

STUDIES OF THE ROOT-NODULE ORGANISM OF LUPINUS¹

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In an earlier paper from this laboratory, Baldwin and Fred (1929a) discussed the characteristics of the root-nodule bacteria and suggested names for five of the common cross-inoculation groups. Specific designation for the organism which forms nodules on the roots of *Lupinus* was not proposed, since the information concerning this cross-inoculation group was so meager.

Schroeter (1886) suggested the name *Phytomyxa lupini* for the organism which formed nodules on *Lupinus luteus* and *Lupinus angustifolius*. Beijerinck (1888) included in a list of seven varieties of *Bacillus radicum*, the organism *Bacillus radicum* var. *Lupini*, which formed nodules on *Lupinus polyphyllus* and *Lupinus luteus*. Later, in 1890, he used the term *Bacillus ornithopi* for the organism forming nodules on serradella. Later results have shown that serradella and lupine cross-inoculate. Dangeard (1926) suggested the species name, *Rhizobium minimum*, for the organism causing nodules on *Lupinus*. His characterization was based largely on the morphological features of the nodule and the organism therein.

In accordance with the rules of nomenclature, it seems that the name *Rhizobium lupini*, Schroeter, *comb. nov.* is the most appropriate for the organism capable of forming nodules on the roots of *Lupinus*. The cultural characters of this organism have not previously been adequately worked out, but most of the

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earlier workers have classified it as a relatively scanty and slow grower. In his original description, Beijerinck (1888) did not include the organism from *Lupinus* in the group characterized by "larger, more hyaline colonies." The nodule organisms were placed in two groups by Hiltner and Störmer (1903), and the lupine-nodule bacteria were placed in the group giving slow, or in certain cases no, growth on a gelatin medium. Greig-Smith (1899) observed the extremely slow growth of the lupine organisms on agar plates, and later, in 1906, their failure to form abundant slime on synthetic media, although production was abundant on plant media. More recently Bialosuknia and Klott (1923) reported slow development of the colonies of lupine organisms on agar plates, and equally slow development on agar slants. Also, Müller and Stapp (1925) included lupine organisms in a group characterized by its slow growth. Fred and Davenport (1918) described two lupine cultures as scanty growers, and three as abundant. The rapid-growing lupine cultures were later discarded from the Wisconsin stock cultures as being of doubtful purity.

In 1917, Burrill and Hansen, however, called attention to differences in the rate of growth of the nodule organisms of the different cross-inoculation groups, and described the bacteria from *Lupinus* as fast growers. Four years later, Löhnis and Hansen confirmed the earlier finding with regard to the lupine bacteria. Schönberg (1929) also characterized these organisms by the abundance and rapidity of their growth.

The significance of this disagreement becomes more apparent when the relatively wide difference between the two groups of Löhnis and Hansen is considered.

Many fast-growing cultures have been isolated from *Lupinus* in this laboratory and found capable of forming nodules. Replating of such cultures, however, has never failed to show two types of colony, one fast-growing and one slow-growing. Inoculation of plants with these two types of cultures has resulted in the formation of nodules by the slow-grower, but never by a pure culture of the fast-grower. It may be that two entirely different groups of organisms are capable of forming nodules on *Lupinus*,

but, since this has been recorded in no other case, it is highly improbable.

The present report is a study of the characteristics of eleven cultures of *Rhizobium lupini*. The source of these cultures is given in table 1.

All of the cultures had been repeatedly tested for purity and ability to form nodules. At the end of these experiments the cultures were again tested for purity and nodule-forming ability. Without exception, the organisms were found to be true to name.

Morphology. Mounts of *Rhizobium lupini* taken from a five-day yeast-water mannitol agar slant showed Gram-negative rods

TABLE 1

LABORATORY NUMBER	YEAR SECURED	SOURCE OF CULTURES	
801	1918	<i>Lupinus albus</i>	Sparta, Wisconsin
804	1927	<i>Lupinus nanus</i>	Madison, Wisconsin
805	1927	<i>Lupinus nanus</i>	Madison, Wisconsin
806	1928	<i>Lupinus albus</i>	Madison, Wisconsin
807	1928	<i>Lupinus albus</i>	Hayward, Wisconsin
808	1928	<i>Lupinus albus</i>	Madison, Wisconsin
809	1929	?	Stockholm, Sweden
810	1929	<i>Lupinus mutabilis</i>	Berlin, Germany
811	1929	<i>Lupinus albus</i>	Hancock, Wisconsin
812	1929	<i>Lupinus albus</i>	Hancock, Wisconsin
813	1929	<i>Lupinus albus</i>	Hancock, Wisconsin

varying in size from 1.1 to 3.3 micra in length and 0.1 to 0.3 micron in width. A few coccoid and a few swollen, vacuolated forms were visible.

Cultural characters (yeast-water mannitol agar).² *Rhizobium lupini* produced a scanty or moderate, white, moist, slightly raised, smooth growth on yeast-water mannitol agar. All of the cultures showed an alkaline reaction, agreeing in this character with the other slow-growers. In a medium containing brom thymol blue, or congo red, there was only a slight absorption of the dye. Although variations occurred, none of the cultures showed a rapid

² The media used in this work were prepared according to the formulae given in Fred and Waksman's Laboratory Manual of General Microbiology.

abundant growth such as is exhibited by *Rhizobium leguminosarum*, *Rhizobium trifolii*, *Rhizobium phaseoli*, and *Rhizobium meliloti*. The nature of the growth of *Rhizobium lupini* on this medium is similar to that of *Rhizobium japonicum* and of the organism causing nodules on cowpeas.

Litmus milk. The slightly alkaline reaction, without the production of a serum zone or the reduction of litmus, which was found to characterize all of the *Rhizobium lupini* cultures on this medium, should serve as a purity test for cultures of this organism. Löhnis and Hansen (1921) and Schönberg (1929) working in Löhnis' laboratory, stated that these organisms produced a serum zone on milk. Miss Schönberg suggested, as had Löhnis and Hansen (1921), that organisms isolated from plants of European origin were characterized by the production of a serum zone in milk; those from plants of American-Asiatic origin, by its absence. The inadequacy of this grouping is apparent when it is considered that, as far as is known, the organisms from all the species of one plant genus, with the exception of *Phaseolus*, will cross-inoculate, but in several genera the different species are not all native to the same continent. In the case of the genus *Lupinus*, certain species are apparently natives of Europe and others of America.

Potato and parsnip slopes. Potato slopes were found by Löhnis and Hansen (1921) to serve as an excellent differential medium to separate the root nodule bacteria from the common contaminant, *Bacillus radiobacter*. The meagre, transparent, slimy growth given by the cultures of *Rhizobium lupini* was typical of the root-nodule bacteria. Some of the cultures showed no apparent growth.

On parsnip slopes, prepared in the same manner as potatoes, the organisms gave a white or colorless, viscid, rather scant growth. Growth was very scant or entirely absent, if the parsnips were not washed for several hours.

Glycerol-phosphate solution. Growth of *Rhizobium lupini* on glycerol-phosphate medium varied considerably. Most of the cultures of *Rhizobium lupini* showed a moderate white turbidity, and two strains also formed a surface ring. Four strains produced little or no growth in this medium.

Carrot agar. Carrot agar inoculated with the cultures of *Rhizobium lupini* showed a scant, dry growth. Observation after thirty days disclosed a slight darkening of the agar by the growth of certain of the cultures. Since carrots gave a positive test for tyrosine, it was thought that the color change might be the result of bacterial tyrosinase causing the conversion of tyrosine to the black pigment melanin. The tyrosine content of carrots is variable, and the color phenomenon is not always observed on this medium.

Tyrosine agar. In 1923, Stapp tested several bacterial cultures, including some of Rhizobia for the production of tyrosinase, as indicated by the darkening of tyrosine medium. His tests with *Rhizobium lupini* were positive.

Similar tests, using both yeast-water mannitol agar and asparagin agar plus 1.5 gram of tyrosine, were made on these cultures of the lupine organism. A comparison of the growth on the two media showed that it was more profuse on the former with a slight indication of tyrosinase production with all cultures, while on the latter some of the cultures failed to give a positive test. Because of the natural color of the yeast-water substrate, the tyrosinase reaction is not as distinct as on the clear mannitol asparagin substrate.

These results confirmed the supposition that the darkening of carrot agar is due to a tyrosine-tyrosinase reaction. It is also possible that the browning of old cultures of *Rhizobium lupini* on yeast-water mannitol agar may be due to the action of the bacterial tyrosinase on tyrosine, present either in the bacterial cells or in the agar.

Beef-extract peptone gelatin. Growth of *Rhizobium lupini* on beef-extract peptone gelatin was found to be very poor. It was slightly favored by the use of Witte's peptone. Slight liquefaction by four of the strains was noted after three months. The poor growth may have been responsible for the lack of liquefaction in the other cultures. Some of the cultures showed a brownish growth, indicating the possibility of a slight tyrosine reaction. These results are in accord with the results of Hiltner and Störmer (1903) and Fred and Davenport (1918) who found that growth

of the lupine organism was poor on gelatin. Later investigators, Stapp (1924) and Müller and Stapp (1925), observed slow liquefaction by cultures of *Rhizobium lupini* after about a month's incubation.

Dye bacteriostasis. A sucrose peptone solution, plus varying amounts of crystal violet, which Stevens (1925) and Wright (1925) used in differentiating strains of alfalfa and soybean organisms, also brought out variations in these cultures of *Rhizobium lupini*. Certain strains were inhibited in concentrations as low as 1:150,000, while others showed growth at a concentration of 1:15,000.

Fermentation characters. The ability of many of the Rhizobia to utilize as an energy source various carbohydrates has received considerable attention, but little work has been done with *Rhizobium lupini*. Greig-Smith (1911) found that organisms from *Lupinus* produced rather luxuriant slimes from sucrose, glucose, fructose, maltose, and mannitol, but seemed incapable of utilizing lactose. Various investigators [Baldwin and Fred (1927), Walker (1928)] have reported differences in the fermentation characters of the root nodule bacteria. Schönberg (1929) recently made a study of the fermentation characteristics of several of the root-nodule bacteria, including *Rhizobium lupini*.³ Her lupine organisms showed no distinguishing characteristics; however, they tended to resemble the fast-growing group more than the others.

The fermentation characters of the various strains of *Rhizobium lupini* on a nitrate medium plus brom-thymol-blue indicator were studied. To avoid the breakdown of sugars in sterilization, the tubes were placed in the autoclave in small batches, sterilized at 15 pounds pressure for 15 minutes, and rapidly cooled. Careful cleansing of the tubes is important in preventing breakdown of the sugar on sterilization. Definite hydrogen-ion readings were not made, but the reaction was recorded as strongly acid, mediumly acid, or slightly acid, as strongly, mediumly, or slightly alkaline, or as no change in reaction. A representative series of pentoses, hexoses, disaccharides, and trisaccharides was studied. Obser-

³ The medium which was used is stated to be that of Baldwin and Fred (1927); however, the composition was changed to such an extent that the results on the two are not comparable.

TABLE 2
Growth and fermentation characteristics of Rhizobium lupini on a nitrate medium containing various pentose and hexose sugars

SUGARS	CULTURES											
	801	804	805	806	807	808	809	810	811	812	813	
Rhamnose:												
Growth.....	++	+	++	+	+	++	++	++	++	++	++	++
Reaction.....	++	++	++	+	+	++	++	+	++	++	++	++
Arabinose:												
Growth.....	+++	+	++	++	+	+	+	++	++	++	++	++
Reaction.....	++	++	++	++	+	+	+	++	++	++	++	++
Xylose:												
Growth.....	+	+	++	++	+	+	+	+	+	+	+	+
Reaction.....	+	+	++	++	+	+	+	+	+	+	+	+
Fructose:												
Growth.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Reaction.....	-	-	-	-	0	-	0	0	0	-	-	-
Glucose:												
Growth.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Reaction.....	++	++	++	++	--	++	--	--	--	--	--	--
Galactose:												
Growth.....	+++	+++	+++	+++	+++	+++	+	+++	++	++	++	++
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--
Mannose:												
Growth.....	+	+	++	+	++	++	+	+	++	+	+	++
Reaction.....	+	+	+	+	--	+	--	--	--	--	--	--

Growth: +, scant; ++, scant to moderate; +++ moderate.

Reaction: +++ strongly acid; ++, mediumly acid; +, slightly acid; 0, no change in reaction; -, slightly alkaline; --, mediumly alkaline; ---, strongly alkaline.

TABLE 3
Growth and fermentation characteristics of Rhizobium lupini on a nitrate medium containing various disaccharide and trisaccharide sugars

SUGARS	CULTURES												
	801	804	805	806	807	808	809	810	811	812	813		
Sucrose:													
Growth.....	++	++	++	++	++	++	++	++	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	
Lactose:													
Growth.....	++	++	+	+	+	+	+	+	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	
Maltose:													
Growth.....	++	++	++	++	++	++	++	++	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	
Trehalose:													
Growth.....	++	++	++	++	++	++	++	++	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	
Raffinose:													
Growth.....	++	++	++	++	++	++	++	++	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	
Melezitose:													
Growth.....	++	+	++	++	++	++	++	++	++	++	++	++	
Reaction.....	--	--	--	--	--	--	--	--	--	--	--	--	

Growth: +, scant; ++, scant to moderate; +++ moderate.
 Reaction: +++ strongly acid; ++, mediumly acid; +, slightly acid; 0, no change in reaction; -, slightly alkaline; --, mediumly alkaline; ---, strongly alkaline.

vations were made at various intervals, but to save space only the two-week readings are reported. In many cases, observations at the end of three or four weeks differed with respect to reaction changes from those reported, apparently depending on the ratio of acid formation to acid breakdown, and the rate of withdrawal of the nitrate or phosphate radicals.

The results of these tests are given in tables 2 and 3. Variations were observed among the cultures, in their growth on, and changes in the reaction of, the various media. *Rhizobium lupini* differed from the fast-growing group of Rhizobia in the fermentation reactions exhibited on several media. Galactose, however, served best, because of the alkaline reaction produced by *Rhizobium lupini*, to differentiate it from all of the fast-growing groups (*Rhizobium phaseoli*, *Rhizobium meliloti*, *Rhizobium trifolii*, and *Rhizobium leguminosarum*). Resembling the slow-growing *Rhizobium japonicum* and the Rhizobium from cowpeas in so many respects, *Rhizobium lupini* could, however, be separated from them by the fermentation reaction on rhamnose and xylose; the initial alkaline reaction is followed much more rapidly with an acid reaction with *Rhizobium lupini* than with the others.

Of considerable interest is the lack of correlation which exists between the fermentative character and the growth of an organism on any one sugar, especially in the disaccharide and trisaccharide groups. Fred (1912) found that growth on sugar media could be very vigorous with less than a 4 to 5 per cent destruction of the total sugar present. A comparison of the growth and reaction changes in the various sugar media with *Rhizobium lupini* is shown in tables 2 and 3. The fermentation of the sugar in the medium with the production of acid is no indication of the extent to which the organism has grown.

PLANT INOCULATION

All of the cultures of *Rhizobium lupini* were tested for nodule production and effect upon the host plant several times during the course of the laboratory experiments. The tests were made on *Lupinus albus*, *Lupinus angustifolius*, *Lupinus hirsutus* and *Lupinus luteus*.

Bacteria-free seeds were planted in sterilized white quartz sand of neutral reaction, which was almost nitrogen-free. The seed was inoculated, after planting and before covering, with a water suspension of the organisms washed from an agar slant. Uninoculated controls were distributed among the inoculated pots. They were watered with sterile water when necessary. Somner's (1928) nutrient solution, minus the potassium nitrate and the silicon salt, was used one or twice a week after the first two weeks of growth.

A representative experiment with *Lupinus albus*, the most frequently used species, is presented in table 4. Variations in plant growth, dry weights, total nitrogen, and to a certain extent in nodulation, were marked. In general, the plants with a few large

TABLE 4
Effect of various cultures of *Rhizobium lupini* on the growth of *Lupinus albus*
Eight weeks of age

	Con- trol	801	804	805	806	807	808	809	810	811	812	813
Appearance of plants...	Poor	Very good	Very good	Very good	Good	Fair	Good	Very good	Very good	Very good	Good	Fair
Grams dry weight per plant.....	0.395	0.733	0.720	0.840	0.6 62	0.613	0.690	0.786	0.726	0.820	0.619	0.478
Percentage of nitrogen.....	1.79	2.93	3.17	3.04	3.18	2.96	3.17	3.07	3.36	2.93	3.19	3.21
Number of nodules per plant.....	0	13	13	8	11	20	17	12	12	8	6	12

nodules concentrated on the tap root were the best; but the variation in nodulation was not definitely proportional to the variation in plant development. It is interesting to note that a high nitrogen percentage does not always indicate thrifty plant growth. Plants inoculated with strain 813 gave a very low dry weight, but contained a high percentage of nitrogen. Similar strain variations in respect to the benefit derived from the host plant have been carefully studied by Stevens (1925) with *Rhizobium meliloti*, by Wright (1925) with *Rhizobium japonicum*, by Helz, Baldwin and Fred (1927) with *Rhizobium leguminosarum*, and by Baldwin and Fred (1929b) with *Rhizobium trifolii*.

Variations in results also occurred on different species of lupinus. The same culture of bacteria caused the formation of round,

medium-sized nodules on the top of the lateral roots of *Lupinus albus*, while on *Lupinus angustifolius* and *Lupinus luteus*, the nodules were extremely large and usually formed a collar around the tap root near the surface of the soil. The nodules on *Lupinus hirsutus* were small and either on the tap root or the lateral roots. As noted by Kirchner (1895), *Lupinus hirsutus*, even when planted in the same inoculated pot with other species which showed nodules, often failed to show any nodulation. It was observed that cultures which gave excellent results on *Lupinus albus* often gave only fair results on other species of lupine, or *vice versa*. Hiltner, as early as 1902, noted that there is a marked difference in the behavior of the same organism on blue and yellow lupines.

SUMMARY

In accordance with the rules of nomenclature, it is proposed that the name *Rhizobium lupini* (Schroeter) *comb. nov.* be given to the organism causing the formation of nodules on *Lupinus* sp.

Rhizobium lupini is a Gram-negative organism, producing a scant to moderate growth and alkaline reaction on yeast-water mannitol agar, a slightly alkaline reaction without reduction and without the formation of a serum zone in litmus milk, a meagre growth on potato and parsnip slopes and on carrot agar. Slight production of tyrosinase by all the strains was noted on tyrosine media. Growth on beef-extract peptone gelatin, and liquefaction of this medium, sensitivity to crystal violet, and the nature of nodulation and the benefit to the host plant varied among the different strains; strain variations also occurred in fermentation characters, but the results with certain sugars were definite enough to serve as a means of separating *Rhizobium lupini* from the other root-nodule bacteria.

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