BACTERIOPHAGE AS RELATED TO THE ROOT NODULE BACTERIA OF ALFALFA[1]

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INTRODUCTION

Bacteriophages virulent for different strains of Rhizobia of leguminous plants have been isolated by various investigators but not much has been learned regarding their significance in the nodules. Laird (1932) established the heterogeneous nature of the Rhizobia with respect to bacteriophage, but his attempt, as well as that of Almon and Wilson (1933), to find a correlation between the effectiveness of different strains in nodulation and sensitiveness to the lytic principle was not successful. Among the theories relative to the means by which atmospheric nitrogen fixed by Rhizobia is made available to the plant, Fred, *et al.*, (1932) have listed the action of bacteriophage, but little or no work has been done in this connection. Demolon and Dunez (1935) isolated a bacteriophage from the soil surrounding the roots of old alfalfa plants and concluded that it interferes with symbiosis between the Rhizobia and the host plant.

Within recent years a decrease in yield of alfalfa, *Medicago sativa*, three to five years after seeding has been noted on irrigated soils in the Yakima and Ellensburg districts in Washington. Marked responses from additions of nitrogen have been obtained in experiments in these districts, pointing to the possibility of the presence of some substance which interferes with normal symbiosis between the nodule bacteria and the alfalfa plant. The object of the work presented here was to determine if Rhizobia bacteri-

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ophage may be responsible for decreases in yield of old stands of alfalfa.

EXPERIMENTAL PROCEDURE

Samples of alfalfa plants, including leaves, stems, and roots, and of soil in contact with, or close to, the tap roots were tested for bacteriophage, using the culture medium of Demolon and Dunez (1935). It contains:

K_2HPO_4	1 gram
$MgSO_4$	0.2 gram
$CaCl_2$	0.1 gram
NaCl	0.1 gram
$FeCl_3$	0.02 gram
Glucose	3 grams
$CaCO_3$	1 gram
Alfalfa root extract	1000 cc.

For the preparation of the root extract 200 grams of finely chopped, fresh, washed alfalfa roots were placed in 1000 cc. of distilled water and boiled gently for two hours. The mixture was kept over night, filtered through cotton, and the filtrate made up to volume. The salts were then added and the reaction of the solution adjusted to pH 7.8.

The soil samples were passed through a 16-mesh sieve and the alfalfa roots and stems and leaves were separated, crushed, and ground in a meat chopper. Portions of 100 grams of soil, root, stem, and leaf tissue were placed in separate flasks containing 500 cc. of the liquid culture medium. The mixtures were kept at 28°C. for 36 to 48 hours, filtered through filter paper, and finally through Berkefeld N candles. Two-cubic centimeter portions of the filtrates were added to 24-hour fluid cultures of *Rhizobium meliloti* (medium 5 of Laird (1932)). After 48 hours of incubation at 28°C., the cultures were filtered through Berkefeld N candles and 2-cc. portions of the filtrate added to fresh 24-hour fluid cultures of *Rh. meliloti*. Clearing of the fluid cultures may be observed following the addition of the initial filtrate or following the second or further serial transfers, depending upon the potency of the lytic principle. At least five serial transfers were made of the filtered extracts of each sample. If no clearing was

effected following the fifth serial transfer, the lytic agent was considered absent and the sample discarded.

The nodules were sterilized with 1:500 $HgCl_2$, thoroughly washed with sterile water, crushed aseptically, and pieces of the crushed nodules placed on streaks made from a suspension of a susceptible strain of *Rh. meliloti* on agar in plates, which were incubated at 28°C.; and lysis, if present, was manifested in 48 to 60 hours by the absence of bacterial growth in the vicinity of the pieces of nodule tissue.

EXPERIMENTAL RESULTS

The extracts of samples of soil, roots, and stems obtained from three-year-old stands of alfalfa in two old fields in the Yakima valley were tested against each of five different strains of laboratory stock cultures of *Rh. meliloti.* The root and stem tissues yielded no lytic principle, but the soil extracts contained a lytic principle that was strongly active against two of the five strains, fairly active against two others, and totally inactive against the fifth after five serial transfers. The roots carried few nodules, but all those tested produced lysis.

Samples of soil and alfalfa obtained in both the Yakima and the Ellensburg districts from fields which have been in alfalfa off and on for many years and also from fields carrying this crop for the first time were used for subsequent experiments. The nodules and the extracts of the samples of soil, alfalfa roots, stems, and leaves were tested against the strain of *Rh. meliloti,* which proved to be most susceptible to lysis in the preliminary tests. The results obtained with the soil extracts and the nodules are summarized in table 1.

As may be noted, bacteriophage was present in several of the soils with old alfalfa stands but not in soils carrying alfalfa less than three years of age. Contrary to the findings of Demolon and Dunez (1935), who isolated bacteriophage from the roots and stems of all alfalfa over one year old, this lytic principle was not found in the roots, stems, or leaves of alfalfa except in one case, in which the roots of three-year-old plants growing on soil containing bacteriophage also showed the presence of the lytic principle.

All the nodules yielded a lytic principle, as in the preliminary tests, and this occurred regardless of the age of the plants carrying the nodules. The presence of the lytic principle was manifested by lysed parts of the streaks on agar plates, as indicated in figure 1.

TABLE 1

Bacteriophage in soils and alfalfa tissues

FIELDS IN ALFALFA OFF AND ON FOR MANY YEARS					FIELDS IN ALFALFA FOR THE FIRST TIME				
Soil sample number	Age of stand	Soil	Nodules	Nodulation	Soil sample number	Age of stand	Soil	Nodules	Nodulation
Yakima District									
	years					*years*			
1	2	−	0	Poor	21	2	−	0	Medium
2	2	−	0	Poor	22	2	−	+	Medium
3	3	+	+	Poor	23	3	+	+	Medium
4	3	+	0	Poor	24	3	−	0	Good
5	3	−	0	Poor					
6	3	+	0	Poor					
7	4	−	0	Medium					
8	4	−	+	Poor					
9	4	+	0	Poor					
10	12	+	0	Poor					
Ellensburg District									
11	1	−	0	Poor	25	2	−	0	Medium
12	1	−	+	Good	26	2	−	0	Good
13	2	−	0	Good	27	3	−	0	Medium
14	3	−	0	Good	28	4	+	0	Poor
15	5	−	0	Medium	29	4	−	0	Medium
16	6	−	0	Poor					
17	6	−	+	Medium					
18	9	+	0	Poor					
19	25	−	0	Good					
20	25	−	0	Medium					

+, bacteriophage present; −, bacteriophage absent; 0, not tested.

These results are in agreement with those of Demolon and Dunez (1935) but at variance with those of Laird (1932), who was able to find bacteriophage only in very young nodules of clover, *Trifolium pratense*, and sweet clover, *Melilotus alba*, in pot cultures or under field conditions.

The results obtained from 29 fields appear to indicate that fields with three- and four-year-old stands of alfalfa are the most common source of bacteriophage, as only two of the eight fields yielding the lytic principle carried alfalfa stands over four years of age,

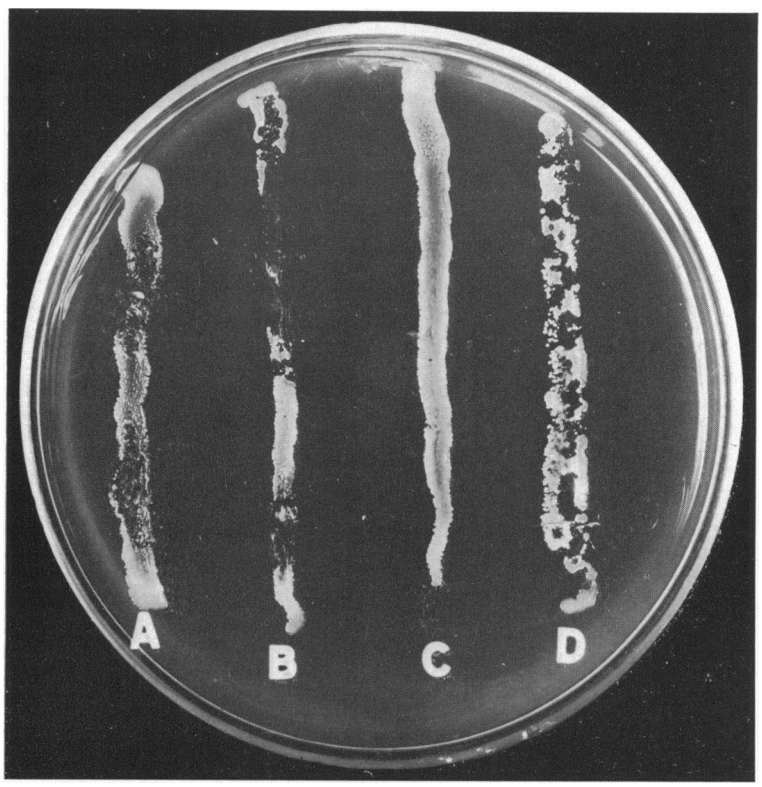

FIG. 1. LYSIS OF RH. MELILOTI ON SOLID MEDIA BY BACTERIOPHAGE FROM ALFALFA NODULES AND BY BACTERIOPHAGE FILTRATE

A and *B*, streaks of *Rh. meliloti* with pieces of crushed nodule; *C*, untreated "control" streak; and *D*, streak impregnated with one loopful of a homologous bacteriophage.

the stands in these cases being nine and twelve years old. On the whole, nodulation on the plants in the affected fields was poor. All except two of these fields are located in the Yakima district and crop records indicate that for many years five of the affected fields in the Yakima district and one of the two in the Ellensburg

district have carried alfalfa off and on for the major part of that time.

VIRULENCE, PLAQUE FORMATION, AND TRANSMISSIBLE LYSIS

Laird (1932), Almon and Wilson (1933), and Demolon and Dunez (1935) demonstrated that different strains of Rhizobia homologous for specific groups of legume plants are not equally sensitive to the same bacteriophage, and conversely that specific strains of Rhizobia are not equally sensitive to different bacteriophages isolated from the nodules or plant tissues of the same species of legumes. The data in table 2 are in agreement with the first observation of these investigators with respect to the sensitiveness of five strains of *Rh. meliloti* to the action of the lytic principle isolated from the alfalfa field represented by soil 3. Their second contention is supported by the results given in table 3, which show the comparative virulence of the lytic principles isolated from a number of different alfalfa fields against the same strain of *Rh. meliloti.*

A quantitative determination of the virulence of the bacteriophage isolated from soil 3 following eleven serial transfers was attempted. The dilution method of Applemans (1921), as modified by Laird (1932), was employed; and the results are summarized in table 4. It is noted that complete dissolution was effected at a dilution of $1:10^9$ and distinct activity manifested at a dilution of $1:10^{11}$. The virulence of this bacteriophage exceeds that reported by Laird (1932) or by Demolon and Dunez (1935) and is of the same magnitude as a bacteriophage against *Staphylococcus aureus* reported by d'Herelle (1917).

Plaque formation was demonstrated, using the technic of Laird (1932). The lytic agent employed was the one isolated from soil 3, using dilutions $1:10^1$ to $1:10^7$ inclusive following the fifth serial transfer. At the end of 48 hours incubation small, distinctly clear, circular areas appeared on the plates on which dilution $1:10^5$ was spread (see fig. 2). A few plaques were present on the plates receiving dilution $1:10^4$ but not on plates receiving lower dilutions, as the concentration of bacteriophage on these plates was too great and caused complete lysis. Dilutions above

TABLE 2

Action of the same lytic principle isolated from soil 3 on different strains of Rhizobium meliloti

STRAIN OF RH. MELILOTI	ORIGINAL FILTRATE		FIRST SERIAL TRANSFER		SECOND SERIAL TRANSFER		THIRD SERIAL TRANSFER		FOURTH SERIAL TRANSFER	
	24*	48	24	48	24	48	24	48	24	48
A-1	−	−	−	−	−	−	−	−	−	−
A-2	+	++	+++	+++	++++	++++	++++	++++	++++	++++
A-3	−	−	−	−	+	+	+	++	++	++
A-4	−	−	−	+	++	++	+++	+++	++++	++++
A-5	−	−	−	−	+	+	+	++	++	++
Control	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

TABLE 3

Comparative strength of lytic principles from different soils tested against Rhizobium meliloti strain A-2

SOIL SAMPLE NUMBER	ORIGINAL FILTRATE		FIRST SERIAL TRANSFER		SECOND SERIAL TRANSFER		THIRD SERIAL TRANSFER		FOURTH SERIAL TRANSFER		FIFTH SERIAL TRANSFER	
	24*	48	24	48	24	48	24	48	24	48	24	48
3	+	++	+++	+++	++++	++++	++++	++++	++++	++++	++++	++++
4	+	++	+++	+++	+++	+++	++++	++++	++++	++++	++++	++++
6	−	+	++	+++	++	+++	+++	+++	++	++++	++++	++++
9	−	−	+	++	−	++	+	++	++	++	+++	+++
10	−	−	−	−	−	+	+	+++	++++	++++	++++	++++
18	−	−	+	+++	++	++	++	+++	++++	+++		
23	−	+	+++	++	++	+++	++	+++	++++	++++	++++	++++
28	−	−	−	++	+++	+++	++++	+++	+++	+++		
Control	−	−	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

$1:10^5$ had too small a concentration of the lytic agent, and so the plates were covered with bacterial growth.

TABLE 4

Virulence of Rhizobia bacteriophage isolated from soil 3 carrying three-year-old alfalfa (eleventh serial transfer)

DILUTION	18*	24	48	72	96	120	144	168	192	216
$1:10^1$	++++	++++	++++	++	+	−	−	−	−	−
$1:10^2$	++++	++++	++++	+++	+++	++	+	+	−	−
$1:10^3$	++++	++++	++++	+++	+++	+++	+++	+++	++	+
$1:10^4$	+++	++++	+++	+++	+++	++	++	−	−	−
$1:10^5$	++++	++++	++++	++++	+++	+++	+++	+++	++	+
$1:10^6$	++++	++++	++++	++++	+++	+++	++	++	+	+
$1:10^7$	+	++++	++++	++++	++	++	−	−	−	−
$1:10^8$	−	+	++++	++++	+++	++	++	++	−	−
$1:10^9$	+	++++	++++	++++	+++	+++	+++	++	−	−
$1:10^{10}$	−	−	+++	+++	+++	+	+	+	−	−
$1:10^{11}$	−	−	−	−	−	−	+	++	+	+
$1:10^{12}$	−	−	−	−	−	−	−	−	−	−
$1:10^{13}$	−	−	−	−	−	−	−	−	−	−
Control	−	−	−	−	−	−	−	−	−	−

* Hours in contact with filtrate.

++++, perfectly clear; +++, almost clear; ++, definitely clearer than control; +, very slight clearing; −, as in control.

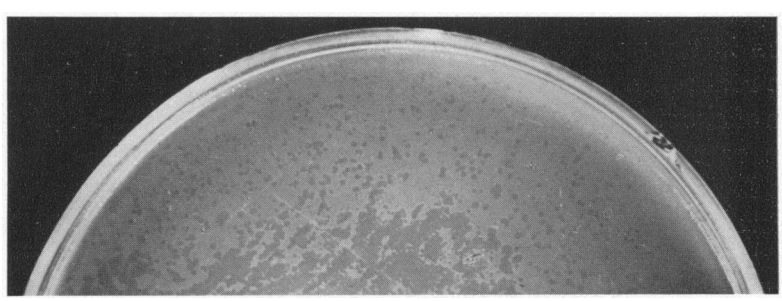

FIG. 2. PLAQUES WITH RH. MELILOTI AFTER 48 HOURS

Inoculum consisted of a 24-hour culture mixed with the lytic agent; dilution, $1:10^5$; magnification, 1.25×.

Bacteriophage was removed from a number of distinct plaques and 24-hour fluid cultures of the susceptible strain used throughout the investigation were inoculated with it. Distinct clearing

of the cultures was evident 24 to 48 hours after inoculation, thus indicating that the lytic agent was transmissible.

DISCUSSION

Although the attempts of Laird (1932) and Almon and Wilson (1933) to discover the significance of bacteriophage in the symbiotic relationship of the nodule bacteria and the leguminous plants were not successful, the recent work of Demolon and Dunez (1935) in this regard has been more fruitful. The latter succeeded in isolating bacteriophage from the nodules, roots, and stems of all alfalfa over one year old on "alfalfa sick" soils or what they call "fatigued alfalfa fields" in a number of districts in France, but experienced difficulty or failed in finding it in the roots of alfalfa over three years old or in those of old alfalfa plants devoid of nodules. They also isolated bacteriophage from the soil carrying old stands of alfalfa but not from soils in other crops or carrying young stands of alfalfa. They demonstrated to their own satisfaction that the presence of this lytic agent in the soil or in plant tissues interferes with normal symbiosis of the nodule bacteria and the host plant, and reached essentially the following conclusions: (1) As long as the root system of alfalfa penetrates deeper in the soil, new nodules may be formed and nitrogen fixed even in the presence of bacteriophage. (2) When expansion of the root system is at a maximum, as when the plant attains the age of about two years, the bacteriophage diffuses through the entire root zone and accomplishes the complete disappearance of the Rhizobia, so that the alfalfa then grows like a non-legume. Thereafter, probably in less than one year, the bacteriophage also disappears from the soil.

These sweeping conclusions imply that as the bacteriophage diffuses through the soil complete destruction of all the nodule bacteria takes place eventually, and little or no possibility remains for the occurrence of secondary growth of the more resistant organisms. Complete bacteriolysis by bacteriophage under natural conditions and especially in the soil under field conditions can hardly be expected in the light of present knowledge. This is confirmed by the results of one of Demolon and Dunez's (1935)

experiments in which they used soils from two old alfalfa fields containing very active bacteriophage for *Rh. meliloti*. The soils were placed in pots in quantities of 10 kgm., and sterilized alfalfa seeds were planted. Two months later the roots were examined, and some nodules were found in the presence of active bacteriophage when according to their own conclusions regarding the action of bacteriophage in "alfalfa sick" soils no *Rh. meliloti* should have been present in those soils.

The fact that in the Yakima district both active bacteriophage and nodules were found in two old alfalfa fields with twelve- and nine-year-old stands of alfalfa respectively probably is best explained on the basis that secondary growth of resistant Rhizobia occurs in the presence of active bacteriophage. Therefore, it is unlikely that alfalfa plants which bear the root nodules during the first years of their growth would ever completely lose the benefit of symbiosis of the Rhizobia in later years even though active Rhizobia bacteriophage was present in the soil or plant tissues.

Rhizobia bacteriophage, according to the results of Demolon and Dunez (1935), is easily destroyed by desiccation, insolation, and irrigation. Assuming that this lytic agent is readily removed from the soil by percolating water, as they contend, it is not likely to be eliminated completely from average soils by the addition of five liters of water to 10 kgm. of soil as reported by these investigators. However, the results of their experiment point out the possibility of rapid distribution of Rhizobia bacteriophage in irrigated districts by means of irrigation water, since in irrigation practices drainage and run-off waters of one field are commonly used for the irrigation of other fields along the water line. This may explain the fact that active Rhizobia bacteriophage was isolated from two young fields of alfalfa on virgin soils in the Yakima and Ellensburg districts respectively.

The irregular occurrence of bacteriophage in the areas here investigated, and especially its presence in certain young alfalfa fields on newly cultivated lands where reductions in yields of the crop have not been apparent, as well as in old alfalfa fields where alfalfa has occupied the land off and on for the major part of the

time for many years and where reductions in yields of alfalfa stands older than three years have been observed is not wholly in accord with the results of Demolon and Dunez (1935). It is entirely conceivable, however, that being present in the soil or in the plant tissues the bacteriophage may multiply at the expense of the Rhizobia and this action, therefore will interfere with symbiosis of the Rhizobia and host plant.

The absence of bacteriophage in soils with one- and two-year-old alfalfa stands and its presence in some of the soils carrying alfalfa three years old and over coincides with the observation of the farmers that decreases in yields of alfalfa usually begin to be manifested from three to five years following seeding. The fact that, on the average, nodulation was poor on the plants in the fields from which active bacteriophage was isolated is additional evidence in favor of the activity of the lytic principle in destroying the nodule bacteria and in reducing the beneficial effect of symbiosis.

Unquestionable proof that the Rhizobia bacteriophage is responsible for the reduced yields of old stands of alfalfa in the Yakima and Ellensburg districts is still lacking, though the observed facts point in that direction. From the standpoint of atmospheric nitrogen fixation and the possibility of rapid dissemination of the lytic principle the problem is one of serious concern in irrigated areas where alfalfa constitutes the major crop, and warrants further investigation, which is anticipated.

SUMMARY

A potent lytic principle active against four out of five laboratory stock cultures of *Rhizobium meliloti* was isolated from each of two soils from the Yakima district carrying three-year-old alfalfa stands.

Further investigation of soil and plant samples from 29 alfalfa fields in the Yakima and Ellensburg districts failed to result in the isolation of bacteriophage from soils carrying alfalfa plants less than three years of age. Bacteriophage was isolated from eight soils with an alfalfa stand three years of age or over and from the root tissue of three-year-old plants in one field. The

most common source of bacteriophage was the soil from fields occupied by alfalfa off and on for many years and carrying three- and four-year-old stands.

Indications of the presence of a lytic principle were found in the nodules, regardless of the age of the plants carrying them.

Nodulation on the whole was poor on plants in fields from whose soils lytic principles were isolated.

One of the bacteriophages isolated caused complete dissolution of a 24-hour fluid culture of *Rhizobium meliloti* at a dilution of $1:10^{9}$ and partial clearing at a dilution of $1:10^{11}$.

The transmissibility of the bacteriophage isolated from one of the soils was demonstrated in fluid cultures and by means of plaques.

The possibility that the bacteriophage is responsible for reduced yields of alfalfa is pointed out; but definite conclusions are reserved pending further investigation.

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