generations.

The efficiency of infected pollen and infected ovule in seed transmission: Healthy and infected green gram plants of the cultivar Golden Glory were crossed in four possible combinations. Healthy female × healthy male (H × H), Healthy female × diseased male (H × D), diseased female × diseased male (D × D), diseased female ×

Table 3. Genetic differences to virus seed passage in five cultivars of *Phaseolus aureus*

Treatment cultivars	Percent virus infected seeds in generations			
	I[a]	II	III	IV
1. Golden Glory	68[b]	52	86	91
2. S/B-16	28	21	54	61
3. K-11/51-S16	18	26	21	20
4. Hi 47/S13 P/S	6	8	11	10
5. Hi 45/S12-3	8	12	9	14
6. MI-3 (Control) Non inoculated	0	0	0	0

a) I, II, III, and IV refer to successive generations raised on infected seeds.
b) Percent seed transmission (decimals deleted) as determined by germination test.

Table 4. Virus infection in F_1 progeny obtained by cross pollination of all possible parental combinations of healthy and diseased plants of *Phaseolus aureus* cultivar Golden Glory

Crosses[a] Ovule × Pollen	Total No. of crosses	No. of fertile pods	No. of seeds	No. germinated	Percent infected[b]
H × H	112	76	354	342	0
H × D	264	102	478	446	41.03
D × D	246	81	314	263	43.99
D × H	230	69	268	228	51.55
Controls					
Healthy selfed	—	164	816	804	0
Diseased selfed	—	93	368	315	52.39

a) H = Healthy plant
D = Diseased plant—Virus inoculated at cotyledonary stage
b) Percent infected plants as determined by germination test

healthy male (D × H). The extent seed set in the virus infected plants was lower than in the healthy controls. The pollen is a carrier of the virus and is an effective agent in the transfer of this virus to the progeny. The efficiency of pollen and ovule in achieving virus transmission to the progeny is about equal. The results of cross pollination studies are in Table 4.

Plant to plant transfer of the virus via infected pollen: In about 30 cross pollinations involving pollen from diseased plants and 15 healthy female parents of the cultivar Golden Glory, incidence of plant to plant transfer of the virus through the medium of infected pollen was not obtained. Seed infection by the virus was confined exclusively to the pods of cross pollinated flowers. No evidence for the presence of infectious virus or its antigen was obtained in the vegetative parts of the female parent.

The possible presence of a gene for non seed passage of the virus in *Phaseolus mungo* cultivar MI. 1 (Black Gram): Black gram although susceptible to systemic infection by the virus did not show any evidence of virus seed passage. Healthy and infected green gram female parents of the cultvar Golden Glory were cross pollinated with black gram cultivar MI. 1 as the donor parent. In a reciprocal cross, black gram

Table 5. Virus infection in F_1 progeny obtained by cross pollination of *Phaseolus mungo* with *P. aureus*

Crosses[a)] Female × Male	No. of crosses	No. of fertile pods	No. of seeds	No. germinated	Percent seed transmission
GG (D) × BG	132	2	3	1	0
GG (H) × BG	124	3	5	3	0
BG × GG (D)	100	0	0	0	0
BG × GG (H)	164	1	2	0	0
Controls					
BG (D) selfed	130	114	454	48 (50)[b)]	0
BG (H) selfed	108	103	394	30 (30)	0

a) GG = Green gram parent cultivar Golden Glory.
BG = Black gram parent cultivar MI. 1.
(D) = Virus infected parent.
(H) = Healthy parent.

b) Numeral in parenthesis refer to number of seeds tested by germination test.

cultivar MI. 1 as the female parent was crossed with green gram cultivar Golden Glory as the male parent. The fertile seeds obtained from these crosses were germinated to determine the rate of seed passage. The results of the inter-specific crosses are in Table 5.

Discussion and Conclusion

The study reported in this paper indicates that the virus causing mosaic and ringspot symptoms in *Phasealus aureus* has a wide distribution in Sri Lanka and its host range includes the commoner weed species and many crop plants. The biological properties of this virus closely resemble those reported for strains of tobacco ringspot virus (4, 8, 13, 14, 15, 17).

A critical and more conclusive evidence for plant virus identification would be the reaction with a known antiserum. The results of the serological tests demonstrates that the 'antigenic patch' in the TRSV strain NC 58 of U.S.A. had close similarities to that of the Sri Lanka strain used in this study or that the Sri Lanka strain of the virus shares similar antigens as in the American strain.

TRSV was recognised for a long time as a soil borne virus as its mode of transmission was not known until recently. It now appears that most if not all soil borne plant viruses are transmitted by a biological vector (3,7) Fulton (1962) obtained evidence for the transmission of TRSV by the nematode vector *Xiphinema americanum*. Thomas (1969) claimed that mites of *Tetranychus* sp. successfully transmitted TRSV from infected to healthy test seedlings. Other workers have implicated aphids, mites and thrips in the transmission of TRSV (6, 15, 16). In vector transmission studies only mites of *Tetranychus* species transmitted the Sri Lanka strain of the virus from diseased to healthy test plants. The nematode vector *Xiphinema americanum* has not been recorded for Sri Lanka and other species of plant parasitic nematodes found associated with green gram plants failed to transfer the Sri Lanka strain of the virus. Similarly the two aphid species *Aphis gossypii* and *Myzus persicae* failed to transmit this virus under the green house conditions. The importance and significance of biological vectors in the epidemiology of this virus has not been adequately investigated in this study.

High rates of seed transmission has been frequently reported for nematode trans-

mitted viruses (2,7,10). Nematodes and mites have poor mobility and hence infected seeds have been considered to be the chief means of long distance spread of several viruses (1, 7, 10). Evidence obtained in this study indicates that some cultivars of *Phaseolus aureus* show very high rates of seed transmission, particularly as a result of passage of virus through successive generations (Table 3). The widespread prevalence of this virus in Sri Lanka may therefore have been due to virus dissemination through infected seeds of cultivated plants and weed species rather than via the agency of biological vectors.

The rate of seed transmission in *Phaseolus aureus* is associated with the ability of the virus to infect developing embryos following invasion of the female gametophyte either directly from the mother plant or through the introduction of the virus through the male gametophyte. This conclusion seems justified from the experiments on the association between time of infection of the mother plant and seed transmission (Table 2) and also from results of cross pollination experiments involving all possible combinations of deseased and healthy male and female plants (Table 4). By contrast *Phaseolus mungo* cultivar MI. 1 (Black gram) though susceptible to the virus does not show seed passage. The freedom from infection of gametes by a systemic virus may be either due to inherent resistance of the gametes to invasion and multiplication through the operation of an effective protective mechanism. It is however unlikely that the differences could be due to sporophytic and gametophytic generations, as there is no evidence to support that ploidy *per se* could affect resistance to plant viruses. The available evidence from cross pollination experiments (Table 5) tend to support the conclusion that gamete susceptibility or immunity to infection by a virus is genetically controlled (9). The useful gene source in *Phaseolus mungo* could therefore be utilized in the production of disease free seeds of *Phaseolus aureus*. This is an important step towards containing this virus until immune varieties are available.

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Discussion

T. Soelaeman, Indonesia: What do you think about the use of crystalline inclusion for diagnosing Tobacco ringspot virus? Because this method offers quick diagnosis, and that, for tobacco mosaic virus and tobacco etch virus, tobacco ringspot virus has rectangular crystallin inclusion in cytoplasm.

Answer: Yes, it is possible to use this for diagnostic purpose. I have, however, used *Luffa acutangular* to index this virus.

E. W. Kitajima, Brazil: 1. You mentioned that the disease is quite widespread in Sri Lanka. Do you think contaminated seeds are responsible for that, since mite, with a low mobility, would not contribute such extensive distribution? Or, do you think there is a winged vector, such as thrips?

2. Would clean seed production solve the problem?

Answer: Our search for a winged vector has been mostly unsuccessful. I would consider infected seeds as a major factor in the dissemination of this virus in Sri Lanka.

Yes, clean seed production will largely check this virus on *Phaseolus aureus* (i.e. green gram). It will however remain a problem in other crops.

N. Yamada, Japan: Does the seed produced on the infected plants exhibit any specific symptoms by which infected seed can be discarded before sowing?

Answer: Yes, seeds from TRSV infected plant have wrinkled seed coat with a slight swelling at the hilum. Environmental conditions could cause similar symptoms, too. Therefore, these symptoms may not be a satisfactory criterion for discarding the infected seeds.

N. Murata, Japan: Is there any correlation between effciency of seed transmission of five cultivars listed in Table 3 and their general symptoms?

Answer: Yes, there is a positive correlation between disease severity and extent of seed transmission. The highly susceptible cultivar, Golden Glory, exhibits severe symptom and has a high seed transmission, while some of hybrid lines show mild symptom with low seed transmission.

M. D. Mishra, India: What is your opinion regarding aphids as the vector of tobacco ringspot virus? Are they an agent of transmission of tobacco ringspot viruse, since many ringspot viruses are reported to be transmitted by aphids and they resemble to tobacco ringspot virus in certain respects?

Answer: Other workers have reported on aphid transmission of TRSV. In our work, we have consistently failed to obtain evidence of aphid transmission using *Aphis gossypii* and *Myzus persicae.*