Effect of Clover Phyllody Virus on Nodulation of White Clover (*Trifolium repens*) by *Rhizobium trifolii*

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(Accepted for publication 23 November 1966)

SUMMARY

When inoculated with a clone of *Rhizobium* that produced large pink effective root nodules in virus-free plants, white clover plants infected with clover phyllody virus (CPV) produced mainly small white nodules characteristic of reduced effectiveness in nitrogen fixation. Bacteria from nodules borne on these CPV-infected plants again produced mainly small white nodules in virus-free clover cuttings and seedlings, but did not transmit CPV. Either exposure to the virus, or culture in CPV-infected tissues, seemed to induce a change in the *Rhizobium*.

INTRODUCTION

Clover phyllody virus (CPV = strawberry green-petal virus) is transmitted in the persistent manner by jassid vectors and causes yellows-type diseases in many legumes and other plants, including a range of annual weeds (Frazier & Posnette, 1957; Chiykowski, 1962; Posnette & Ellenberger, 1963). The disease is particularly prevalent in white clover and in this species, as in other clovers, the inflorescences become transformed into leafy structures which set no seed. Other symptoms include: chlorosis, vein clearing and bronzing of the foliage, diminished leaf size, a degree of stunting and axillary proliferation. Expression of floral symptoms is associated with hormonal unbalance induced by the virus (Carr, 1961) and is linked with zinc metabolism (Carr & Stoddart, 1963).

Surveys (Carr & Large, 1963) and work with experimental plots (Carr & Catherall, 1964) have established the extent of infection and loss of seed and herbage due to CPV. While much of this loss is undoubtedly attributable to the direct effect of virus on host, many of our unpublished observations suggest an indirect effect also—through the interaction of CPV with the clover/*Rhizobium* symbiosis leading to a degree of ineffective nodulation. The ultimate criterion of ineffectiveness is total or partial failure of nitrogen fixation, but certain associated phenomena are known (Fred, Baldwin & McCoy, 1932). These include: absence of pink pigmentation and the production of many small round nodules over the entire root area rather than of a few large obovate ones restricted mainly to the upper part of the root system. Vanderveken (1964) noted that in CPV-infected white clover, the reaction between an effective bacterial strain and the host was similar to that produced by an ineffective strain on healthy plants, and reported that, when bacteria were re-isolated from infected and transferred to healthy plants, full effectiveness was restored.

The present paper reports on the relationship between CPV and nodulation by a normally highly effective isolate of *Rhizobium* from white clover, with specific reference to residual effects on transference of bacteria from infected to healthy plants.

METHODS

Sources of materials. The four CPV isolates (here designated B, C, E, F) were chosen from among several obtained originally from a single field-infected white clover plant. Transfer by the natural insect vector, *Euscelis plebejus*, to a range of white clover genotypes randomly selected from the variety S. 100 has established that these isolates vary in the severity and in the type of vegetative symptoms produced, and that there is a large host genotype/virus isolate interaction (Joshi & Carr, unpublished). Throughout this paper the term 'infected' refers to virus infection exclusively and not to infection with *Rhizobium*. The culture of *Rhizobium trifolii* was derived by dilution plating and re-isolation of a single colony from the highly effective w4 strain in the Welsh Plant Breeding Station collection. This procedure should have given reasonable assurance of a clonally pure line free from accumulated mutants, although there is a chance that the colony may have arisen from more than one cell. In the latter event, the procedure would still have served to limit the initial genetic variation. Cultures of the *Rhizobium* w4 were maintained on yeast mannitol agar.

Nodulation techniques. All nodulation studies were made in a nitrogen-free agar culture (Jensen, 1942) according to the general method of Nutman (1954). CPV is not seed-borne and seedlings could therefore not be used in experiments involving infected plants. Instead, vegetatively propagated cuttings of virus-free and CPV-infected plants were used. However, the seedling technique was used for determining residual effects due to the previous *Rhizobium* source. Cuttings proved to have an advantage over seedlings in limiting host genetic variation, but root growth was more erratic and commonly adventitious roots developed from the end of the stolon in addition to normal growth from the root initial.

Two host genotypes, Nos. 33 and 43, selected on the basis of major differences in their reaction to CPV, were clonally propagated in pots of soil in an insect-free glasshouse (*a*) in healthy condition and (*b*) infected with one of the four virus isolates. Cuttings were obtained by detaching from each plant young, actively growing stolons, bearing a single apical growing point and incipient root not yet broken through the outer tissues. After thorough washing, the cut end of each was sealed with paraffin wax and the whole surface-sterilized in 80% ethanol in water for 1 min. After a rinse in sterile distilled water, they were further sterilized by immersion in 0.1% (w/v) mercuric chloride solution for 3 min. and washed for 5 min. in each of 6 changes of sterile distilled water. They were then planted singly and aseptically on 15 ml. slopes of sterile Jensen agar contained in 20 × 2.5 cm. tubes to which 10 ml. of sterile liquid Jensen medium of quarter strength had been added. Tubes were maintained in a glasshouse with the lower portions shaded, and when, after about 15 days, viable cuttings had begun to develop roots each was inoculated and returned to the glasshouse.

In the first experiment, in which the nodulating ability of infected and healthy cuttings of the two genotypes was compared, all were inoculated in the root area with a single loopful of an aqueous suspension of an actively growing *Rhizobium* w4 culture. Ten tubes represented each of the 5 'isolates' (4 infected and 1 uninfected) for each of the 2 genotypes, and the whole was arranged as a randomized block. In the second experiment involving cuttings, in which the effect of *Rhizobium* source as well as plant condition (healthy or infected) on nodulation patterns was under

investigation, transfer was made from a suspension of the bacteria obtained by crushing directly into sterile distilled water three previously surface-sterilized nodules of appropriate origin. Uninfected cuttings, and those infected with CPV-isolate B only, were inoculated with Rhizobium derived both from uninfected and from CPV-isolate B-infected cuttings from the first experiment; each cutting was inoculated with Rhizobium from its own genotype only. Three tubes represented each individual treatment and all treatments were replicated three times in a randomized block.

For the seedling experiment, seeds of the variety S. 100, surface-sterilized previously with 0.1% (w/v) mercuric chloride solution, were planted singly and aseptically on 7 ml. slopes of sterile Jensen agar contained in 15×1.5 cm. tubes, to which 2 ml. of quarter strength sterile Jensen liquid medium had been added. After about 7 days, the young seedlings were inoculated in the root region with a loopful of an aqueous suspension of the appropriate Rhizobium culture. The various Rhizobium cultures were derived from single colonies arising from plating out the contents of nodules borne on cuttings from the first experiment; owing to the failure of an earlier attempt at inoculation, these cultures had been maintained on yeast mannitol agar for more than 4 months, with four successive transfers to fresh medium. Cuttings of genotype 43 only, both in uninfected condition and infected with all four CPV isolates, provided the sources of Rhizobium. Since it seemed possible that the size of nodule from which the rhizobia were isolated might influence subsequent nodulation behaviour and so confuse the main effects, separate isolations of rhizobia were made from large, medium and small nodules for each treatment. Direct inoculation from an original Rhizobium w4 culture of comparable age served as an overall control, but was excluded from the final analysis as it proved not to differ significantly from the treatment of greatest mean value. Two tubes represented each treatment and these were replicated 3 times in a randomized block in the glasshouse.

Collation of data. Observations of the time of nodule development were made initially at daily intervals and subsequently every 2 weeks up to harvest time 6–8 weeks after inoculation. At these times, nodules were classified into large, medium and small on a purely visual estimate and the number in each class recorded. The statistical technique used in the analysis was such that the variances of individual components (time of sampling, genotype, plant condition, source of Rhizobium) were a measure of variation in the total number of nodules produced, whereas the interaction of any one of these components with size was a function of the frequency of distribution of the nodules over the three classes, large, medium and small. Note was taken of host-plant appearance, of the distribution, shape and colour (white or pink) of the nodules and of the time taken to develop pigmentation. Finally, all plants were removed from the tubes, air dried at 80° for 6 hr and the dry weight recorded. Initially, a chemical determination of total nitrogen was also made, but since dry weight and nitrogen content were found to be correlated completely ($r = +0.97$) this practice was subsequently discontinued.

Possible transfer of virus. In the second cutting experiment four additional tubes were included for each healthy genotype inoculated with rhizobia from an infected source, and two additional tubes inoculated with rhizobia from a healthy source served as controls. These additional tubes did not feature in the main experiment, but at harvest time the plants were transplanted from them into pots of steam-sterilized soil and maintained in an insect-free glasshouse until the onset of flowering, when

each was inspected for symptoms of CPV. A representative number of plants from the seedling experiment received similar treatment.

RESULTS

Effect of CPV on growth and nodulation

The data for dry weight (Table 1) show that healthy plants reared from cuttings significantly outyielded infected ones ($P = 0.001$), but differences between individual virus isolates and the host genotypes were not significant. Observations of leaf size and mortality in healthy and infected plants indicated that the latter produced a greater number of much smaller leaves ($P = 0.05$) which, however, became senescent more rapidly ($P = 0.001$). Genotypes differed significantly in this respect, no. 43 producing more leaves of greater longevity than no. 33 ($P = 0.01$).

Table 1. *Mean dry weight (in mg.) of healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Host Genotype	no.	Uninfected	Infected with CPV isolate:				Mean	L.S.D. at 5%
			B	C	E	F		
33		52.1	30.4	38.6	36.7	34.8	38.5	N.S.
43		54.4	36.9	39.8	30.8	29.4	38.3	
Mean		53.2	33.6	39.2	33.7	32.1	—	
L.S.D. at 5%			8.9					12.5

Table 2. *Mean time in days to appearance and subsequent colour-change of the first-formed nodule produced on healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Nodule type	Genotype	Uninfected	Infected with CPV isolate:				Mean	L.S.D. at 5%
			B	C	E	F		
White	33	9.1	14.8	17.8	16.2	18.5	15.3	1.6
	43	12.2	19.4	18.7	18.9	23.5	18.5	
	Mean	10.6	17.1	18.2	17.5	21.0	—	
L.S.D. at 5%			2.6					3.7
Pink	33	13.0	17.7	18.9	18.0	20.5	17.6	1.7
	43	15.9	21.3	19.9	20.9	25.9	20.8	
	Mean	14.4	19.5	19.4	19.4	23.2	—	
L.S.D. at 5%			2.7					3.8
White to pink (by difference)	33	3.9	2.9	1.1	1.8	2.0	2.3	N.S.
	43	3.7	1.9	1.2	2.0	2.4	2.2	
	Mean	3.8	2.4	1.1	1.9	2.2	—	
L.S.D. at 5%			0.7					1.0

There was a significant delay ($P = 0.01$) in the time taken for infected cuttings to produce nodules, as compared with that taken by healthy cuttings (Table 2). This was true both for the initial stages and for the overall time taken from inoculation to

the development of pigmentation. However, the time taken to progress from white to pink was substantially shorter for infected than for healthy plants ($P = 0.01$). In this essentially continuous process, proportionately fewer nodules produced by infected plants developed pigmentation. Isolate F had a significantly greater effect in delaying nodulation than the other isolates. Genotype 43 took a significantly longer time to nodulate than 33, but there was no genotype/plant condition (healthy versus infected) interaction; differences between genotypes were of the same order in infected and in uninfected plants.

Table 3 presents an analysis of the main components of variation influencing the total number of nodules formed on the plants and their distribution into 3 sizes. To avoid additional complexity of computation, replicates were pooled and the basic error was taken into the third-order interaction. Neither time of sampling nor genotype had any significant influence on nodulation, but plant condition (healthy versus infected) clearly had a major effect both on total number and on distribution. Smaller differences between individual virus isolates were, in this instance, not significant.

Table 3. *Analysis of total number and size distribution of nodules produced by healthy and CPV-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 culture of Rhizobium trifolii*

Source of variation	<i>N</i>	Mean square
Time of sampling	2	35,846
Genotype	1	6,934
Time $\times$ genotype (Error A)	2	3,418
Size	2	55,759 *
Size $\times$ genotype	2	7,143
Size $\times$ time	4	7,596
Size $\times$ time $\times$ genotype (Error B)	4	3,547
Isolate		
Healthy v. infected	1	3,901 *
Between infected	3	924
Isolate $\times$ size		
Healthy v. infected	2	18,786 ***
Between infected	6	526
Isolate $\times$ size $\times$ time		
Healthy v. infected	4	2,933 *
Between infected	12	1,204
Residual error†	44	854

† Residual error is compounded of the third order interaction, together with the remaining non-significant first- and second-order interactions.

The nature of the difference in nodule size distribution is illustrated for genotype 33 in the histograms comprising Fig. 1. Fewer total nodules were produced by healthy than by infected plants, but whereas the former had a preponderance of large effective nodules, the latter had a great many small ones which were probably largely ineffective. A high degree of correlation was shown between the number of large nodules produced and dry weight ($r = +0.78$), and in subsequent experiments the value for r was even higher. There was some indication that isolates, notably B and E, differed in their effect on nodulation and, on an individual comparison basis, these differences were often significant. However, the general distribution was similar throughout the sampling period.

Fig. 1. Size distribution of nodules produced by healthy and CPV-infected cuttings of white clover genotype 33, grown in Jensen's agar culture and inoculated with effective (w4) culture of *Rhizobium trifolii*.

Persistence of ineffectiveness following bacterial transfer. Seedlings. Table 4 shows that both the size of the original nodule and the source plant (healthy or infected) from which the *Rhizobium* cultures were obtained, influenced significantly the subsequent dry weight of healthy white clover seedlings inoculated with them ($P = 0.001$ and 0.01 , respectively). Differences between individual isolates were not significant. There was a highly significant interaction between nodule source in respect of size and the source-plant condition, which reference to Table 4 shows to have been due entirely to CPV-isolate E. For the remaining sources (uninfected and infected with

Table 4. Mean dry weight (in mg.) of healthy *S. 100* white clover seedlings grown in Jensen agar culture and inoculated with w4 cultures of *Rhizobium trifolii* derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43

Nodule source (size)	Source of <i>Rhizobium</i> culture: plants infected with CPV isolate:					Mean	L.S.D. at 5%
	Uninfected	B	C	E	F		
Large	14.2	10.1	10.2	9.9	12.7	11.4	1.4
Medium	11.2	9.8	8.7	3.6	4.6	7.6	
Small	3.6	3.9	2.9	11.1	3.4	5.0	
Mean	9.7	7.9	7.3	8.2	6.9	—	—
L.S.D. at 5%		1.8					3.1

CPV-isolates B, C, F) seedling dry weight was conditioned independently both by source-plant condition and the size of the original nodules from which the *Rhizobium* cultures were derived. Of these factors, original nodule size clearly exercised the major influence, indicating the importance of taking it into account in estimating the effects of other variables. However, the mean values taken over all nodule sizes show that seedlings inoculated with rhizobia from an infected source produced a significantly lower yield than those inoculated with rhizobia isolated from healthy plants: this despite the fact that the cultures had been maintained on agar through four successive transfers before use.

Similar effects were evident in the size distribution of nodules produced by these seedlings (Table 5; Fig. 2). For the sake of simplicity in computation, replicates were again pooled in the detailed analysis but a separate analysis of the main components, which included replicates, showed that error variation was of a low order and that sampling date (time) and the size distribution of nodules were both highly significant.

Table 5. *Analysis of total number and size distribution of nodules produced by healthy S. 100 white clover seedlings grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43*

Source of variation	N	Mean square
Time of sampling	3	539.6
Size	2	538.6
Time $\times$ size (error A)	6	135.8
Treatment		
Nodule source (size)	2	523.4 ***
Isolate		
Healthy v. infected	1	2.7
Between infected	3	133.1 ***
Isolate $\times$ nodule source		
Healthy v. infected	2	112.7 **
Between infected	6	176.4 ***
Treatment $\times$ size		
Nodule source $\times$ size	4	41.1
Isolate $\times$ size		
Healthy v. infected	2	387.8 ***
Between infected	6	19.8
Isolate $\times$ nodule source $\times$ size	16	32.0
Treatment $\times$ time	42	10.8
Time $\times$ treatment $\times$ size (residual error)	84	21.2

Since the relative differences between treatments remained the same whatever the date of sampling (see treatment $\times$ time-interaction), only the final (8 week) data are illustrated in the histograms comprising Fig. 2. As with dry weight, so also the total number and size distribution of nodules produced by the seedlings was influenced by the size of nodule from which the rhizobial cultures had been isolated originally but there was no interaction of nodule source with distribution. There was a large degree of nodule source/isolate interaction which reference to the histograms shows to have been due, again, to CPV-isolate E, and in particular to the effect of bacteria isolated from small nodules formed on plants inoculated with this isolate. The anomalous behaviour of CPV-isolate E also accounts for the lack of significance

between the total number of nodules produced on seedlings inoculated with rhizobia from healthy and infected sources. This single observation of virus isolate interaction with rhizobium source should, clearly, be treated with caution. In considering the overall effect of CPV isolate on the distribution of nodule size, differences between healthy and infected sources were highly significant. Although the high between-isolate variation is also due, in part, to the effect of rhizobia isolated from plants infected with CPV isolate E, many individual comparisons between other CPV isolates showed a high order of significance. Figure 1 and Pl. 1, fig. 1, show that these differences were due to the fact that seedlings inoculated with rhizobia from infected plants

Fig. 2. Size distribution of nodules produced by healthy S. 100 white clover seedlings, grown in Jensen agar culture and inoculated with effective (w4) cultures of *Rhizobium trifolii* derived from large, medium and small nodules developed on healthy and on CPV-infected plants of genotype 43.

produced a larger total number of nodules, but the number of large effective nodules was smaller. Within the virus-infected group, somewhat lesser differences in distribution are also evident, notably between CPV isolates C and F.

Cuttings. In the previously described seedling experiments the nature of the plant material allowed only the effect of *Rhizobium* source (whether from infected or from healthy plants) on nodulation to be investigated. The use of cuttings has enabled a direct comparison to be made in the same experiment between effects attributable to *Rhizobium* source and those attributable to plant condition. Table 6 (a) shows that the dry weight of these cuttings was affected significantly both by the genotype ($P = 0.05$) and by treatment.

Table 6. Mean dry weight (in mg.) of healthy (H) and CPV-isolate B-infected (P) cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with effective w4 cultures of *Rhizobium trifolii* obtained from healthy (h) and CPV-isolate B-infected (p) plants

(a)						
Genotype	Treatment				Mean	L.S.D. at 5 %
	Hh	Hp	Ph	Pp		
33	44.4	31.1	14.6	13.7	25.9	5.7
43	43.1	38.5	35.2	32.0	37.2	
Mean	43.7	34.8	24.9	22.8	—	—
				6.2		8.8
L.S.D. at 5 %						

(b)				
Rhizobium source	Plant condition		Mean	L.S.D. at 5 %
	H	P		
h	43.7	24.9	34.3	4.4
p	34.8	22.8	28.8	
Mean	39.2	23.8	—	—
		4.4		6.2
L.S.D. at 5 %				

Table 7. Analysis of total number and size distribution of nodules produced by healthy and CPV-isolate B-infected cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with w4 cultures of *Rhizobium trifolii* obtained from healthy and from CPV-isolate B-infected plants

Source of variation	N	Mean square
Genotypes	1	3990.2 *
Replicates	2	49.7
Error A	2	71.8
Size	2	2086.4 ***
Size × genotypes	2	453.4 **
Error B	8	40.3
Treatment		
Plant condition	1	355.6 **
Rhizobium source	1	16.0
Condition × source	1	112.5
Treatment × genotype		
Plant condition	1	128.0
Rhizobium source	1	122.7
Condition × source	1	364.5 **
Treatment × size		
Plant condition	2	534.3 ***
Rhizobium source	2	160.8 *
Condition × source	2	95.5
Treatment × genotype × size		
Plant condition	2	91.8
Rhizobium source	2	54.3
Condition × source	2	212.0 *
Error C	36	46.4

It is evident from the analysis and from Table 6 (*b*), where the two genotypes have been pooled, that although plant condition was the greater source of variation ($P = 0.001$), Rhizobium source also had a major effect on plant growth in a nitrogen-free medium ($P = 0.05$). This is illustrated in Pl. 1, fig. 2. There was no interaction of plant genotype with Rhizobium source, but only a genotype with plant condition which would appear to have been due to the greater effect of the virus on genotype 33 than on 43. There was also no plant condition/Rhizobium source interaction.

Table 8. *Mean number and size distribution of nodules per plant produced by healthy (H) and CPV-isolate B-infected (P) cuttings of 2 white clover genotypes, grown in Jensen agar culture and inoculated with w4 cultures of Rhizobium trifolii obtained from healthy (h) and from CPV-isolate B-infected (p) plants*

(a)								
Treatment	Genotype 33				Genotype 43			
	Small	Medium	Large	Total*	Small	Medium	Large	Total*
Hh	8.6	3.7	8.4	20.7	9.6	5.0	11.2	25.8
Hp	3.9	1.6	4.7	10.2	15.9	7.0	6.6	29.5
Ph	3.6	3.0	0.0	6.6	15.2	5.7	5.1	26.0
Pp	7.3	2.7	0.0	10.0	15.7	4.3	5.7	25.7
Mean	5.8	2.7	3.3	11.9†	14.1	5.5	7.1	26.8†
L.S.D. at 5%	2.0				3.6			

† L.S.D. at 5% for comparing these 2 means = 8.7.

(b)								
Rhizobium source	Healthy cutting (H)				Infected cuttings (P)			
	Small	Medium	Large	Total*	Small	Medium	Large	Total*
h	9.1	4.3	9.8	23.2	9.4	4.3	2.6	16.3
p	9.9	4.3	5.6	19.8	11.5	3.5	2.8	17.8
Mean	9.5	4.3	7.7	21.5†	10.4	3.9	2.7	17.0†
L.S.D. at 5%	1.8				2.6			

* L.S.D. applies to columns asterisked in respective tables.

† L.S.D. at 5% for comparing these 2 means = 3.0.

The data for nodule number and size distribution 8 weeks after inoculation (Tables 7, 8) show that, as for plant weight, plant condition and Rhizobium source both had a major effect. Inevitably, plant condition exerted the greatest influence on nodulation, but the source of Rhizobium, although not affecting total number, had a significant effect on size. There was, however, no interaction between genotype and either plant condition or Rhizobium source; genotypes appear largely to have influenced nodule size independently of the main effects attributable to either treatment. There was a significant interaction between plant condition and Rhizobium source in relation to genotype. Table 8 (*a*) shows this interaction to have been expressed in the production of small nodules. No such interaction is evident in the data for large nodules where, although a greater number was produced consistently by plant genotype 43, this genotypic effect was independent of either plant condition or

Rhizobium source. Of the two genotypes no. 43 produced a significantly greater number of nodules of all classes.

Table 8 (*b*) compares the effect of plant condition and Rhizobium source on the mean combined number and size of nodules produced by both genotypes. In this experiment, healthy plants unexpectedly produced a significantly greater total number of nodules than did the infected plants. However, this was due entirely to an abundance of large effective nodules. Rhizobium source had no significant effect on the total number of nodules, but healthy cuttings produced a significantly greater number of large ones when inoculated with rhizobia from healthy plants, and CPV infected cuttings produced a significantly greater number of small nodules when inoculated with rhizobia from CPV infected plants. Thus, transfer of formerly effective Rhizobium from CPV-infected plants produced on both healthy and infected cuttings nodulation patterns indicative of a shift towards ineffectiveness.

Possible transfer of virus All plants of genotype 43 from the second cutting experiment, which were transferred from tubes to pots of soil and maintained in the glasshouse, flowered by mid June. None showed symptoms of CPV. No plants of genotype 33 had flowered by this date, neither had 60% of the plants from the seedling experiment. However, the remainder produced normal inflorescences without CPV symptoms. Thus it appears that the rhizobia did not act as a vector of this virus.

DISCUSSION

When inoculated with a normally highly effective strain of Rhizobium, white clover plants infected with clover phyllody virus (CPV) gave a smaller yield than similarly inoculated uninfected plants, and exhibited a partially ineffective type of nodulation. The high correlation between plant weight as a measure of nitrogen fixation and the number of large nodules produced, provides a clear indication that the nodule characters noted (increased time to nodulation, less pigmentation, a generally greater total number of nodules but fewer large ones) are valid criteria of Rhizobium ineffectiveness. Although these results were obtained under the precise but artificial conditions obtaining in test-tube culture, it is reasonable to assume that they would be also applicable to natural field conditions.

Contrary to the contention of Vanderveken (1964), transference of rhizobia from virus-infected to healthy plants, either directly from nodules or after several agar transfers, did not restore full effectiveness. Even on virus-infected plants, nodulation was less effective with Rhizobium from a virus-infected plant than from an uninfected one.

The occasionally significant between-isolate variation, which occurred in experiments with several different virus isolates, suggests that the virulence of a particular virus isolate influences the degree of Rhizobium ineffectiveness, both in terms of the host plant itself and the plant as a source of Rhizobium. Time of sampling, host genotype, and the size of the nodule from which rhizobia were isolated originally, all had a major effect on growth and nodulation. The results obtained for the effect of Rhizobium isolated from nodules of different sizes are contrary to the reports of Holding & King (1963). None of these three factors exerted a major influence on the degree of ineffectiveness induced by CPV, but were not studied in all experiments; hence no firm conclusion can be drawn from this apparently small order of interaction.

particularly for genotype, where the only two genotypes included could not be entirely representative of the range of possible genotypic expression. Size of nodule which serves as a source of *Rhizobium* showed marked interaction with plant condition, but as this was due entirely to one isolate it cannot be assumed that this was a major factor influencing induced ineffectiveness.

The failure of *Rhizobium* to transmit CPV parallels the results of Johnson & Jones (1943), who were similarly unsuccessful in transmitting pea enation mosaic, pea severe mosaic and white clover mosaic viruses by this means, except in one unsubstantiated instance. The persistence of this partial ineffectiveness after transfer of rhizobia from CPV infected to healthy plants was clearly not due to transfer of virus, but to some modification of the rhizobia, induced either by virus-infected tissue or by the virus itself. A parallel example, though of opposite effect, is to be found in the change towards greater nodulation potential induced in *Rhizobium* inoculum following passage through graft and sexual hybrids of *Trifolium ambiguum* with *T. hybridum* (Evans & Jones, 1964). At the present time, the precise mechanism of this induced change is a matter of speculation. Since the rhizobial culture was derived from a single colony it is unlikely to have been due to selection of accumulated mutants, although in view of the technique of isolation used this possibility cannot be excluded entirely. Even in the event that the colony derived from more than one cell, the variation available for such selection would be strictly limited. There remains the possibility of simple adaptation (Cohen & Monod, 1957), or of genetic chromosomal, episomal (Jacob, Schaeffer & Wollman, 1960) or extra-chromosomal (Beale, 1958; Jinks, 1964) change induced either as a direct mutation or by transfer of genetic material from virus to bacterium (Zinder & Lederberg, 1952; Stocker, Zinder & Lederberg, 1953). The latter possibility (a phage-type transductive mechanism) would be unique for such a virus and would require explanation for the apparent failure of ultimate reconstitution of the independent virus. Further, the maintenance in the *Rhizobium* stock of a high level of adaptive change exemplified by its behaviour after repeated agar transfer precludes the possibility of 'unilinear transmission' (Stocker, 1956) unless a high selective advantage during agar passage is assumed.

Whatever the mechanism of induction, it is evident that *Rhizobium* is capable of a great deal of adaptive variation, and it is not improbable that this could also be towards greater effectiveness. In addition to virus, host genotype, soil and other nutritional and biochemical factors might well prove capable of inducing such adaptive change. In this connexion the contention of Nutman (1949), that in red clover the cytoplasmic and nuclear gene complex which conditions resistance to nodulation has no adaptive influence on the rhizobia involved, seems worthy of reinvestigation.

In assessing the significance of this indirect effect of CPV (through its influence on nodulation) it is important to consider the possible outcome of competition between fully effective *Rhizobium* and those induced to a degree of ineffectiveness. If, as is true of certain strains of ineffective *Rhizobium* (Nicol & Thornton, 1942), those induced to partial ineffectiveness as a result of virus action compete better than fully effective ones for invasion sites on the host roots, then even uninfected plants in a white clover sward infected with CPV might be expected to show a depression in yield as a result of nodulation with rhizobia from infected plants. The magnitude of this effect would be dependent on whether effectiveness can be fully restored following serial passage through healthy plants.

We express our gratitude to Professor P. T. Thomas, Director of the Welsh Plant Breeding Station, for facilities and helpful advice and criticism, to Dr E. L. Breese and W. Ellis Davies for advice on the statistical presentation, to Miss Alison Spinney for technical assistance, Mr H. Richards for the photographs and Mrs E. Horzelska for help in the preparation of the manuscript.

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EXPLANATION OF PLATE

Fig. 1. Root systems of healthy S. 100 white clover seedlings grown in Jensen agar and inoculated with rhizobia from: L., a healthy plant, showing large, obovate, effective nodules confined to the upper root area; R., a CPV-infected plant, showing small, round, ineffective nodules distributed over the entire root system.

Fig. 2. Healthy (H) and CPV-infected (P) cuttings of S. 100 white clover (genotype 33), grown in Jensen agar culture and inoculated with rhizobia from healthy (h) and CPV-infected (p) plants. L. to R.: Hh, Hp, Ph, Pp.