PANTOTHENIC ACID AND THE NODULE BACTERIA - LEGUME SYMBIOSIS

by

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A THESIS
submitted to the
OREGON STATE AGRICULTURAL COLLEGE

in partial fulfillment of
the requirements for the
degree of
MASTER OF SCIENCE

May 10, 1935
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PANTOTHENIC ACID AND THE NODULE BACTERIA - LEGUME SYMBIOSIS

INTRODUCTION

Since Greek and Roman times it has been known that the Leguminosae are soil improvers and are extremely valuable as green manures. The explanation of their value was, however, unknown. It was not until 1881 that a partial explanation was forthcoming; this from a German agriculturist, Schultz-Lupitz (14), who stated that leguminous plants could utilize atmospheric nitrogen in some form other than that required by non-leguminous plants.

In the early years of the 19th century many eminent chemists were investigating the sources of nitrogen available for green plants. Boussingault's (6) work in this field is classical. In 1838 he found that peas and clover grown in pots could apparently take nitrogen from the air, while wheat and oats could not; that is, he found that the leguminous plants contained more nitrogen than could be accounted for as coming from the seed, soil and water used. Atwater (3), in America, reached the same conclusions independently by similar experiments. Neither of these workers appreciated the significance of their data. Rather were they inclined to discount them as "being contrary to the best opinions and evidence of the present time".

Berthelot (5) in 1885 demonstrated that clay soils were able to fix atmospheric nitrogen, and he made the suggestion, being the first to do so, that possibly bacteria were responsible. Schloesing and Muntz (13) also performed experiments which indicated that perhaps bacteria were the responsible agents in the nitrification process.

It had been known for several years that the nodules on the roots of leguminous plants contained bodies whose nature was in dis-
Lachmann (11) in 1858 was the first to recognize them and called them "vibrio-like". Woronin (16)(1866-67) spoke of them as "bacteria-like". However, other workers were of the opinion that they were fungi.

Such was the status of the problem when Hellriegel (8) reported his great discovery on September 20th, 1886. Since his conclusions clarified the situation rather completely and form the basis of the opinions held today they are given here:

1. "The behaviour of the Leguminosae is fundamentally different from the Gramineae in regard to their nitrogenous nutrition."
2. "The Gramineae are solely dependent upon the assimilable nitrogenous compounds present in the soil, and their development always stands in direct relation to the nitrogenous supply available in the soil.
3. "To the Leguminosae a second source is available beside the nitrogen of the soil, from which they are able in a highly efficient manner to cover their needs entirely or in part, in case the first source is insufficient.
4. "The free elemental nitrogen of the air is this second source.
5. "The Leguminosae do not themselves possess the ability to assimilate the free nitrogen of the air, but the active participation of living microorganisms in the soil is absolutely necessary.
6. "In order to make the free nitrogen of the air serviceable to the Leguminosae for the purpose of nourishment, the mere presence of lower organisms in the soil is not sufficient, but it is necessary that certain kinds of the latter enter into a symbiotic relationship with the former.
7. "The root nodules of the Leguminosae are not to be considered merely as reserve storehouses for albuminous material, but stand in a causal relationship to the assimilation of free nitrogen."

Beijerinck (4) at this time contributed to the elucidation of the problem by isolating the bacteria from the root nodules and growing them in the laboratory. Ward (15) completed this work by demonstrating conclusively that nodule formation was caused by bacterial inoculation.

Having investigated the main aspects of the peculiar prop-
erty of the Leguminosae, namely, the ability to fix atmospheric nitrogen through the cooperation of the nodule bacteria, the workers in the field turned their attention to an examination of the mechanism of the nitrogen fixing process. The accepted opinion today is expressed by Fred (7), who states, "The fact that leguminous plants are unable to assimilate atmospheric nitrogen in the absence of nodule bacteria would seem to indicate that the bacteria are the active agents responsible for this process." The mechanism of fixation is unknown. It is, however, thought that the bacteria fix the nitrogen in the nodules and that some compound of nitrogen is then utilized or assimilated by the plant.

It is reasonable to expect that, if the bacteria in the host plant fix nitrogen in the nodules, they should fix nitrogen in the laboratory when grown in suitable media. Such is not the case. There is no creditable evidence available that indicates nitrogen fixation by bacteria apart from the host plant. On the contrary the most reliable data demonstrate no nitrogen fixation whatever when the bacteria are cultured in the laboratory. Allison (1) in 1929 published an extensive study of nitrogen fixation by nodule bacteria, using a wide variety of media. He states, "The experiments in no case gave any evidence that rhizobia can fix atmospheric nitrogen when grown apart from the host." Hopkins (9) and Löhnis (12) reached the same conclusions, namely, that there is no evidence of the nodule bacteria alone being able to fix atmospheric nitrogen. None of these investigators are willing to take the results as final. They evidently believe that sometime a new medium or a new set of experimental conditions will be obtained which will enable the bacteria to fix nitrogen apart from the host.

So in summarizing these various results, we may say that the attitude today is: (1) that the rhizobia can, under the special con-
ditions prevailing in the nodules of the host plant, fix atmospheric nitrogen and in some manner present it to the plant in a readily utilized form; and (2) that the principal benefit accruing to the plant from the symbiosis is the utilization of atmospheric nitrogen.

Allison (2) and other workers have, in studies on the rhizobia, succeeded in growing them on a synthetic medium consisting of commercial sucrose, calcium sulfate, potassium nitrate, magnesium sulfate, mono- and di-potassium phosphate, sodium chloride, and ferric sulfate. When they attempted to grow the bacteria in the same medium using highly purified sucrose they were unsuccessful. Investigation led to the discovery of a chemical compound, or a mixture of compounds, of unknown composition associated with the commercial sucrose, which is indispensable to the growth of the rhizobia. While the unknown substance has been found to occur widely in nature, being found in many diverse sources such as liver, kale and malt extract, it is most abundant in leguminous plants. With this evidence the mechanism of the symbiosis is clarified a bit, for it is seen that the bacteria pass some chemical entities to the plant, which in turn supplies, among other things, the indispensable growth substance to the bacteria.

INVESTIGATION OF THE RELATIONSHIP BETWEEN THE RHIZOBIUM AND THE HOST PLANT.

The demonstrated fact that rhizobia, even though grown in the most favorable media which should contain all the food materials and growth promoting substances found in the plant, do not fix nitrogen, made it appear to the writer that the accepted view of their beneficial action presented above was improbable. Recent work done in this laboratory by Williams and Rohrman (19) indicated that "pantothenic acid"
(17) stimulates green plants. Further evidence as to the stimulatory power of pantothenic acid was obtained from the work of Robbins on excised corn root tips, decided positive results being obtained. These facts, coupled with the experimental evidence obtained by the writer (see Experimental Part) showing that the organism *Rhizobium meliloti*, in common with several other bacteria and molds, synthesizes and excretes pantothenic acid as it grows, initiated this research. The following hypothesis, tentatively explaining the role of pantothenic acid in the symbiosis, was formulated to explain the above mentioned facts:

1. **Leguminous plants** are inherently capable, under suitable conditions, of fixing atmospheric nitrogen.

2. The function of the nodule bacteria is not nitrogen fixation, but production of an indispensable chemical compound, which is passed on to the host plant, enabling it to carry out the nitrogen fixation process.

3. "Pantothenic acid" is the chemical compound, furnished the plant by the bacteria, which enables the plant to utilize atmospheric nitrogen.

**EXPERIMENTAL PART.**

In testing the ability of *Rhizobium meliloti* to synthesize pantothenic acid the following procedure was used: 25 c.c. of Allison's medium were placed in each of four 125 c.c. erlenmeyer flasks, which were then plugged with cotton and sterilized. These were each seeded with 0.3 c.c. of a uniform suspension of the organism grown at 30 degrees C. for 24 hours in the same medium. They were then placed in the 30-degree incubator, and one flask was sterilized each successive 24 hours. The medium was filtered free of suspended cells
by means of Kieselguhr, and a 2 c.c. portion of the filtrate was tested for its pantothenic acid content in the usual manner (18).

In testing the effect of pantothenic acid on alfalfa the following procedure was used: twelve Pyrex test tubes (2½" x 24") were used for growing three sets of cultures in quadruplicate. 500 gms. of white quartz sand and 150 c.c. of Crone's solution were placed in each tube, which was then plugged with cotton and sterilized at 15 pounds pressure for 6 hours. Four tubes served as controls, with nothing added except the Crone's solution, four were used for inoculated plants, and four for pantothenic acid treated plants. Alfalfa seeds were sterilized in the usual manner with mercuric chloride and sprouted in sterile Petri dishes. To each tube were added under aseptic conditions 6-8 uniformly sized sprouts. The inoculated sprouts were first dipped in a heavy suspension of *Rhizobium meliloti*. Two micrograms (0.000002 gms.) of a pantothenic acid preparation were added to each of the four pantothenic acid tubes. The tubes were then removed to the greenhouse. Ten days later 10 micrograms of pantothenic acid were added to each of the four pantothenic acid tubes. The plants were allowed to grow for one month, when they were removed from the tubes and washed free from the adhering sand particles. The length of each plant was then measured in millimeters, measuring from the first pair of leaves to the longest tip of the plant. The plants were then air dried to constant weight. The roots were then cut off, and the total weight of roots and of stems was determined for each tube. After this had been done, the total nitrogen of the plants in each tube was determined by the modified method of Koch and McMeekin (10). In order to determine the amount of nitrogen fixed, if any, the nitrogen content of alfalfa seeds was determined by the same method.
Table I

Production of Pantothenic Acid by *Rhizobium meliloti*.

<table>
<thead>
<tr>
<th>Flask</th>
<th>Time</th>
<th>Units/flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>24 hr</td>
<td>0.39</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>0.78</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>1.63</td>
</tr>
<tr>
<td>4</td>
<td>96</td>
<td>5.46</td>
</tr>
</tbody>
</table>
### Table II

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Numbers of plants</th>
<th>Ave. length of stems (mm)</th>
<th>Total wt. of roots (mg)</th>
<th>Total wt. of stems (mg)</th>
<th>Total wt. of whole plants (mg)</th>
<th>Total N (mg)</th>
<th>N/plant (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>30</td>
<td>9.5</td>
<td>19.5</td>
<td>29.0</td>
<td>0.882</td>
<td>0.146</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>34</td>
<td>12.5</td>
<td>14.5</td>
<td>27.0</td>
<td>0.750</td>
<td>0.125</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>39</td>
<td>14.5</td>
<td>27.5</td>
<td>42.0</td>
<td>1.382</td>
<td>0.173</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>20</td>
<td>7.5</td>
<td>12.5</td>
<td>19.5</td>
<td>0.790</td>
<td>0.158</td>
</tr>
<tr>
<td><strong>Inoculated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>50</td>
<td>7.5</td>
<td>22.5</td>
<td>29.5</td>
<td>0.832</td>
<td>0.208</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>54</td>
<td>12.5</td>
<td>31.0</td>
<td>43.0</td>
<td>1.304</td>
<td>0.208</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>36</td>
<td>8.5</td>
<td>19.5</td>
<td>28.0</td>
<td>0.864</td>
<td>0.173</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>41</td>
<td>8.5</td>
<td>16.0</td>
<td>24.5</td>
<td>0.722</td>
<td>0.180</td>
</tr>
<tr>
<td><strong>Pantothenic acid treated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>50</td>
<td>19.5</td>
<td>35.0</td>
<td>54.0</td>
<td>1.030</td>
<td>0.172</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>55</td>
<td>20.5</td>
<td>35.5</td>
<td>55.5</td>
<td>1.030</td>
<td>0.172</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>53</td>
<td>13.5</td>
<td>33.5</td>
<td>47.0</td>
<td>1.076</td>
<td>0.178</td>
</tr>
<tr>
<td>12</td>
<td>6</td>
<td>47</td>
<td>10.5</td>
<td>27.0</td>
<td>37.0</td>
<td>0.930</td>
<td>0.153</td>
</tr>
</tbody>
</table>

* All plants under 7 mm. were discarded.*
Table III

Grand average of values from Table II.

<table>
<thead>
<tr>
<th>Length/plant</th>
<th>wt/plant</th>
<th>Total N/plant</th>
<th>% N/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>31 mm</td>
<td>4.70 mg</td>
<td>0.152</td>
</tr>
<tr>
<td>Inoculated</td>
<td>45 mm</td>
<td>7.10 mg</td>
<td>0.196</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>51 mm</td>
<td>8.05 mg</td>
<td>0.169</td>
</tr>
</tbody>
</table>
DISCUSSION OF RESULTS:

Heretofore the favorable effects resulting from inoculating alfalfa and other legumes have been explained solely by the nitrogen fixation theory. On the basis of our results this position is no longer tenable. Perusal of Tables II and III shows that minute amounts of pantothenic acid exert a striking effect on alfalfa, and, since it has also been shown (Table I) that Rhizobium meliloti produces significant amounts of pantothenic acid, it is therefore likely that a considerable part of the stimulatory effect is due to the pantothenic acid synthesized and excreted by the organism in the nodule and passed on to the host plant.

The nitrogen content of the pantothenic acid treated plants, while not as great as that of the inoculated plants, is greater than that of the controls. From this, and the fact that the weight of the pantothenic acid treated plants is greater than that of the controls and inoculated plants, it may be inferred that pantothenic acid alone is not the deciding factor in the nitrogen fixation process, but rather that it plays an important part in the carbohydrate anabolism of the plant. This is in keeping with the fact that it has been shown to exert a powerful effect on both the aerobic and anaerobic respiration of yeast (20).

It is possible that another factor (or factors) elaborated by the bacteria, when supplemented by pantothenic acid, enables the plant to grow vigorously and carry on the nitrogen fixation process. The fact that the controls contained more nitrogen than that present in the seed might be taken, provided that no ammonia was absorbed from the air, as an indication that the plants themselves possess to a limited degree the ability to utilize atmospheric nitrogen. However,
the point is to be considered in a new experiment to be undertaken shortly. The writer believes that while the hypothesis advanced above is not completely true, pantothenic acid does play a supplementary part in the process, and undoubtedly a decisive role in the carbohydrate anabolism of the plant.

DISCUSSION OF AN EXPERIMENT NOW IN PROGRESS.

If the true function of the nodule bacteria in the nodule bacteria-legume symbiosis is the elaboration of stimulating substances which enable the plant to thrive and utilize atmospheric nitrogen, then it should be possible to obtain this effect under controlled conditions in the absence of the living organisms. By growing the bacteria in suitable quantities in liquid medium, from which they can be filtered and washed, it should be possible to prepare an extract of the bacterial cells which will contain any stimulating substances synthesized by them. This procedure is now being carried out. The plants will be treated as in the first experiment; that is, the technique of the experiment will be the same, using an extract prepared from the bacterial cells. This experiment, which will also contain pantothenic acid treated plants, will be allowed to continue for a longer period than the first experiment.

CONCLUSIONS.

(1) A large part of the increased growth of alfalfa caused by Rhizobium meliloti is due to pantothenic acid, shown to be synthesized and excreted by this organism.

(2) The total beneficial effects of Rhizobium meliloti can no longer be interpreted as due to its ability to fix atmospheric nitrogen
when existing in symbiosis with the alfalfa plant.

(3) Pantothenic acid plays an important part in the carbohydrate anabolism of the alfalfa plant, and a secondary or supplementary part in the process of nitrogen fixation.
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