# NITROGEN FIXATION IN LEGUMINOUS PLANTS. I.

## GENERAL CHARACTERS OF ROOT-NODULE BACTERIA ISOLATED FROM SPECIES OF MEDICAGO AND TRIFOLIUM IN AUSTRALIA.

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(Plate ii; three Text-figures.)

[Read 29th April, 1942.]

### *Introduction.*

The establishment of leguminous crops in the general agricultural systems in Australia has received widespread attention in recent years. In the wheat-producing districts this must be considered a matter of great importance, in view of the insufficiency of non-symbiotic nitrogen fixation, as compensation for the nitrogen demands of the cereal crops, as previously discussed (Jensen, 1940). As a sequel to this work, investigations on symbiotic nitrogen fixation have therefore been undertaken; the present paper deals with the general biological properties of root-nodule bacteria from those species of medics and clovers that are likely to be of most importance in the Australian wheat districts. In spite of some valuable contributions in recent years, e.g., by Strong (1937, 1940) and Vincent (1941), our knowledge of the rhizobia of local occurrence is still very incomplete. Particular importance attaches to the question of their nitrogen-fixing capacity, since "ineffective" types of rhizobia have often been stated to occur frequently in "wild" leguminous plants (Wilson, 1940); such organisms can sometimes be so richly represented in the soil that they constitute a problem of practical significance by suppressing the effective rhizobia supplied as inoculum to the seed (Nicol and Thornton, 1941). Since artificial inoculation of leguminous seeds has not yet reached very large proportions, "wild" rhizobia are presumably responsible for most of the nodule-formation in legumes in the Australian wheat districts, particularly in the prevalent self-sown legumes like burr-trefoil (*Medicago hispida* var. *denticulata*) and ball-clover (*Trifolium glomeratum*), and to some extent probably also in sown annuals which, like subterranean clover, maintain their stand by self-sowing. Furthermore, since the soils in large areas of the wheat-belt of New South Wales are more or less acid, it is important to get information on the influence of reaction on growth and infectiveness of the rhizobia, and nitrogen-fixing power of the plant-rhizobia complexes. This acquires particular significance in view of the earlier statements of Stevens (1925) and Wright (1925) concerning the existence, within the same inoculation-group, of different types of rhizobia with different pH-limits and -optima, allegedly correlated with differences in general cultural characters, serological properties, and nitrogen-fixing efficiency. Later studies (Fred *et al.*, 1932; Wilson, 1940) have failed to trace definite correlations within the last group of characters, but the question of pH-tolerance in relation to efficiency does not seem to have been followed up, in spite of its obvious significance, which becomes even greater in view of the later discovery of "host-plant specificity" (Strong, 1937; Wilson, 1940) and the far-reaching observations by Nicol and Thornton (1941) on the relative growth-rates of rhizobia as determining the infection of legume roots by competing strains of rhizobia. To find out whether any correlations

exist between adaption to host plants, tolerance of acidity and other physiological characters in rhizobia obtained from different localities, has been the aim of the experiments discussed here.

## *Material and Methods.*

Altogether twenty-four strains of *Rhizobium meliloti* and twenty-four of *Rh. trifolii* were isolated from the following host plants and localities:

*Rhizobium meliloti.*—From lucerne (*Medicago sativa*), 3 strains (Canberra, A.C.T.; Lawes, Qd.; Crawley, W. Aust.).—From burr-trefoil (*M. hispida* var. *denticulata*), 6 strains (Sydney, N.S.W. (2); Finley, N.S.W.; Temora, N.S.W.; Canberra, A.C.T.; Lawes, Qd.).—From *M. hispida* var. *apiculata*, *confinis*, and *lappacea*, 1 strain from each (Canberra, A.C.T.).—From *M. falcata*, 2 strains (Canberra, A.C.T.; Lawes, Qd.).—From *M. lupulina*, 2 strains (Canberra, A.C.T.; Crawley, W. Aust.).—From *M. arabica*, *coerulea*, *gaetula*, *minima*, *murex*, *orbicularis*, *scutellata*, and *truncatula*, 1 strain of each (Canberra, A.C.T.).

*Rhizobium trifolii.*—From *Trifolium glomeratum*, 9 strains (Temora, N.S.W. (2); Narrandera, N.S.W. (2); Parkes, N.S.W.; Stockinbingal, N.S.W.; Canberra, A.C.T. (2); Crawley, W. Aust.).—From *T. repens*, 4 strains (Sydney, N.S.W.; Canberra, A.C.T.; Lawes, Qd.).—From *T. subterraneum*, 4 strains (Sydney, N.S.W.; Canberra, A.C.T.; Crawley, W. Aust.).—From *T. pratense*, 1 strain (Lawes, Qd.).—From *T. arvense*, *cernum*, *dubium*, and *procumbens*, 1 strain from each (Canberra, A.C.T.).

Two isolates from root nodules of burr-trefoil (from Cowra, N.S.W., and Crawley, W. Aust.) proved incapable of infecting their host plant as well as lucerne, and turned out actually to be *Rhizobium trifolii*, producing normal nodules and inducing good growth in both white and subterranean clover; culturally and physiologically they resembled the other clover-rhizobia. They probably represent contaminants that had gained entrance to the nodules together with the proper symbionts, such as *Bacterium radiobacter* frequently, and other bacteria occasionally, will do (Fred *et al.*, 1932).

All isolates were obtained by plating on yeast-extract mannite agar (Fred *et al.*, 1932) from contents of healthy-looking nodules externally sterilized with alcohol and mercuric chloride. The pure cultures were maintained on the same medium or on potato-extract agar with 1% sucrose; this medium seemed even more favourable. All subsequent data refer to cultures grown at 28–30°C., unless otherwise stated.

## *Experimental Results.*

*Cell morphology.* In this respect all strains of both species showed a normal appearance (Fred *et al.*, 1932). The prevalence of motile forms varied to some extent, and a few strains of *Rhizobium trifolii* showed branched, bacteroid-like forms already in quite young (2–3 days) cultures on potato-extract agar, but no definite subgroups could be distinguished.

*Growth on agar* was, in all freshly-isolated strains, true to type: in *Rhizobium meliloti* raised, smooth, soft and white, somewhat varying in abundance and degree of opacity, and in *Rhizobium trifolii* very voluminous and fluid, whitish, semi-transparent, in a few strains extremely viscid. In neither species was there any clear-cut separation into subgroups, as observed by Stevens (1925) and Wright (1925). Variant colony-types arose in some strains after prolonged cultivation, as discussed below.

*Growth in liquid media* (yeast-extract mannite solution, or soil extract with 1% sucrose) started in both species as a faint uniform turbidity gradually collecting into a slimy sediment. *Rhizobium meliloti* also gradually formed a more or less coherent surface pellicle. In synthetic solution (1% mannite, 0·05% $NaNO_3$, and mineral salts) no visible growth was produced by any strain of *Rhizobium trifolii*, while the *meliloti*-strains gave only a feeble growth which tended to disappear in subsequent transfers. An authentic strain of *Bacterium radiobacter*, obtained from the Department of Agricultural Bacteriology, University of Wisconsin, grew well on continued transfer in this medium; apparently it is independent of biotin, the essential growth-factor for the rhizobia (Wilson, 1940). Upon the whole the differences between this organism and the rhizobia seem, as shown by subsequent tests, to be more of a quantitative than a

qualitative nature (cf. also Hofer, 1941); indeed, there is a good deal to suggest the idea that *Bacterium radiobacter* may be neither more nor less than a rhizobium which has lost its infective power and adapted itself to a purely saprophytic existence.

*Growth on potato* was very scant (often absent), whitish-grey at first, but in a few strains of both species it assumed after 2–3 weeks a faint brown colour similar to, but much less intense than, that of *Bacterium radiobacter*, which showed rapid, abundant growth of the typical rust-brown colour (Hofer, 1941). Isolates from such brown growth of the rhizobia showed unaltered cultural characters and nodule-forming capacity.

*Growth in milk.* All strains of *Rhizobium trifolii* showed the normal behaviour (Fred *et al.*, 1932): early formation of a clear surface-zone becoming 1–2 cm. deep after 5–6 weeks, and change of the initially acid reaction (pH 6·2–6·3) to faintly alkaline (pH 7·0–8·2).* A very faint brownish colour of the milk was noticeable after long incubation, again similar to, but much weaker than, *Bacterium radiobacter*, which rapidly produced a clear zone, alkaline reaction, and light coffee-brown pigment. The strains of *Rhizobium meliloti* fell into two fairly distinct groups. One of these comprised eleven strains (two from lucerne, two from *M. falcata*, the rest from *M. coerulea*, *gaetula*, *lupulina*, *minima*, *orbicularis*, *scutellata*, and *truncatula*), which, as expected, formed a clear surface-zone after 1–2 weeks, and with one exception a more or less acid reaction (pH 4·8–5·6 after 5–6 weeks). In some cases the milk was actually coagulated, and no contaminants could be detected in such cultures; previously, coagulation of milk by rhizobia seems to have been recorded only by Topley and Wilson (1936). The exception was represented by a strain from lucerne, in all other respects typical, which rendered the milk faintly alkaline (pH 7·1–7·6). Another group of twelve strains, viz., the nine from the different varieties of *M. hispida*, and three from *M. arabica*, *lupulina*, and *murex*, failed regularly to produce a clear zone, even after two months, and left the reaction unchanged; growth was not macroscopically visible, but tests on agar showed large numbers of viable rhizobia after incubation. Only by the use of extra heavy inoculum (a big loopful of cell-material from agar slope) could some strains be induced to form a zone after 4–5 weeks; in these cases the reaction was changed towards neutrality (pH 6·4–6·7). Finally, one strain from lucerne showed an inconstant but mostly negative zone-formation which, when positive, was accompanied by acid formation. The appearance of some typical cultures is shown in Plate ii, fig. 2.

The lack of zone-formation, which thus seems characteristic of the rhizobia from *M. hispida*, showed no correlation with other cultural characters or with the ability to ferment lactose, but may be connected with a lesser ability to utilize the nitrogen-compounds of the milk. Tests on plates of milk-agar (equal parts of skimmed milk and tap-water with 3% agar, sterilized separately and mixed in Petri dishes) showed a heavy, opaque, white to pale yellow growth of the zone-positive, and a much weaker, semi-transparent, colourless growth of the zone-negative strains.

*Fermentation of carbohydrates* was tested in a solution containing 1% of the compound to be tried, 1% of yeast extract (of dry yeast autoclaved with 10 times its amount of tap-water), 0·05% $K_2HPO_4$, 0·02% $MgSO_4$, 0·01% NaCl, and 0·00025% bromo-thymol-blue; initial pH 6·7–6·9. Readings of duplicate test-tube cultures with 4 c.c. of medium were taken after 7 days, frequently checked by electrometric pH-determinations. The results (Table 1) show no definite subgroups within each species, except perhaps in regard to the fermentation of dulcite by *Rhizobium meliloti*, but this is not correlated with any other character or the origin of the strains. Compared with the results of Baldwin and Fred (1927), the present data show *Rhizobium trifolii* to have definitely stronger fermenting power than *Rh. meliloti*, as regards both the degree of acidity produced and the number of compounds attacked; for instance, Baldwin and Fred's *trifolii*-strains fermented dulcite only feebly, and sucrose and raffinose not at all. It remains uncertain whether this discrepancy is due to inherent differences in the organisms or to differences in the medium, such as the circumstance that the present basal medium is richer in growth-compounds, especially biotin, than the nitrate-agar chiefly used by Baldwin and Fred. The former possibility seems the more likely, since Baldwin and Fred found only weak acid-formation by *Rhizobium trifolii* in their

* All pH-determinations were carried out by means of the glass electrode method.

supplementary tests with medium containing yeast extract. Verification of the results by means of Baldwin and Fred's technique (agar slopes) unfortunately had to be given up, owing to the necessity of conserving agar under prevailing war conditions.

TABLE 1.
*Fermentation Reactions of Rhizobia.*

| | *Rhizobium meliloti.* | | | | *Rhizobium trifolii.* | | | | Lowest pH observed in |
|---|---|---|---|---|---|---|---|---|---|
| | ++ | + | 0 | − | ++ | + | 0 | − | |
| Glycerine .. | 2 | 20 | 2 | | 10 | 13 | 1 | | *Rh. meliloti* in : |
| Mannite .. | 2 | 22 | | | 19 | 5 | | | Mannite .. 6·06 |
| Dulcite .. | | 11 | 13 | | 3 | 18 | 2 | | Glucose .. 5·04 |
| Glucose .. | 15 | 9 | | | 22 | 2 | | | Galactose .. 5·22 |
| Galactose .. | 17 | 7 | | | 23 | 1 | | | Sucrose .. 6·51 |
| Sucrose .. | | 22 | 2 | | 18 | 5 | 1 | | |
| Maltose .. | | 24 | | | 10 | 14 | | | *Rh. trifolii* in : |
| Lactose .. | 6 | 12 | 6 | | 17 | 5 | 2 | | Mannite .. 4·67 |
| Raffinose .. | | | 24 | | 2 | 19 | 3 | | Glucose .. 4·77 |
| Dextrine .. | | | | 24 | | | | 24 | Galactose .. 5·13 |
| Ca-Gluconate | | | | 24 | | | | 24 | Sucrose .. 4·90 |
| Control .. | | | 1 | 23 | | | | 24 | |

Figures indicate number of strains showing the following reactions : ++ : strongly acid, indicator pure yellow (pH $\leqq$ 6·0). + : faintly acid, indicator between yellow and green. 0 : unchanged. − : alkali formation, indicator blue.

*Influence of reaction* on the growth of rhizobia was first tested by cultivation in a liquid medium rich in nutrients and of high buffer-content, containing mannite 1%, yeast extract 5·0%, asparagine 0·2%, $K_2HPO_4$ 0·2%, Na-succinate 0·5%, $MgSO_4$ 0·02%; reaction varied between pH 4·8 and 8·7 by addition of $H_2SO_4$ and NaOH. Duplicate test-tube cultures with 5 c.c. of medium were incubated for 14 days; several controls showed no growth after longer incubation in cases where it had not appeared within that time. The results are seen in Table 2.

TABLE 2.
*Growth of Rhizobia in Yeast-Extract Mannite Solution.*

| pH of Medium. | Number of Strains showing Growth : | | | |
|---|---|---|---|---|
| | *Rhizobium meliloti.* | | *Rhizobium trifolii.* | |
| | + | − | + | − |
| 4·8 | 0 | 24 | 0 | 24 |
| 5·0 | 0 | 24 | 0 | 24 |
| 5·2 | 0 | 24 | 10 | 14 |
| 5·4 | 8 | 16 | 24 | 0 |
| 5·6 | 20 | 4 | 24 | 0 |
| 5·9 | 24 | 0 | 24 | 0 |
| 6·4 | 24 | 0 | 24 | 0 |
| 6·7 | 24 | 0 | 24 | 0 |
| 7·1 | 24 | 0 | 11 | 13 |
| 7·5 | 24 | 0 | 0 | 24 |
| 8·2 | 24 | 0 | 0 | 24 |
| 8·7 | 24 | 0 | | |



These figures indicate only a slight difference in the pH-tolerance of the various *meliloti*-strains, for which the limits of acidity lie between those found by Fred and Davenport (1918) and Stevens (1925). *Rhizobium trifolii* also shows little difference between strains, but generally a slightly higher resistance to acidity. In the alkaline range we notice the surprising fact that growth is suppressed at pH 7·1–7·5. Further tests showed that this inhibition at alkaline reaction also existed in cultures on agar of corresponding composition, but was less marked in solutions with less buffer-effect, and especially when the succinate was omitted.

| Growth of *Rhizobium trifolii* in : | At initial pH : | | |
|---|---|---|---|
| | 7·0 | 7·5 | 7·9 |
| I. Complete medium (Table 2) .. .. .. | ± | − | − |
| II. Do., without succinate .. .. .. .. | ± | ± | ± |
| III. 0·5% yeast extract, 0·5% succinate .. .. | | − | |
| IV. Do., without succinate .. .. .. .. | + | + | + |

(The buffer-capacity of these 4 solutions is seen in Fig. 1.)

Since a pH of 7·9 does not *per se* prevent growth, the effect could hardly be due to destruction of some vital factor, like thiamine. Besides in yeast-extract solution, the effect was also displayed in soil extract of pH 7·5, and was not specific to succinate; 0·5% sodium salt of malic, citric, tartaric, lactic, pyruvic, propionic and formic (but not acetic*) acid suppressed the growth of all strains of *Rhizobium trifolii* in a 0·5% yeast-extract solution of pH 7·5 more or less completely, while having no effect on *Rhizobium meliloti* and *Bacterium radiobacter*. Inability to start growth in a highly buffered solution of faintly alkaline reaction, especially in the presence of organic sodium salts, thus seems a characteristic feature of *Rhizobium trifolii*.

Since the composition of the medium apparently influences the pH-tolerance considerably, another series of experiments was made in a solution of less buffer-capacity and without organic salts, thus avoiding the change to alkalinity which was very marked with many acids besides succinic, owing to decomposition of the acid radicle. The new medium consisted of extract from a rich humus-soil autoclaved with 5 times its amount of water; to the filtrate were added 1% sucrose, 0·05% asparagine, 0·1% $K_2HPO_4$, 0·1% $Na_2HPO_4$ and 0·05% $MgSO_4$. Incubation for 14 days gave the following results with *Rhizobium meliloti*:

| Number of strains growing at pH : | | | | | |
|---|---|---|---|---|---|
| 5·1 | 5·3 | 5·5 | 5·7 | 5·9 | 6·9 |
| 24− | 11+<br>13− | 23+<br>1− | 24+ | 24+ | 24+ |

If growth took place at pH 5·3 it was only feeble; this reaction seems thus, as in the previous series, to represent the lower limit for growth of *Rhizobium meliloti*, and about half the strains seem to be inhibited at pH 5·5. Although this difference is in itself very small, it may assume significance through the fact that it is correlated with the kind of

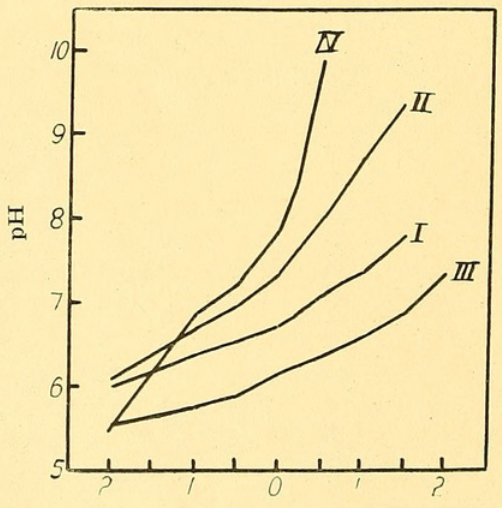


Figure 1.

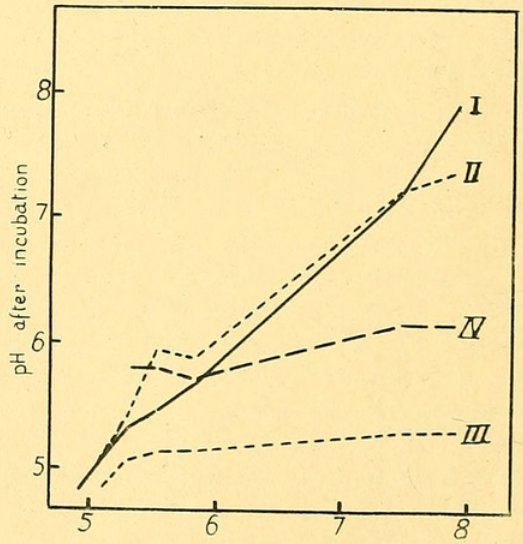


Figure 2.

Fig. 1.—Buffer-effect of nutrient solutions I-IV; c.c. n/28 $H_2SO_4$ or NaOH per 10 c.c. of solution.

Fig. 2.—Change of reaction in soil extract sucrose cultures of *Rhizobium trifolii*. I, Sterile medium; II, Non-acidifying strain (from *T. cernum*); III, Strongly acidifying strain (from *T. glomeratum*); IV, Strain typical of the majority (from *T. subterraneum*).

* According to Tam and Wilson (1940), acetic acid is not readily dehydrogenated by *Rhizobium trifolii*.

host plant and the zone-formation in milk. The strains failing to grow at pH 5·3 were largely those isolated from *M. hispida* and failing to produce a clear zone in milk, as shown thus (omitting the strain from lucerne that gave variable results):

| | Zone Formed. | No Zone Formed. |
|---|---|---|
| Growth at pH 5·3 .. .. .. | 8 | 2 |
| No growth at pH 5·3 .. .. | 3 | 10 |

The value of $\chi^2$ (Fisher, 1935) in this fourfold table (n = 1) is 7·738, which is beyond the 1% point of significance. Determinations of pH after 14 days showed only minor changes which, however, indicated a certain shifting of the reaction towards middle pH-values (5·8–5·9), as shown in Table 3.

TABLE 3.

*Change of Reaction in Soil-Extract Cultures of* Rhizobium meliloti.

| pH Initially. | | pH after Incubation. | | |
|---|---|---|---|---|
| Calculated. | Found. | Lowest. | Highest. | Mean. |
| 5·1 | 5·13 | | (No growth) | |
| 5·3 | 5·26 | 5·58 | 5·94 | 5·77 |
| 5·5 | 5·50 | 5·50 | 6·04 | 5·82 |
| 5·7 | 5·68 | 5·72 | 6·10 | 5·92 |
| 5·9 | 5·92 | 5·70 | 6·22 | 5·93 |
| 6·9 | 6·86 | 6·28 | 7·04 | 6·55 |

A corresponding experiment with *Rhizobium trifolii* showed the following numbers of strains being capable of growth at pH:

| 4·7 | 4·9 | 5·1 | 5·3 | 5·5 | 5·8 | 6·1 | 7·5 | 7·9 |
|---|---|---|---|---|---|---|---|---|
| 24 – | 1 + | 10 + | 22 + | 24 + | 24 + | 24 + | 24 + | 24 + |
| | 23 – | 14 – | 2 – | | | | | |

There is also here some indication of two groups separated by a very narrow pH-interval, with limits near pH 5·1 and 5·3, besides two slightly more sensitive strains, and finally one which, like *Bacterium radiobacter*, resists pH 4·9. This strain, however, was found to be non-infective, although typical in every other respect. The change in reaction was less uniform than in *Rhizobium meliloti*, as might be expected in view of the more vigorous sucrose fermentation; some strains constantly lowered the pH, especially in the alkaline range, a few others caused very little change, and the majority had, like *Rhizobium meliloti*, a more or less pronounced tendency to equalize the reaction to a range of pH 5·6–6·2. Examples of these three types are seen in Fig. 2.

Generally the limits of acidity for cell multiplication thus seem to lie at pH 5·3–5·5 in *Rhizobium meliloti*, and at pH 5·1–5·3 in *Rh. trifolii*. A somewhat stronger acidity can apparently be tolerated by cells already developed, as suggested by the lowest pH-values observed in the fermentation tests (Table 1). In addition, in a physiologically acid solution (soil extract with 1% sucrose and 0·1% $(NH_4)_2SO_4$) of initial pH 6·4, many strains of *Rhizobium trifolii* lowered the pH to 4·6–4·9. *Rh. meliloti* produced only small changes (pH 5·5–5·6 lowest).

The appearance of the cultures suggested that the optimal reaction for *Rhizobium meliloti* was approximately neutral, but for *Rh. trifolii* in the buffered solution definitely on the acid side (pH 5·6–5·9). Some quantitative experiments were made in order to check this. Three strains of *Rhizobium meliloti* and three of *Rh. trifolii* were grown for 7 days in mannite-solution with 5% yeast extract but no Na-succinate, adjusted to 9 pH-values between 4·5 and 8·7; the change in pH was then measured, and the bacterial substance was removed by sharp centrifugation, dried, and weighed. The pH-change was mostly slight, not exceeding 0·5 unit, and rarely 0·25. The weights of bacterial mass are shown in Fig. 3. In this well-buffered solution *Rhizobium meliloti* grows best at pH 7–8, somewhat varying according to the strain; the one from *M. hispida* even fails to grow at pH 5·9. *Rhizobium trifolii* grows decidedly best at acid reaction, viz., pH 5·5–6·5; the most vigorous strain (T.g.–44) shows some growth even at pH 4·5. A supplementary

experiment was made with the two most rapidly growing strains in a less buffered medium: soil extract with 1% mannite, of 8 pH-values from 5·0 to 8·0. The results, after 14 days, are included in Fig. 3. *Rhizobium meliloti* again grows best at neutral reaction, but *Rh. trifolii* is not much influenced; the yield, indeed, is highest at initial pH 6·5–7·0, but these values had dropped to 5·3–5·6 during growth.

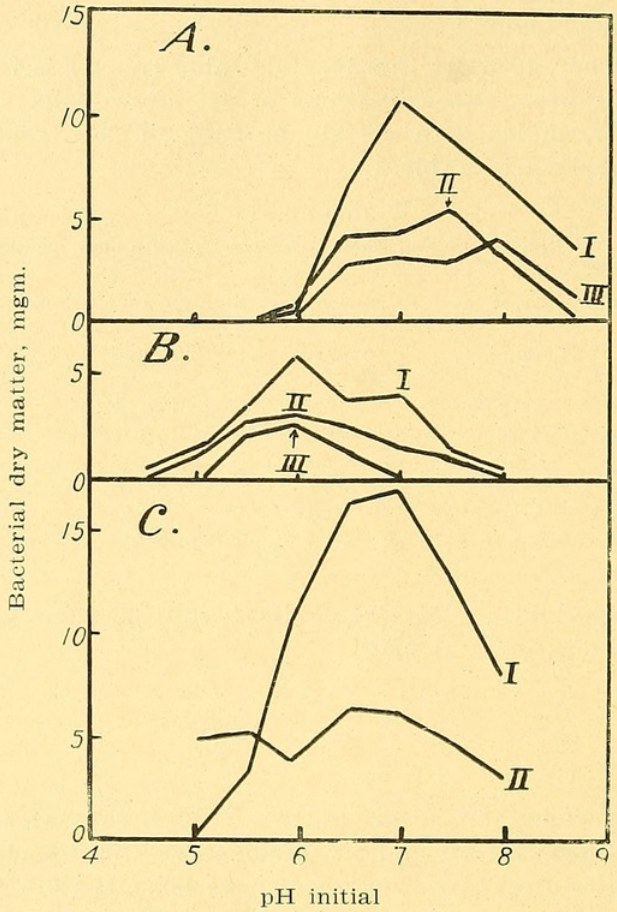


Fig. 3.—Growth of rhizobia in solutions of different reaction. A. *Rhizobium meliloti* in yeast-extract mannite solution. I, from *M. lupulina*; II, from *M. sativa*; III, from *M. hispida* var. *denticulata*. B. Same medium, *Rhizobium trifolii*. I, from *T. glomeratum*; II, from *T. subterraneum*; III, from *T. repens*. C. Soil extract. I, *Rhizobium meliloti*; II, *Rhizobium trifolii*. Yield of dry bacterial substance, mgm. per 25 c.c. of medium.

Generally there seem to be only minor differences in the pH-tolerance of the strains within each species; if anything, the *meliloti*-strains from *M. hispida* and a few others appear slightly more sensitive to acidity than the rest. *Rhizobium trifolii* is not only more acid-tolerant, but actually displays an optimum at moderately acid reaction—a point of obvious significance in the infection of roots under natural soil conditions; it is important to note that this optimum applies to total cell-growth and is different from the pH-optima for respiration and dehydrogenase-activity (Tam and Wilson, 1941). Experiments on the influence of reaction on the ability of rhizobia to infect their host plants, as well as on the resulting nitrogen fixation, are in progress.

*Infective power of the rhizobia* was tested in aseptic agar cultures. Seeds externally disinfected with alcohol and $HgCl_2$ were sown, after washing with sterile water, in large test-tubes containing 20 or 40 c.c. sterile medium of the following composition: $CaHPO_4$ 0·1%, $K_2HPO_4$, $MgSO_4$ and NaCl each 0·02%, $FeCl_3$ 0·01%, washed agar 0·6%. Two or three seeds were sown per tube, and the part filled with agar was wrapped in paper to protect the roots from light. When the seedlings had produced the first true leaf, they were inoculated with the rhizobia to be tested, and left in a greenhouse for observation. Nodules usually appeared within 1–2 weeks after inoculation, and after 4–5 weeks the difference in appearance between inoculated and control plants became conspicuous if "effective" nodules had been produced (Pl. ii, fig. 1). By this test all strains of *Rhizobium trifolii* proved capable of forming nodules on white clover, but a few (one

from *T. dubium*, two from *T. subterraneum*) produced small scattered nodules which did not seem to benefit the plant. These proved effective on subterranean clover, while conversely one strain from this plant failed to benefit white clover. The strains from *T. glomeratum* seemed equally effective on this species and on *T. repens*, *pratense*, *subterraneum*, and *incarnatum*. The exceptional strain, isolated from red clover, did not produce nodules on any plant, although it appeared normal in other respects. All strains of *Rhizobium meliloti* formed nodules on lucerne, which constantly showed improved growth as a result. A remarkable contrast to this was seen in burr-trefoil: all strains produced nodules, but only those isolated from varieties of *M. hispida*, and *M. murex*, appeared to be beneficial. All the rest gave typical "ineffective" nodulation (Fred *et al.*, 1932): innumerable tiny nodules distributed over the whole root system, sometimes so dense as to appear as continuous swellings of the lateral roots; the leaves and stems after two months were stunted, yellowish, showing every sign of nitrogen starvation, and looking entirely like the uninoculated control plants. A special test was made with *Medicago tribuloides*, of which a specimen from the Institute of Agriculture, University of West Australia, Crawley, was found devoid of root nodules, although these were plentiful on roots of lucerne and *M. lupulina* taken from the same plot. All *meliloti*-strains formed nodules on *M. tribuloides*, but only those from *M. hispida* appeared to be effective, as shown by a length-measurement on plants 10 weeks old:

| Inoculum. | Height and Standard Error, mm. |
|---|---|
| ex *M. hispida* (8 strains, 22 plants) | 74·0 ± 3·05 |
| ex other *Medicago* spp. (15 strains, 40 plants) | 60·8 ± 2·14 |

Although only tentative, these data suggest that *M. tribuloides* with regard to host-plant specificity belongs to the same group as *M. hispida* (Burton and Erdman, 1940).

Nitrogen determinations in a number of clover- and lucerne-cultures about 2 months old showed such marked gains in plants with the "effective" type of nodulation (limited number of big, elongated nodules, usually with pink pigment) as to suggest that this simple method, which is exceedingly convenient for testing numerous strains simultaneously and under identical (even if highly artificial) conditions, might be useful for testing not only the identity but also the effectiveness and host-plant specificity of the rhizobia. A cross-inoculation experiment was made with five strains of *Rhizobium trifolii* on white and subterranean clover, the former grown for 96 days in 20 c.c. of agar, the latter for 98 days in 40 c.c. The results are given in Table 4.

TABLE 4.

*Cross-inoculation Experiments with* Rhizobium trifolii *and* Rh. meliloti.

| Inoculum. | White Clover. | | Subterranean Clover. | |
|---|---|---|---|---|
| | Repl. | mgm. N, mean. | Repl. | mgm. N, mean. |
| From white clover (C–5) | 4 | 1·37 | 4 | 3·05 |
| ,, ,, ,, (H–1) | 4 | 1·14 | 4 | 1·51 |
| From subterranean clover (67–3) | 4 | 1·23 | 4 | 3·80 |
| ,, ,, ,, (H–2) | 4 | 0·94 | 4 | 3·87 |
| From cluster-clover (Tg–44) | 4 | 0·63 | 4 | 3·90 |
| Uninoculated | 5 | 0·22 | 5 | 1·55 |
| | Lucerne. | | Burr-trefoil. | |
| From lucerne (94–16) | 4 | 4·00 | 2 | 1·52 |
| From *M. lupulina* (ML–34) | 3 | 3·96 | 2 | 1·08 |
| From *M. murex* (MMu–2) | 5 | 3·69 | 2 | 1·79 |
| From *M. h.* var. *denticulata* | 3 | 4·01 | 2 | 2·96 |
| From *M. h.* var. *apiculata* | 4 | 3·50 | 2 | 3·62 |
| Uninoculated | 4 | 0·72 | 2 | 1·00 |

White clover is benefited by all strains, but analysis of the variance shows that the one from *T. glomeratum* is less effective than the rest, including two from subterranean clover. In this species one strain from white clover is completely ineffective, giving

the characteristic picture of many tiny scattered nodules; the differences between the other strains are not significant. As mentioned elsewhere (Jensen and Vincent, 1941), this and the preliminary tests indicate that the reciprocal specificity of white and subterranean clover with respect to their nodule bacteria is not, as maintained by Strong (1937), a rule without exception.

A corresponding experiment was made with lucerne, grown for 4 months in 40 c.c. of agar, and *M. hispida* var. *denticulata*, grown for 107 days in 75 c.c. of agar. These results are included in Table 4. In lucerne all strains are effective without showing significant differences, but in burr-trefoil only those of homologous origin show high efficiency; one of the others is completely, and two partly, ineffective. These results, as well as those found in the preliminary tests, agree fully with the findings of Burton and Erdman (1940), but only partially with those of Strong (1940), who found all *meliloti*-strains more or less effective on various medics but not always on *Melilotus*. On the other hand the results found both with clovers and with medics support Strong's contention that it is doubtful whether there exist any rhizobia that are not effective on some host plant or other.

Upon the whole the agar-culture method appears most helpful when it is desired to test a large number of rhizobia for host-plant specificity as well as genuineness, and to some extent also for effectiveness with a given host plant, although detection of smaller differences in this respect may require cultivation under more natural conditions permitting the production of a larger crop. Wilson *et al.* (1931) found the agar-method suitable only for detecting gross differences in effectiveness, owing to the slow diffusion of carbon dioxide through the cotton plugs and consequent low rate of photosynthesis. Yet in the present tests it appeared that at least in the early stages of growth, nitrogen supply and not carbon dioxide tension was the limiting factor, as suggested by the considerably faster growth of seedlings in agar with addition of sodium nitrate.

*Dissociation.* After cultivation for about 18 months, several strains of *Rhizobium meliloti* showed by plating on potato-extract agar an aberrant colony-type which superficially resembled *R. trifolii* by its very voluminous, slimy, semi-transparent growth. Two strains, from *M. sativa* and *M. falcata*, produced in addition a second aberrant type: very opaque, of a firm, almost cartilaginous consistency, with a finely rugose surface, later deeply wrinkled, and quickly forming a coherent pellicle in liquid media where the original type, as well as the slimy variant, grew with diffuse turbidity. Superficially the phenomenon looks like a production of "mucoid" and "rough" variants from an originally "smooth" type; whether it is an actual "S→R variation" in the true sense, which implies antigenic changes (Topley and Wilson, 1936), remains uncertain. Preliminary serological tests on a few of the variants have shown no differences in antigenic structure.* Very few observations have hitherto been made on dissociation in the rhizobia. Almon and Baldwin (1933) mention the arising of variants, sometimes pigmented, which had lost their infective power. Israilsky and Leonowitsch (1933) describe rough, smooth and intermediate variants of *Rhizobium meliloti* and *Rh. leguminosarum* from vetch; these were stated to arise both *in vitro* and in the nodules. Serological tests gave no clear results, but the rough types of vetch-rhizobia had less fermentative power and produced no zones in milk. The variants encountered here produced zones in milk and changes of reaction like their mother-cultures; both the slimy and the rough variant of the strain from lucerne had retained the distinctive character of producing alkaline reaction in milk. On lucerne in agar culture they all formed normal-looking nodules from which they could be recovered in unaltered form after an interval of about a month. Experiments on the effectiveness of these variants are being continued.

## *Discussion.*

The results suggest that strains of *Rhizobium meliloti* and *Rh. trifolii*, which are *per se* ineffective, are not commonly found in Australian soils, but on the other hand the host-plant specificity presents a very complicated problem in both species. In *Rh. trifolii* the host-plant relations are obviously much less simple than would appear from the

* These tests were kindly made by Mr. J. M. Vincent, School of Agriculture, University of Sydney.

results of Strong (1937), and *Rh. meliloti* seems to comprise at least two groups of strains with a kind of non-reciprocal host-plant specificity, one of them wholly or partly ineffective on *M. hispida*, but both effective on lucerne. From a practical point of view this implies that in localities where burr-trefoil grows well ("trefoil country"), lucerne is likely to find effective rhizobia even without artificial inoculation; if, on the other hand, it is intended to introduce varieties of *M. hispida* into localities where other medics have previously grown, it is important to apply artificial inoculation with the proper rhizobia, since otherwise there is every likelihood of an ineffective nodulation being established. In view of the complicated nature of the problem, caution in drawing final conclusions is certainly indicated, but a complete investigation of the phenomena of host-plant specificity in *Medicago* as well as *Trifolium* appears a matter of equal importance from a scientific as well as a practical point of view.


## Summary.

Twenty-four strains of *Rhizobium meliloti* and twenty-four of *Rh. trifolii* were isolated from host plants grown in Australia. As a whole they conformed to the general descriptions of the two species. Fermentation of carbohydrates did not reveal any subgroups within the species, but was stronger in *Rhizobium trifolii*. The limit of acidity for growth of *Rh. trifolii* was pH 5·1–5·3, of *Rh. meliloti* pH 5·3–5·5. In well-buffered nutrient solutions, particularly in the presence of organic sodium salts, *Rh. trifolii* failed to grow at pH 7·1–7·5 and had an optimum at pH 5·5–6·5, whereas *Rh. meliloti* had an optimum at pH 7·0–8·0 and could grow at pH-values above 8·7. All strains of *Rh. trifolii* (except one) produced nodules on sterile seedlings and appeared to be effective on some species of clover; inefficiency of rhizobia from white clover on subterranean clover and *vice versa* was not found to be a constant phenomenon. All strains of *Rhizobium meliloti* produced effective nodules on lucerne, but in burr-trefoil this only happened with strains isolated from varieties of *Medicago hispida*. These rhizobia belonged to a group of strains that seemed to differ from the rest by being slightly more sensitive to acid reaction and by not readily forming a clear zone in milk. Some strains of *Rhizobium meliloti* showed phenomena of dissociation which gave rise to two types of variants, one producing a mucoid and the other a firm and wrinkled growth.



## *Acknowledgement.*

The collaboration of Mr. R. C. Betty, M.Sc., who has carried out much of the analytical work, is gratefully acknowledged. Thanks are also due to Mr. F. W. Hely, B.Sc.Agr., and several other Research Officers of the Council for Scientific and Industrial Research for assistance in obtaining plant material, and to Mr. S. Woodward-Smith, Department of Medical Artistry, University of Sydney, for the photographs.


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## EXPLANATION OF PLATE II.

Fig. 1.—White clover in agar culture, 2 months old. Left and centre, inoculated with *Rhizobium trifolii*; right, uninoculated.

Fig. 2.—Cultures of rhizobia in milk, 6 weeks old. From left to right: *Rhizobium trifolii* (from *T. glomeratum*), *Rhizobium meliloti* from lucerne, *Rh. meliloti* from burr-trefoil, Sterile control.