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FACTORS RESPONSIBLE FOR THE CURLING AND BRANCHING OF CLOVER ROOT HAIRS BY RHIZOBIUM

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SUMMARY

The branching of clover root hairs was due to a factor (or factors) readily extracted from cells of *Rhizobium trifolii* and found in seedling solution supporting clover roots inoculated with this organism. Part of the active material was retained within a dialysis sac but a smaller, escaping fraction evoked the same response. The branching fraction was stable at 100°C and, partly, to nuclease and periodate, but was unstable when left in contact with the cells and when treated with trypsin. The dried residue of active filtrate contained 2% nucleic acid, 20% protein and about 34% carbohydrate which included antigenic lipopolysaccharide (approximately 1.6%). The branching response could not be attributed to either of these polysaccharides or to indole acetic acid. The capacity to cause branching was absent from all non-invasive mutants, but was increased in ineffective invasive forms, compared with the effective parent strain.

Moderate curling was quantitatively much less striking and was generally found in the same preparations as showed branching. Notable exceptions were the lack of significant moderate curling in the filtered inoculated seedling solution and retention of this capacity in avirulent mutants.

Marked curling occurred only when viable virulent rhizobia were present. Avirulent mutants were incapable of causing marked curling but the response to ineffective variants was rather better than to their effective parent strains. A dialysis membrane separating bacteria and plant roots prevented marked curling.

INTRODUCTION

An earlier report from this laboratory [17] showed, in agreement with those of Haack [6] and, so far as comparison is possible, Li and Hubbell [9] that the markedly curled condition of root hairs of leguminous plants was almost entirely the result of direct association between host plant and rhizobia able to form nodules with it. Ex-

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ceptions were restricted to cases where there was other evidence of close taxonomic relationship, *e.g. Rhizobium trifolii* with Pisum[6] and *R. leguminosarum* with Trifolium [17]. Lower categories of root hair deformation (moderate curling and branching) were generally less specific being commonly found also with legumes belonging to different nodulating groups. They were not commonly found with nonrhizobia. All of several avirulent forms of *R. trifolii* had lost the capacity to cause either marked curling or branching, though most were still able to cause some moderate curling. Filtrate from virulent *R. trifolii* failed to make good the deficiency of the avirulent bacteria so far as the marked curling condition was concerned but had an additive effect in connection with the otherwise deficient branching response.

Reports on the influence and nature of cell-free filtrates, and preparations therefrom [7 12 13 15 16] are confusing because sufficient care has seldom been taken to record kinds and degree of root hair deformation. Although we have regularly observed branching and moderate curling in filtrates obtained from rhizobia grown on laboratory media, or as a result of diffusion from those associated with roots, we have not observed the tightly curled appearance under these conditions. Plant auxins have generally been given a role in the curling of root hairs: a view that has tended to persist despite evidence and cogent arguments to the contrary [5 13 15].

Because in our experience the markedly curled condition is limited to roots directly associated with the rhizobia, we have had to depend largely on the lower categories of plant response (branching and moderate curling) to study the nature of any cell-free factor. However, we have attempted to obtain some leads by the establishment of isolated microcolonies using sparse inocula, and by the use of a dialysis barrier *in situ*. The recent findings of Solheim and Raa [14] which were reported after most of our own work was completed, needed additional investigation.

MATERIALS AND METHODS

Plant tests, methods of plant culture, incorporation of rhizobia or filtrate, and grading of plant response were as already described [17], except when otherwise stated. The rhizobia were strains of *R. trifolii* and included ineffective and some non-invasive sub-strains. Results, based on 4 replicate slides, each with three seedlings, have been analysed in the logarithmic form.

Filtrate from cultured bactaria was prepared as in the earlier account, but, for the more detailed investigations, the distilled water extract of the bacteria, grown in quantity on yeast mannitol agar, was concentrated by evaporation at 30°C while constantly rotated under reduced pressure, and then lyophilized.

The main constituents were detected and estimated as follows:

Nucleotides. Concentrated filtrate was scanned using a Unicam SP800 spectrophotometer and total nucleotide calculated by absorbance at 260nm.

Carbohydrate. Usual paper chromatography methods were used to characterise the major sugar components in the hydrolysed product. Hexose was estimated [3] in every fourth tube of a sample obtained by the addition of 13 ml 0.2*M* ammonium acetate to 300 mg of dried filtrate residue, which was then passed through a 6% (w/v) gelarose column, bead size 50–200μm. 1 ml 5% (w/v) phenol was added to 1 ml of the fraction followed by 5 ml concentrated H_2SO_4 and the colour read on a spectrophotometer at 490 nm.

Protein. Protein was indicated by high 290/260 nm aborbance ratio, and was determined, after allowance for nucleotide at 280 nm.

Lipopolysaccharide antigen. Gel diffusion was carried out to determine the antigenicity of the filtrate product compared with purified antigen of the same Rhizobium [8]. A comparison of the extinction dilution with that of pure antigen enabled the concentration to be estimated.

The dried filtrate residue was subjected to 3 methods of selective destruction and compared with buffer controls. The treatments, each involving 20 mg filtrate residue (equivalent to 10^9–10^{10} rhizobia), and incubation for 24 h at 37°C were:

For nucleotides. 1 mg ribonuclease and 1 mg deoxydribonuclease (Sigma), dissolved in 20 ml tris-HCl–$MgCl_2$ buffer (pH 8.05).

For carbohydrate. 20 ml 0.05 *M* sodium periodate shaken in the dark.

For protein. 4 mg trypsin (once-crystallised Calbiochem) dissolved in 20 ml tris-HCl–$MgCl_2$ buffer compared with boiled enzyme control.

After each treatment the material was dialysed, heated at 100°C for 10 min. made up to 50 ml, passed through 0.2 μm millipore filter and used in the plant test at the rate of 8 ml added to 13 ml of the seedling solution.

'Extracellular polysaccharide' (EPS)

Crude EPS was obtained from the filtrate of invasive strains of *R. trifolii*, adhering closely to the procedure described by Hubbell [7].

Filtrate from solution bathing inoculated plants was obtained by growing the clover seedlings supported on a stainless steel grill over a petri dish of seedling solution and filtering through a bacteria-retaining sintered glass filter (Grade 5, Baird and Tatlock (London) Ltd.).

RESULTS

Relationship of root hair response to symbiotic capacity

The earlier investigation included strains which differed in their capacity to form an effective symbiosis with *T. glomeratum* and some which had lost their capacity to nodulate the test plant. Whereas the first provoked a full root hair response, members of the second group had completely lost their ability to cause the markedly curled condition, and, virtually, any branching. With one exception a reduced but significant capacity to cause moderate curling was retained. In the present work it has been possible to compare 3 groups of substrains which differed from the parent type in their effectiveness and two other cases of lost invasiveness (Table 1).

TABLE 1

Response of root hairs of *Trifolium glomeratum* to substrains of differing symbiotic capacity

Strain or substrain	Colony form[2]	Symbiotic capacity	No of root hairs per 12 plants[1]					
			Branched		Moderately curled		Markedly curled	
			(i)	(ii)	(i)	(ii)	(i)	(ii)
SU297/31	Gummy	Effective	236		104		48	
SU297/32	Non gummy	Ineffective	380		64		172	
SU298/534	Gummy	Effective	136		48		64	
SU298/536	Gummy	Ineffective	176		48		72	
SU298/531	Non gummy	Ineffective	320		104[5]		116	
SU304/7[3]	Gummy	Effective	312	488	60	44	132	108
SU304/12	Non gummy	Ineffective	508	724[5]	88	88[5]	132	220
SU304/11				812[5]		104[5]		232
SU304/3	Gummy	Avirulent		4		20[6]		0
SU304/9				4		24[6]		4
SU469	Gummy	Avirulent		16		8		0
SU3054*				16		12		0
Uninoculated			24	12	24	4	8	0

[1] Based on pooled 5–8 day results;

[2] Gummy colonies, 3–5 mm after 5 days at 26°C on yeast mannitol agar; Non-gummy, approx. 1 mm under the same conditions;

[3] SU304 substrains are single colony re-isolates of NA34; small, non-gummy forms are probably the same as NA34S used by Hubbell;

[4] Originally SU46, returned from Rothamsted Experimental Station after several years separate cultivation;

[5] Significantly greater than related gummy substrains ($P \leqslant 0.05$);

[6] Significantly greater than uninoculated plants and less than virulent substrains ($P \leqslant 0.05$).

Ineffectiveness was not associated with any reduction of root hair response; in fact the reverse situation generally applied in the case of the small non-gummy variants (numerically in 13 of 15 comparisons; statistically significant in 5). The gummy ineffective variant of strain SU 298 did not show this trend to any significant degree. Lost virulence was again characterised by loss of capacity to cause marked curling and branching, but again some capacity to cause moderate curling remained.

It seemed possible that a strain of *R. leguminosarum* (ineffective for its own host and able to cause marked deformation of clover root hairs [17], though not nodulation) might complement avirulent *R. trifolii* and so result in infection. However, no such infection was obtained in combination with two of the avirulent strains. The root hair response to each was unaffected by the presence of the other.

Response with sparse seedings

The interaction between the plant root and rhizobium has generally been studied under conditions where the bacteria are free to colonise the whole root surface and form a massive overlay. It is however possible, by using the modified Fåhraeus method with more concentrated agar (0.6%), to restrict the distribution of the preseeded rhizobia to relatively few well isolated microcolonies. This in turn opens up the possibility of relating the behaviour of individual root hairs to the presence or absence of rhizobia in their vicinity.

Reduction of the inoculum to provide 5×10^6/ml of the seeded agar medium (about one per root hair) did not cause any delay or reduction for any category of deformation. Such effects were observed only when the inoculum was reduced well below that level (Table 2) and even then root hair branching, although it developed more slowly, was equally marked at all levels by 5 days. On the other hand the curling response (particularly marked curling) was significantly reduced when the inoculum provided less than one microcolony per root hair. With the most dilute inoculum there was virtually no marked curling, and no infected root hair. In other experiments extremely sparse inocula which severely reduced or prevented marked curling were yet able to cause abundant branching. In these cases incubation of the inoculated agar slides for 3 days prior to planting the germinated seedlings permitted the early development of microcolonies and so speeded up the onset of branching and, when

TABLE 2

Influence of inoculum size of *R. trifolii* (SU297/32) on root hair response with *T. glomeratum*

Inoculum size†	Number of root hairs per 12 plants*			
	Branched	Moderately curled	Markedly curled	Infected
High	424[a]	44[a]	188[a]	37[a]
Medium	444[a]	12[b]	104[b]	35[a]
Low	436[a]	8[b]	1[c]	0[b]

* Data for root hair response based on pooled 5–8 day results; infections on 7 and 8 day observations; values in each column not sharing the same letter index are significantly different ($P \ll 0.05$).

† Medium level approx. 5×10^5/ml (1 per 10 root hairs) high and low calculated to provide one hundred times and one hundredth this concentration.

reduction in inoculum size was not extreme, marked curling. An additional condition which was common when the inoculum was sparse was that the tips of the hairs (generally those that were already branched) took on a striking bulbous appearance. Direct phase microscopy of the sparsely seeded slides confirmed the fact that the microcolonies were well spaced and showed that, on the one hand, branching, but not marked curling, regularly occurred in hairs clearly away from microcolonies, whilst many hairs growing right through a mass of rhizobial growth failed to show any response.

It can be concluded from these experiments that root hair branching can be caused by an extremely diffusible, evidently very potent product of rhizobial growth, but that root hairs differ greatly amongst themselves in their ability to respond to this factor or to give the markedly curled condition when in close contact with the specific bacteria.

Separation of rhizobia from roots by a dialysis barrier

The experiments with sparse seedings of rhizobia suffered from several disadvantages particularly because one could not be sure of the actual absence of rhizobia from the vicinity of the few cases of markedly curled hairs, due to optical difficulties associated with the material. There was moreover, the possibility that on occasions the agar assembly did not completely restrict the inoculum to isolated microcolonies, but permitted some spread in liquid between agar and glass surface, or along the advancing root itself. For these rea-

sons the Fåhraeus-Nutman slide assembly was modified so as to have a dialysis barrier between the roots and nearby virulent homologous rhizobia. Such a barrier prevented any marked curling, but permitted abundant branching and some moderate curling (Table 3). This result, showing ready diffusion of branching factor through the dialysis membrane combined with other well established evidence of some retention (*e.g.* Table 6), suggests that either the one factor occurs in a larger and smaller molecular form (possibly involving an aggregation-disaggregation phenomenon) or that two factors are involved that have a similar effect though different in molecular size.

TABLE 3

Blockage and passage of root hair deforming factors by a dialysis membrane*

Treatments		Number of root hairs per 12 plants*		
		Branched	Moderately curled	Markedly curled
Outside sac	*Inside sac*			
Plant + Rhizobium	Nil	408**	12**	28**
Plant	Rhizobium	488**	4	0
Plant	Rhizobium + Plant	644**	16**	2
Plant	Nil	4	0	0

* *T. glomeratum* and *R. trifolii* SU297/32; observations on plant *outside* dialysis sac.
** Significantly > control ($P \leqslant 0.05$).

Response to rhizobia-free filtrates

The deformation of root hairs with filtrates obtained by suspending the homologous rhizobium in distilled water for one hour was restricted to branching and moderate curling, and was not appreciably reduced by sixteen-fold dilution. An attempt to increase the effect of fourfold concentration at low temperature resulted in loss of activity (Table 4). Heating the filtrate had little adverse effect but 24 h exposure of bacteria to distilled water prior to filtration resulted in considerable loss of activity. One hour extraction was sufficient to remove all of the filtrate factor, in that cells which had been so extracted and then broken were without effect when added in equivalent concentration to the test plant.

Unheated filtrate obtained as the membrane-filtered liquid from

TABLE 4

Influence of concentration of filtrate from cells of *R. trifolii* (SU297/32) on root hair response†

Treatment	Number of root hairs per 12 plants*			
	Branched		Moderately curled	
	Exp. (i)	Exp. (ii)	Exp. (i)	Exp. (ii)
Filtrate × 4		16^{bc}		44^{b}
1	132^{a}	124^{a}	84^{a}	32^{b}
$\frac{1}{2}$	152^{a}		28^{a}	
$\frac{1}{4}$	296^{a}	128^{a}	44^{a}	36^{b}
$\frac{1}{16}$		76^{ab}		28^{bc}
Homologous rhizobium	432^{a}	528^{a}	36^{a}	120^{a}
Control	28^{b}	4^{c}	4^{b}	12^{c}

† with *T. glomeratum.*

* Values in each column not sharing the same index letter are significantly different from each other ($P \leqslant 0.05$).

around the roots of a dense growth of inoculated clover plants in seedling solution caused a significant level of branching which was in fact increased by a twofold dilution (Table 5). No significant moderate curling was obtained with this filtrate.

TABLE 5

Influence of filtrate from seedling solution bathing inoculated plants*

Treatment	Root hairs per 12 plants**	
	Branched	Moderately curled
Filtrate	52^{b}	0.8^{b}
Filtrate (diluted twofold)	352^{a}	0.8^{b}
Homologous rhizobium	574^{a}	24^{a}
Control	0^{c}	0^{b}

* *T. glomeratum* and filtrate of *R. trifolii* SU297/32.

** Values in each column not sharing the same index letter are significantly different from each other ($P \leqslant 0.05$).

The nature of the filtrate factor

The residue obtained from the dried filtrate contained 2% nucleic acid (based on dried product), 35% carbohydrate (which inincluded about 1.6% lipopolysaccharide somatic antigen) and 20% protein. The branching and moderate curling factors were heat-stable

and sufficiently retained in the dialysis sac for that fraction to cause root hair response. Treatment with the nuclease mixture or periodate reduced the branching activity; trypsin, but not a boiled trypsin control, removed it all (Table 6). One experiment with purified lipopolysaccharide somatic antigen appeared to give some branching and moderate curling response, but this was not substantiated in a second experiment.

TABLE 6

Effect of various treatments on the activity of filtrate*

Treatment†	Number of root hairs per 12 plants showing branching**	
	Exp. (i)	Exp. (ii)
Filtrate	416[a]	160[a]
Filtrate nuclease	112[b]	
Filtrate periodate	120[b]	
Filtrate trypsin	16[c]	8[b]
Filtrate boiled trypsin		60[a]
Control, without filtrate	8[c]	16[b]

* Filtrate from *R. trifolii*, SU297/32 tested on *T. glomeratum*.
† All dialysed and heated (100°C, 10 min) after specified treatment.
** Values in each column not sharing the same index letter are significantly different from each other ($P \leqslant 0.05$). Moderately curled hairs showed a similar trend but numbers were too small to justify detailed consideration.

Following a recent report [7] which attributed marked root hair responses to 'extracellular polysaccharide' (EPS), we obtained a similar crude fraction from three fully invasive strains of *R. trifolii*, which included one used by Hubbell. Our results, shown in Table 7, yielded no case where the crude EPS, even when considerably concentrated, caused the markedly curled condition. The EPS from two of the three strains (including a preparation from that used by Hubbell) caused a significant degree of branching and moderate curling. Some hairs exposed to these bacteria-free treatments were swollen and irregularly curved, but none was markedly curled as we interpret that description. The precipitated gum of the third strain (SU297/31) was without effect. Moreover in the two positive cases the supernatant, from which gum had been precipitated, was just as potent as the reconstituted crude polysaccharide.

TABLE 7

Deformation of root hairs of *T. glomeratum* by 'extracellular polysaccharide' (EPS) from several strains of *R. trifolii*

Strain*	Treatment**	Number of root hairs per 12 plants		
		Branched†	Mod.† curled	Markedly curled
A				
TA1	Seeded with bacteria	358[ab]	91[a]	76
	EPS (100 μg/ml)	186[b]	19[b]	0
	EPS (50 μg/ml)	308[ab]	18[b]	0
	EPS (100 μg/ml) + supernatant	471[a]	43[b]	0
SU297/31	Seeded with bacteria	388[a]	124[a]	44
	EPS (100 μg/ml)	4[c]	4[c]	0
	EPS (33 μg/ml)	1[c]	5[c]	0
	EPS (100 μg/ml) + supernatant	1[c]	6[c]	1
Control		21[c]	2[c]	0
B				
SU304/12 (NA34)	Seeded with bacteria	351[a]	117[a]	68
	EPS (100 μg/ml)	236[a]	16[b]	0
	Supernatant	170[a]	1[c]	1
	EPS (100 μg/ml) + supernatant	216[a]	26[b]	3
TA1	EPS (100 μg/ml)	318[a]	8[b]	0
	EPS (1400 μg/ml)	274[a]	43[b]	0
Control		9[b]	2[c]	0

* SU304/12 is a small non-gummy invasive, but ineffective, variant derived from our culture of NA34. It is similar in cultural and symbiotic behaviour to NA34S used by Hubbell [7]. TA1 and SU297/31 are gummy invasive effective strains.

** EPS is crude extracellular polysaccharide [7]; supernatant is the fraction from which the EPS had been precipitated, which was then dialysed, lyophilised and reconstituted quantitatively so as to permit a direct comparison with the EPS (100 μg/ml) fraction.

† Values in each column in each sub-table, not sharing the same index letter are significantly different from each other ($P < 0.05$).

Solheim and Raa [14] have presented results which run counter to our experience in that they report the 'markedly curled' condition as one of the responses to cell-free exudates of the rhizobia growing in liquid medium or associated with clover roots. It seemed desirable therefore to attempt to secure a similar result by modifying our technique to conform as closely as possible to that used by Solheim

and Raa and to include *T. repens*, as well as *T. glomeratum* which we have mostly used in the interest of greater seedling uniformity. The results of this comparison with both hosts are given in Table 8, mostly using the Fåhraeus slide with liquid between slide and coverslip (as was done by Solheim and Raa) but including one comparison with agar (as has been general in our work). Unlike Solheim and Raa we have maintained distinction between as many categories of root hair deformities as possible, and have added others including the condition which these workers described as 'helical', but which is better described as zig-zag because it is apparent from the focussing situation that the back and forth curvature takes place chiefly in the one plane (Fig. 1b of Solheim and Raa).

TABLE 8

Deformation of root hairs of *T. glomeratum* and *T. repens* by filtrate of *R. trifolii*, SU297/32[1]

Treatment	Host[3]	Number of root hairs per 12 plants[2]					
		Branched	Moderately curled	Markedly curled	Bent	Zig-zag	Bulbous
Seeded with bacteria	TG	357[a]	72[a]	33	48	17	3
	TR	74[a]	81[a]	13	18	126[4]	2
Filtrate	TG	18[b]	42[b]	0	22	6	1
	TR	754[b]	33[b]	0	5	62[4]	32[4]
	TR(agar)	22[b]	41[b]	0	10	24	0
Control	TG	1[c]	1[c]	0	0	0	0
	TR	7[c]	1[c]	0	3	3	1

[1] Except where otherwise specified with the original Fåhraeus technique (liquid under cover-slip) and using procedure as detailed by Solheim and Raa [14].

[2] Branched and curled categories as described previously; 'bent': slightly curved at tip (>90°); 'zig-zag'; equivalent to 'helical' of Solheim and Raa [14] 'bulbous': swollen along or at tip of hair. Treatments in these columns not sharing the same index letter are significantly different ($P \leqslant 0.05$).

[3] TG: *T. glomeratum*; TR: *T. repens*.

[4] High value due to single aberrent replicate.

This detailed analysis completely failed to reveal any condition of marked curling resultant on exposure to bacterial filtrate. The less specific categories of branching and moderate curling were regularly encountered as were the zig-zag and bulbous conditions, irregularly, in the case of *T. repens*.

One experiment testing indole acetic acid over a concentration range from $3 \times 10^{-6}M$ to $3 \times 10^{-16}M$ failed to reveal significant increase over the control plants in any category, a second gave two cases ($P \leqslant 0.05$) but so randomly a function of concentration as to throw doubt on their real significance.

DISCUSSION

Four conditions are characteristic of the early stage of the reaction of root hairs to the invasive homologous rhizobium: branching, moderate curling, marked curling and the formation of infection threads. Our use of the three categories of root hair deformation has been defined in an earlier communication [17] and it is essential that they be clearly distinguished and their separate occurrence recorded quantitatively if further confusion is to be avoided in reporting and interpretation. This is particularly important for those classed as 'markedly curled' which should have the tip in a tight, generally deformed, 360° curl.

The results we have obtained with extremely sparse seedings of the Fåhraeus slide are a timely reminder that it is not necessary to have the root surface swamped with rhizobia, as is the common practice in experiments of this kind. The number of affected root hairs reached its maximum even before the inoculum provided one microcolony per root hair and the overriding effect exercised by the individual root hair was shown on the numerous occasions when hairs grew right through a dense microcolony without being affected by it.

It is worth considering the occurrence of the several kinds of response in relation to the nodulating specificity and invasive capacity of rhizobia. The invasive rhizobia with their homologous host produce all four conditions. The virulent strains of *R. leguminosarum* we have tested on clover stop short of infection threads, though commonly showing, in the deformed region of markedly curled roots hairs, a bright refractile area similar to that associated with the origin of an infection thread and which could represent an early aborted invasion. Other virulent rhizobia are unable to cause marked curling with a heterologous host and non-invasive *R. trifolii* fail even to cause branching of clover root hairs. Thus the three conditions: branching, marked curling and formation of an infection thread, seem to require increasing degree of host compatibility in that order.

The failure of *R. leguminosarum*, which on its own is capable of causing marked curling and abundant branching, to complement the avirulent *R. trifolii* could indicate a failure of the latter at the 'last' infection step as well as in those concerned with the branching and the marked curling response. The moderately curled condition is harder to place because it is generally retained by non-invasive mutants.

In all our experience the marked curling response demands the close proximity of viable invasive rhizobia. The results reported by Solheim and Raa appear to offer the most serious contradiction of this statement, but interpretation is confused by the pooling of 'helical' with the 'curling' category and the possibility of rhizobial carry over in the filtered medium or on the 'surface-sterilised' seed. We have encountered both these technical difficulties, in our first attempts to repeat the work, surprisingly due to the failure of the membrane filter to clear the filtrate of bacteria and, not so surprisingly, due to inadequacies of 'seed sterilisation' [1]. Any such difficulty is exacerbated with the liquid (comp. agar) Fåhraeus assembly and apparently by greater sterilisation difficulty associated with white clover (*T. repens*) compared with *T. glomeratum*. Unfortunately Solheim and Raa did not report results with controls that would be needed to safeguard against such flaws in technique and the absence of statistical data makes the quantitative aspects of their results difficult to interpret. It has been our experience that variation between replicates can be considerable, so that two to fourfold differences are likely to be required for significance, even when adequate replicates are included. The position is worst when many cases of a particular condition occur sporadically from plant to plant (as noted in Table 8).

Because no cell-free preparatation we have tested has been able to cause the marked curling it has not been possible to make much progress towards defining the nature of any substance responsible for this condition. There are two main possibilities. Either a poorly diffusible factor is able to operate only when produced close to the hair, or direct contact between the specific virulent bacterium and the root hair is required. In either case the asymmetric nature of the markedly curled condition would seem to reflect localization of stimulating bacteria (comp. [5]) or of a poorly diffusing factor. There could be a place here for specific localised induction of enzyme production,

such as the disputed polygalacturonase model [4 10 11]. Difficulties in obtaining definitive confirmation or rejection of such a hypothesis are to be expected in a reaction as localised as is suggested by the present investigation, particularly when the available techniques are unsuitable to the investigation of such a condition *in situ*. However, if direct contact between rhizobium and host is always involved, the markedly curled condition that is not accompanied by infection thread might reflect, not a prerequisite to invasion, but a very early stage in invasion itself. In that case markedly curled hairs without an infection thread would be interpreted as an early aborted infection process. Marked curling, without full infection, of clover by *R. leguminosarum* [17] and of pea (Pisum) with *R. trifolii* [6] could similarly reflect failure of infection to go beyond the first step, in the case of these taxonomically related rhizobial species.

The filtrate responsible for branching and moderate curling contains nucleic acid, carbohydrate (including bacterial lipopolysaccharide) and protein. In our experience it has been heat-stable whether contained in the filtrate extracted from cells grown on yeast extract agar medium or from the vicinity of inoculated roots. Solheim and Raa obtained a similar result in the latter case, but found that the defined liquid medium filtered from the bacteria lost its activity after relatively mild heating (80°C, 30 min). The extremely potent branching factor is readily extracted from bacteria, and, although some form of it is retained, another fraction diffuses and passes readily through a dialysis membrane. It is relatively stable to nucleases and periodate but is susceptible to trypsin. These observations are, as far as comparison is possible, not incompatible with the leads given by adsorption and elution data [14] although the undoubted complexity of the system demands more rigourous investigation for its elucidation. None of the responses we have observed can be attributed to externally operating indole acetic acid, although an associated role in infection, as distinct from root hair deformation, is not precluded, particularly if this were to involve internal plant-produced auxin [2].

The water soluble gum ('extracellular polysaccharide') appears not to have any essential causal effect, so far as root hair deformation is concerned. Our conclusion which supports a brief reference to a wholly negative result by Fåhraeus and Ljunggren [5] is based on these observations: (i) marked root hair responses have always

been obtained with non-gummy invasive mutants; (ii) avirulent, but still gummy, mutants have lost virtually all capacity to deform root hairs; (iii) crude 'extracellular polysaccharides' from different strains of gummy virulent strains have differed widely in their deforming capacity; (iv) supernatants, from which crude extracellular polysaccharide have been precipitated, are as active as the precipitated material. Such effect as could be observed in any precipitated fraction could better be attributed to a potent, but still undefined, material which would be markedly concentrated in any attempt to secure a workable amount of crude polysaccharide from a non-gummy strain such as the NA34S used by Hubbell. Nor do our results give any support to lipopolysaccharide as a responsible factor.

Although the minor nature of the 'moderate curling' condition with clover and *R. trifolii* and its diminution when working with cell-free preparations have militated against a fuller investigation of this factor, it has to be noted that it has a more important place in other host: rhizobium combinations (*e.g. Medicago sativa* and *R. meliloti*; *Macroptilium atropurpureum*; *Phaseolus atropurpureus*) and slow-growing rhizobia [17]. The cause of other less regular categories of deformation is even less defined at present; their separate recognition and recording is the more necessary because of that.

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