Table 1. Cross reaction of rhizobiophages with rhizobial isolates

Rhizobial solates	PR 1	PR 2	PR 3	PR 1	PR 2	PR 3	PR 4	PR 5	PR 1	PR 2	PR 3	PR 4
CRR 6	*	*	-	-	-	*	-	*	-	*	-	-
CRR 4	-	*	-	*	-	-	-	*	-	*	-	*
CC 1	*	-	*	*	-	-	-	*	-	*	-	*
CRU 15	-	-	-	*	-	-	-	-	*	-	-	*
CRU 7	-	*	-	-	*	*	-	-	*	*	-	*
RS 1	*	-	-	-	*	*	-	-	-	*	*	-
COC 10	-	*	-	-	*	-	*	-	-	-	-	-
BMBS	-	*	-	-	*	-	*	-	-	-	-	-
CRM 6	-	*	-	*	-	*	-	-	*	-	-	-
CRM 7	*	*	*	*	*	-	-	*	-	*	-	*
CRM 13	-	-	-	-	-	*	-	-	-	-	*	-
COG 15	-	-	*	*	*	*	-	-	-	*	-	*

* Lysis - No Lysis

Table 2. Isolation of rhizobiophages from different soils, their homologous rhizobial cultures and lytic efficiency

Rhizobiophages	Host strains	*Average titre of stock phage (x 108 pfu ml^{-1})	Number of cultures lysed	Number of cultures tested	Lytic efficiency (%)
Redgram					
PR 1	CRR 6	14.0	4	12	33.3
PR 2	CRR 4	45.0	7	12	58.2
PR 3	CC 1	65.0	3	12	25.0
Blackgram					
PB 1	CRU 15	3.0	6	12	50.0
PB 2	CRU 7	45.0	5	12	41.6
PB 3	RS 1	51.0	6	12	50.0
PB 4	COC 10	60.0	1	12	08.3
PB 5	BMBS	12.0	5	12	41.6
Greengram					
PG 1	CRM 6	15.0	3	12	25.0
PG 2	CRM 7	12.0	8	12	66.6
PG 3	CRM 13	23.0	2	12	16.0
PG 4	COG 15	45.0	6	12	50.0

PR - Phage isolatd from corresponding redgram host strains
PB - Phage isolated from corresponding blackgram host strains
PG - Phage isolated from corresponding greengram host strains
* - The titre of each phage stock was determined by titration in YSM medium using host strain as indicator

Table 3. Resistance of rhizobial isolates to rhizobiophages

Rhizobial isolates	Number of phages		Number of phages tested	Percentage of resistance to phages
	Lytic	Non-lytic		
CRR 6	4	8	12	66.6
CRR 4	7	5	12	41.6
CC 1	3	9	12	75.0
CRU 15	6	6	12	50.0
CRU 7	5	7	12	58.3
RS 1	6	6	12	50.0
COC 10	1	11	12	91.6
BMBS	5	7	12	58.3
CRM 6	3	9	12	75.0
CRM 7	8	4	12	33.3
CRM 13	2	10	12	83.3
COG 15	6	6	12	50.0

Schwinghamer (1960) and the conditions were found to be satisfactory for rhizobiophage isolation. The same medium was used, for the preparation of double layered agar plates for assaying of the rhizobiophages susceptibility of rhizobia.

Enrichment, isolation and purification of rhizobiophages

Rhizosphere soil was mixed with equal quantity of water and incubated in an incubator shaker at room temperature for 1 h and filtered through a cheese cloth. Chloroform 0.5 per cent was added and shaken for 30 minutes. Chloroform mixed filtrate was again centrifuged at 5,000 rpm for 15 minutes. Decanted supernatant was filtered twice through 0.45 gm and 0.2 gm membrane filters to remove bacterial cells. Suspensions were taken and serially numbered. The soil filtrate was again separately inoculated with actively growing mid-log phase cultures of respective rhizobia and further incubated with equal volume of yeast sucrose broth for 2-5 days. The enriched mixture was centrifuged at 10,000 rpm for 30 minutes. Supenatant was decanted and used for rhizobiophage isolation (Patel and Craig, 1984).

Two ml of supernatant (phage suspension) and 1 ml of mid-log phase appropriate rhizobial cultures were mixed into 5 ml of 0.7 per cent molten agar Schwinghamer medium. This mixture was overlaid on the solid medium (1.5 per cent) so as to form a double layer. The petridishes were incubated at room temperature for 7 days (Hashem and Angle, 1990). Single plaques were isolated from the plates and kept for further purification.

The single plaques isolated were again placed on double layer agar plates containing actively growing mid-log phase rhizobial cultures to get plaques (lytic growth) on incubation. This procedure was repeated 4 times as the plaques were considered pure after four successive cycles of isolation. At every stage of rhizobiophage incubation, to avoid host modification of phages, it was ensured that the cultures of the original ones were employed for the phage enrichment. Phages were designated to represent the rhizobial cultures from which they were isolated.

Cross reaction of rhizobiophages and rhizobial cultures

Three red gram isolates, five black gram isolates and four green gram isolates of the rhizobial cultures were taken for cross reaction study against the isolated phages. Mid log phase rhizobial cultures seeded in soft agar was overlaid on the solid agar medium kept in sterile petridishes. Phage dilutions were made from the respective

ihage stocks and spotting was done on the oft seeded agar layer, using sterile multiple noculator. The phage spots on the soft agar urface were air dried by keeping the plates :xposed in laminar flow chamber for 10 minutes, ıefore the plates were incubated at room emperature. The susceptibility *of Rhizobium* o a given phage was judged by the confluent ysis seen on a spotted area. No lysis or growth of rhizobial culture on the spot area was considered as resistance. In this experiment ıll the isolated and purified phages were cross-:hecked with all the rhizobial cultures isolated rom various crops of pulses. The following ormulae were used to assess the resistance of the rhizobial culture and lytic efficiency of rhizobiophages as described by Murugesan, 1997.

'ercentage of phage resistance of a culture =

$$\frac{\text{Number of non-lytic phages}}{\text{Number of phages tested}} \times 100$$

'ercentage of lytic efficiency of a Rhizobiophage =

$$\frac{\text{Number of rhizobial cultures lysed}}{\text{Number of rhizobial cultures tested}} \times 100$$

Results and Discussion

All the 12 cultures of rhizobia were used s hosts for phage isolation and enrichment. During the study, using the filtrates of rhizosphere oil, all the rhizobial cultures established a isible lytic growth in 2-5 days of incubation hen compared to their respective control (Table). This study has clearly indicated that the ıizosphere soil samples contained rhizobiophages. esley (1982) isolated 15 specific bacteriophages or *Rhizobium meliloti* by enrichment of soils. hage population was high in the rhizosphere gion and around root nodules and this region as considered to be the site for phage multiplication ecause of the presence of metabolically active ıizobial populations (Lotz and Mayer, 1972). owalski *et al.* (1974) reported that the phages ere found in all soils and nodule samples. hizobiophages were present almost in all the fields but rarely in non rhizosphere soils (Dhar and Ramakrishna, 1987).

From the lysed suspensions, the phages were isolated and designated as PR1, PR2, PR3, PB I, PB2, PB3, PB4, PB5, PGI, PG2, PG3 and PG4 which were specific to rhizobial hosts CRR6, CRR4, CC1, CRUI5, CRU7, RSI, CoClO, BMBS, CRM6, CRM7, CRM13 and CoGI5 respectively (Table 1). Each phage produced a clear lytic zone and inhibited the rhizobial growth. Susceptibility of individual strain to each phage produced a distinctive pattern and identified 80 different groups in the soils and found that typing system was reliable and reproducible (Lesley, 1982). Sometimes extended incubation also enhanced the plaques formation. The titre of individual phages varied from 3.0 to 65.0 x 10^8 pfu ml^{-1} (Table 2). Lawson *et al,* (1987) reported that fluctuations, in the population of *Rhizobium leguminosarum bv. trifolii* and its phage in different soil types. For obtaining the higher titre of phages, the carbon source in YEMA medium was modified to sucrose instead of mannitol. Since with mannitol, excess of polysaccharides were produced and resulted in weak positive reaction and the modification was carried out.

The phage cross reaction pattern was established on the basis of susceptibility or resistance of rhizobial cultures i.e. by different phages and their interaction with various rhizobial strains. The percentage of phage resistance among the rhizobial strains varied from 33.3 to 9.16 (Table 3). The rhizobial strain CoC 10 (Standard black gram isolate) showed higher resistance (91.6 per cent) and CRM7 (Green gram isolate) showed the lower resistance (33.3 per cent). Other standard rhizobial isolates CCI (Red gram) and CoG15 (greengram) showed 75.0 and 50.0 per cent resistance only. The lytic eiticiency of rhizobiophages on rhizobial strains were checked and was found that it varied much (Table 2). PB 4 exhibited the minimum lytic efficiency (8.3%) whereas the maximum lytic efficiency was noticed in PG 2 phages (66.6%). The cross reaction showed specificity among the rhizobial strains. Dhar and Ramakrishna (1976) reported that none of the 6 phages could infect the strains of other *Rhizobium* spp. but were highly infectious to chickpea *Rhizobium*. Rhizobiophages

often have a narrow host range. De Laujdie and Bogusz (1984) reported that 2 phages RS 1 and RS 2 were found to be restricted to a single *Rhizobium* strain US 5971 which nodulates on *Sesbania rostrata*. Cross reaction of phages and phage typing has been used for the characterization of strains of several *Rhizobium* spp. (Lesley, 1982; Lindstorm and Lehtomaki, 1988) and to determine the indigenous bacterial population (Thurman and Bromfield, 1988). This study indicates that the rhizobiophages have a form of coexistence, surviving along with bacterial strains in soil environment.

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