Microbiology. — "*The significance of the tubercle bacteria of the Papilionaceae for the host plant*". By Prof. BEIJERINCK.

(Communicated in the meeting of April 26, 1918).

As there is no reason to doubt of the accuracy of HELLRIEGEL's [1]) experiments, it appears certain that the bacteria of the nodules on the roots of the Leguminosae are indispensable for the fixation of atmospheric nitrogen by these plants. [2]) But I shall prove that the theory, at present generally adopted, according to which this process takes place only within the tubercles, cannot be correct. But previously some remarks on the occurrence of the tubercles and the cultivation of bacteria from them.

For some plant species such as serradella (*Ornithopus sativus*) and the yellow lupine (*Lupinus luteus*), it cannot be doubted that only the tubercle-bearing specimens grow vigorously in nitrogen-poor soils and consequently, after the theory, fix the atmospheric nitrogen. It is therefore easy on poor heath fields to find languishing, stunted lupine plants, always devoid of nodules, amid the luxuriantly growing tubercle-bearing ones. Never did I find there well-developed lupine or serradella plants quite without them. But the number of tubercles is of no consequence, it evidently suffices if only few come to development..

In garden experiments on open sandbeds, without supply of nitrogen, but where inevitably more nitrogen compounds occur than in heath soils, also in peas and beans (*Vicia faba*), plants with nodules grow better than those devoid of them.

In fertile garden soil such as in the laboratory garden at Delft, yellow lupine and serradella do not fully develop, and especially their roots make the impression of sickliness; tubercles do not grow on them, not even when the soil has been abundantly provided

[1]) H. HELLRIEGEL und H. WILFARTH, Untersuchungen über die Stickstoffnahrung der Gramineën und Leguminosen, Zeitschrift für Rübenzuckerindustrie, Beilageheft November 1888. See further the excellent treatise of HILTNER, Bindung von freiem Stickstoff in höheren Pflanzen, in Handbuch der technischen Mykologie, Bd. 3, 1903—1905.

[2]) For the objective proof that here free atmospheric nitrogen is fixed see, besides HELLRIEGEL (l. c. p. 191), SCHLÖSING et LAURENT, Fixation de l'azote libre par les plantes, Ann. de l'Institut Pasteur, Tome 6, pag. 65, 1892.

with the concerned bacteria. Whether the latter die in the soil or are not attracted by the roots of the plant is not yet clear. Most other leguminous plants, such as clover, *Vicia*, peas and *Vicia faba*, bear also in fertile soil many nodules, and it is not easy to find specimens wholly devoid of them, unless the soil has been previously sterilised.

On the roots of *Genista anglica* and *Genista pilosa*, growing on poor heath fields, I found after long seeking only very few tubercles, although they and in particular the former, bore many pods with good seeds; the tubercles are, however, never quite absent. When sown in my garden at Delft or brought there as plants, they die after some few years. On the other hand, *Genista tinctoria* thrives as well at Delft as along the highway of Zutphen to Vorden and at both places bears a small number of nodules.

For *Robinia pseudo-acacia* the favourable influence of *B. radicicola* only on the young plant, has been stated by NOBBE. [1]) As to full-grown specimens on poor heath soil at Gorssel I could after long digging find but few tubercles, while at a small distance, but on somewhat better soil more tubercles occurred, but still so little numerous, that nobody would attribute to them any direct significance for such a large tree, had not the fixation of nitrogen in the tubercles become an inveterate belief. *Sarothamnus vulgaris* and *Ulex europaeus* behave in the same way as *Robinia*. On *Phaseolus vulgaris* on sandy soils I found but few nodules, and then only on thin rootlets and nearly always enclosed by plant remains; in the pure sand the nodules are very rare. In garden soil at Delft *Phaseolus* produces no nodules, but it does in a there arranged sandbed; *Lupinus luteus* and *Serradella* behave likewise.

When comparing the various mentioned plants, all noted in agriculture for their power of ameliorating the soil, as they contain in their dry substance nearly double the quantity of nitrogen found in other plants, for example the grasses, we come to the conclusion that only for lupine and serradella the number and weight of the tubercles is of some significance in regard to the whole weight of the plant. For other species they are of so little volume that even if within them free nitrogen were fixed with great intensity, only an extremely little quantity of fixed nitrogen could be expected, whilst in reality this amount is very considerable. Hence the theory, at present generally accepted, after which the fixation takes place in the

[1]) HILTNER l.c. Also BÜSGEN, Bau und Leben unserer Waldbäume, 2te Aufl., Pag 246, 1917.

nodules only, requires reconsideration. Also other experiences make this reconsideration necessary. But previously a few remarks on the isolation of the bacteria from the nodules and from other materials, and on the question of their specificity.

A very convenient medium for isolation was already described in 1888, [1]) namely pea leaves- or clover-extract-gelatin with 2 % cane sugar. *B. radicicola* grows thereon in soft, white, non-liquefying colonies, while *B. ornithopodis* from *Ornithopus perpusillus*, *O. sativus* or *Lupinus luteus*, when isolated in the autumn or in March, liquefy somewhat, as does *B. herbicola*. [2])

As a solid medium, poor in nitrogen compounds, I recommend a plate of: Tapwater 100, agar 2, cane sugar 1, starch 1, bipotassiumphosphate 0,05, in which, because of the albuminous matter of the agar, enough fixed nitrogen is present to cause a distinct growth of *B. radicicola*, but the colonies remain small. Later a little saltpetre or ammoniumsulphate may be added locally, which makes the tubercle bacteria like the other saprophytes thrive well, showing that they do not assimilate the free atmospheric nitrogen. If on such a plate eventually germs of *Azotobacter*, which is able to assimilate free atmospheric nitrogen, are present, these will grow quite well if no nitrogen compounds are added. Such nitrogen-poor plates are also useful to recognise the spore-bearing soil bacteria, which almost constantly appear at the isolation of *B. radicicola*.

I only call tubercle bacteria those species which develop mutually identic colonies by thousands or hundreds of thousands from the externally well-sterilised and cautiously crushed nodules. These bacteria derive for the greater part from within the cells. I consider the deviating and less numerous colonies obtained at the culture experiments as the product of germs accidentally present in the intercellular cavities of the rind of the nodules. [3]) That the full-grown bacteroïds cannot develop on the plates is well-known; hence bacteria may be expected from the tubercles only in the beginning of their development.

It is an important and until now not yet sufficiently investigated circumstance that from the tubercles of the same plant not always the same bacteria are obtained. So I found for *Ornithopus perpusillus*

[1]) Botan. Zeitung. 1888 Pag. 764.

[2]) Occasionally a great number of colonies of *B. herbicola* are obtained from the tubercles; whoever is unacquainted with this species may make mistakes in the isolation of *B. radicicola*. But even with this knowledge the isolation of serradella- and lupine-bacteria is difficult. Good descriptions of these forms do not exist.

[3]) Besides *B. radicicola* *B. herbicola* can also occur *within* the living cells.

the bacteria I had isolated in March different from those grown in October, whilst the tubercles came from plants growing side by side and being in the same state of development. With the yellow lupine and serradella I had similar results. In most other cases, however, for example with *Pisum*, *Lathyrus*, *Vicia*, and *Trifolium*, the similarity of the various mutually independently isolated stocks is so complete and the image of *B. radicicola* can so distinctly be recognised, that the above observation requires nearer confirmation. But we cannot now enter upon this point.

When trying to isolate *B. radicicola* from materials other than the nodules, for example from the soil and from the dying surface cell-layers of the root, it proves very difficult to recognise this species amid the numerous other saprophytes, especially when the number of the germs of the different species is to be determined quantitatively. *B. fluorescens liquefaciens* causes much trouble by the liquefying of the gelatin plates, and yet it is necessary to use these plates as on them the colonies of all the species lie free from one another, while on agar they are overgrown and rendered unrecognisable by *B. fluorescens*, which extends strongly sideways.

Concerning the question if only one or more species of tubercle bacteria exist the following.

Already in 1892 experiments thereabout were made by the late HELLRIEGEL [1]) in the experimental station at Bernburg with pure cultures of the bacteria made by myself at Delft. Of his results HELLRIEGEL sent me two reports. In the first, dated 24 July 1892, he gives as "Augenblickliches Hauptresultat: "Es gelingt mit den Reinkulturen von *B. radicicola* var. *Pisi* oder von *Vicia faba*, die Erbsen und Bohnen, und mit denen des *Bac. radic.* var. *Lupin.* oder *Ornithopodis* Lupinen und Serradella erfolgreich zu infiziren und zum Wachstum resp. der Assimilation des freien Stickstoffs zu bringen, und das ist was unsere anfängliche Behauptung bestätigt". Already earlier HELLRIEGEL had arrived at the conclusion that the bacteria of *Lupinus* and *Ornithopus* belong to a species different from that of *Pisum* and *Vicia*, which was also my own opinion.

In later years many interesting experiments were made in this direction, especially by HILTNER. Yet the evidence is unsatisfactory as it proved hitherto impossible in the sand cultures [2]) to bring Leguminosae to complete development by infection with

[1]) He died 24 September 1895 of a stomach disease and was already suffering when I visited him at Bernburg in 1892.

[2]) It is a well-known fact that the Papilionaceae, when cultivated in liquids, do not fix the atmospheric nitrogen indifferently whether they produce tubercles or not.

B. radicicola only and with exclusion of all other microbes. Such cultures are always at the end of the vegetation period rich in various other species, in particular in *B. fluorescens liquefaciens* and the nitrogen-fixing spore-forming *Granulobacter* (*Clostridium*) *pasteurianem* and *Helobacter cellulosae.* This observation holds good as well for the first experiments made by myself as for those of others, and this should never be lost sight of when reading the descriptions of the infection experiments with the so-called "pure cultures". It had not escaped HELLRIEGEL's attention, and we see it in all the photographs of his above mentioned treatise at the film of the glass vessels, wherein he cultivated his plants (in bright daylight), which film consisted of Chlorophyceae and various other species of microbes, but he thought it of no consequence (l. c. p. 169). For myself I have observed in nitrogen-free sand, besides the mentioned species, *Chlorella* and *Cystococcus* and sometimes also *Palmella cruenta* and many Cyanophyceae. Many of my later efforts to bring clover plants to complete growth on agar with nutrient salts and *B. radicicola* in large cotton-plugged ERLENMEYER-flasks, failed as the plants ceased to grow before they blossomed, although the nodules developed very well.

The tubercle bacteria do not fix the atmospheric nitrogen when cultivated in nutrient media.

I will now call attention to my chief subject namely the want of power of the tubercle bacteria to fix the free atmospheric nitrogen. They do this neither when cultivated out of the plant nor within the nodules.

Regarding the first point the experiment is very simple. We have but to crush the nodules and bring the thus obtained material into culture soils used for the ordinary experiments to fix free nitrogen and then cultivate at 20° to 30° C.; or we use the pure cultures for infection of the same media. A convenient medium is: Tapwater 100, Glucose 2, Dikaliumphosphate 0,05, lime 2, fresh garden soil 2. This liquid, to which the garden soil is added as a catalyst, must previously be sterilised to kill the germs of *Azotobacter*, *Granulobacter* and *Helobacter*; notwithstanding the sterilisation, the soil preserves its catalytic power very little impaired. The spores of the nitrogen-fixing *Helobacter* and *Granulobacter* often adhere to the nodules and, when present, fermentation phenomena show that the experiments cannot be relied upon, *B. radicicola* not causing fermentation. Commonly, however, these fermenting and nitrogen-

fixing microbes can be removed by thoroughly washing of the nodules with alcohol and water. In the course of many years I have experimented in this way with numerous species of tubercle bacteria, and with many modifications in the nutrient media as well in the temperature as in the source of carbon. Moreover I have, as said, tried to grow pure cultures of the bacteria themselves in the liquid culture medium as also on solid culture soils of various compositions, and at first I thought I had observed a rather considerable increase of these organisms. This increase, however, proved to be really very slight, so slight that gain of atmospheric nitrogen is not proved, whilst the obvious augmentation of dry weight of the sown bacteria derives from the formation of thick slime walls, that is of nitrogen-free, cellulose-like substances around the hardly augmented original protoplasmic material. [1])

Only when cultivating the microbes in plant extracts with cane sugar, wherein nitrogen compounds are evidently present, I could observe a very slight and by no means convincing increase of the total nitrogen rate of the liquid in consequence of the growth of *B. radicicola*. But when performing these experiments I was not yet acquainted with the circumstance that laboratory air contains sufficient carbon and nitrogen compounds to be made perceptible by the growth of microbes which can feed on them. This was afterwards demonstrated by Ir. A. VAN DELDEN and myself in our investigation on *Bacillus (Actinobacillus) oligocarbophilus.* [2])

There exists moreover an aërobic spore-producing bacterium [3]), hard to kill by sterilisation of the nutrient liquids, which fixes free nitrogen; at that time it was still quite unknown to me and even now it is very imperfectly understood. It may have been present at my experiments likewise as at those of other investigators who think they have observed fixation of free nitrogen out of the plant in the pure cultures of *B. radicicola*.

With sufficient precautions the results of such experiments are however always the same: The bacteria of the nodules do in no way fix the free atmospheric nitrogen. When the experiments are performed, not with

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[1]) The slime formation is of importance for the explanation of the "slime threads" (erroneously called "infection threads") within the nodules. See "Die Natur der Fäden der Papilionaceënknöllchen." Centralbl für Bakteriologie. Bd. 15, pag. 928, 1894.

[2]) Ueber eine farblose Bakterie deren Kohlenstoffnahrung aus der atmosferischen Luft herrührt. Centralbl. f. Bakteriologie 2te Abt. Bd. 10, pag. 33, 1903.

[3]) *Bacillus danicus*, T. WESTERMANN and F. LÖHNIS, Centralbl. f. Bakteriologie, 2te Abt. Bd. 22, pag. 250, 1909).

nutrient liquids, but with a solid medium, the results are quite the same: fixation of nitrogen does not take place then either. Stress must be laid on the latter fact as it seems impossible to fix free nitrogen by the Papilionaceae when cultivated in liquid media even under the best circumstances and whether tubercles are produced or not. So it seems probable that for this process a direct contact with the air is necessary, which cannot be realised in the liquid culture media, but very well in solid ones.

Further it must be observed that the plate cultures of some of the nodule organisms,[1]) for example the forms from *Pisum*, *Vicia*, and *Trifolium*, on glucose-agar-potassiumphosphate plates, in absence of purposely added nitrogen compounds, at superficial view make the impression of being quite able to develop, but here too, it is only the formation of much wall substance, as already described above, and not of nitrogen-rich protoplasm, which explains the voluminosity of the colonies.[2]) With other slime-producing bacteria, as *B. radiobacter* and *Aerobacter viscosum*, of which it is quite certain that they cannot live on the atmospheric nitrogen, extensive colonies may likewise be grown on the said nitrogen-poor medium with fit carbon food. By a better nitrogen nutrition such colonies may even be greatly reduced in volume, the wall substance then serving as food under a strong increase of the bacterial protoplasm, which gives rise to very interesting experiments. It is only when being acquainted with these facts by personal observation that we can understand how in the literature so many statements can occur on the nitrogen fixation by the nodule bacteria, which does not take place.

Within the nodules the atmospheric nitrogen is neither fixed.

The preceding gives rise to the question, whether the protoplasm of the host plant might be the catalyst that enables the invading bacteria, in their bacteroidal state, to fix the free nitrogen. However improbable this hypothesis may appear, being in contradiction with the laws of heredity, still it deserves attention because the rate of

[1]) The wonderful "experiments" of Mazé (Annales de l'Institut Pasteur T. 11, pag. 44, 1897, T. 12, pag. 1 and pag, 128, 1898), who asserts that on broth gelatin plates at the same time ammoniumcarbonate is produced and fixation of free nitrogen by *B. radicicola* takes place, need not be considered, although they are taken up uncritisised in the handbooks of Plantphysiology.

[2]) Likewise for the ordinary saprophytic bacteria the want of nitrogen compounds varies very much: the large-celled *Bacillus megatherium* requires very little, the small celled *Bacterium fluorescens* very much.

albuminous matter in the nodules is so very high. I myself found about 4 % nitrogen, which is about 25 % albumen in the dry matter of pease-nodules. Others found 5 to 6 % nitrogen. It is noteworthy that the bacterial colonies on agar plates, grown out of the plant, contain but 1 to 2 % nitrogen of the dry weight, which consists for the greater part of carbohydrates. So it is certain that the bacterial body is very much modified by its entrance into the plant cell as well morphologically as physiologically. Therefore it was tried gazometrically to state nitrogen absorption in the tubercles. If the hypothesis is founded it must be possible, with a great quantity of tubercles in a closed space and under favourable physiological conditions, easily to observe that absorption. For the number of tubercles, for example of the woody papilionaceae, being as said very small, while yet these plants are noted in agriculture for their considerable nitrogen-fixing power, the action of the tubercles must necessarily be very intense.

To test the hypothesis we acted as follows. [1]) First small, later larger quantities of lupine and serradella tubercles were placed in wide glass tubes which could readily be connected with the gas burettes, then put in thermostats at about 25° C. The tubercles respiring vigorously we had to keep account with a rapid assimilation and supply of the oxygen. Further it was only necessary to determine the quantity of nitrogen still present after deduction of the carbonic acid and the oxygen. The only difficulty we met with was that the nodules, which by their abundant content of albuminous matter are an excellent food for bacteria, when they touch each other and get moist, easily give rise to fermentations in particular by *Bacterium aërogenes*. Hereby hydrogen and much carbonic acid are produced, so that it is then necessary also to determine the hydrogen. But this fermentation may be prevented by introducing the material very loosely into the burette, so that there are but few points of contact between the nodules, and the air can freely pass between. Under such conditions there is no danger that free nitrogen will be formed; this only occurring through the action of the denitrifying bacteria on nitrates, which substance is in the nodules completely absent.

Of the tubercles of yellow lupine we used in our experiments quantities of 100 grs., 500 grs., and later even of 1 kil. In some

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[1]) In some of these experiments I was assisted by Ir. D. C. J MINKMAN, formerly assistant to the Laboratory for Microbiology of the Technical High School at Delft.

experiments we had the root tubercles cut off, in others the roots with the tubercles were left united with large pieces of the stem, so that eventually formed nitrogen compounds might be able to flow into the stem. All our estimations, however, showed that not in a single case the slightest fixation of nitrogen by the tubercles was observable. As at first we doubted of the accuracy of our results obtained with relatively little material, we afterwards used the just mentioned larger quantities, but this did not make any difference either. Besides the two said species we still examined several times 10 to 20 grs. of the nodules of *Vicia faba*, and once about 15 grs. nodules of *Robinia pseudo-acacia*, but other results were not obtained.

As our researches did not last longer than 12 to 20 days it might be objected that we have not sufficiently imitated the conditions of the plants in the field. Further, that in these experiments the growth of the tubercles, together with that of the whole plant, was excluded. Although these objections have not been refuted in the preceding, it is still highly improbable that nitrogen fixation would be associated with the growth of the tubercles and not with the augmentation of the bacteria out of the plant. Principal, however, is the fact that if within the nodules nitrogen fixation were to take place, which might have escaped our attention, the concerned quantity must certainly be extremely small. When we now consider how difficult it is to collect a few grams of tubercles for example of *Robinia*, it is clear that if this material is to be of any significance for such a great tree, its nitrogen-fixing power must be enormous. The experiments, however, show that the tubercles are wholly inactive or nearly so, hence there can be no question of attaching to them any importance concerning the nitrogen nutrition, whilst yet nitrogen fixation by this tree is as certain as for lupine and serradella and even on a much larger scale. So the nitrogen nutrition of the Papilionaceae can only be indirectly connected with the bacteria of the nodules. In my opinion this relation can only exist in the herbaceous species and in the germ plants of the shrubs and trees of that plant order, but in full-grown specimens of the woody species such as *Robinia pseudo-acacia* the presence or the absence of the nodules is wholly indifferent. Likewise on the roots of shrubs, such as *Sarothamnus vulgaris*, *Spartium scoparium*, *Genista anglica*, and *Genista pilosa* in full-grown condition, the number of tubercles is so small, their volume so insignificant to that of the whole plant, that even if they were able to assimilate some free nitrogen their slight activity could not possibly explain the rich nitrogen store of the whole plant.

Hence, the at present generally accepted explanation of the peculiar

behaviour of the Papilionaceae cannot be correct. New researches, especially with *Phaseolus*, are desirable.

From the preceding follows:

For various Papilionaceae, excelling by their abundance of nitrogen compounds, even when cultivated in media without such compounds, the number and volume of the tubercles is so small, that if only within them the fixation of free nitrogen should take place, the intensity of the process in these tubercles must necessarily be very great. We have not, however, succeeded gazometrically in observing the process in the tubercles at all.

Neither do the tubercle bacteria fix the atmospheric nitrogen when cultivated out of the plant in nutrient liquids or in plate cultures, nor enclosed in solid media.

The contradictory statements in the hand books of Plantphysiology are erroneous.