

BACTERIA PRODUCING TUBERCLES ON LEGUMINOUS PLANTS.

THESIS.

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Bacteria are very beneficial organisms to the agriculturist especially certain parts of them. Bacteria are small living organisms that live as parasites on organic material, dead or alive. They are composed of a single cell having a nucleus, cytoplasm, and a cell wall. The organisms are so minute in size that they cannot be seen without the aid of a very powerful microscope. Their size varies considerably with the different species.

The principal legumes to be spoken of in this work are alfalfa, peas, beans, clover and lupines. The legumes in general are such plants that have a seed vessel of two valves, which are fixed to one suture only. In some localities where attempts have been made to grow such legumes as alfalfa, peas, beans, etc., the results have shown very little success, the plants having made but very slow growth and their color being pale and sickly. Many a farmer has given up saying that these crops would not grow in his locality, not knowing the reason why. This fact has led to experimentation upon the growth of these plants, both with and without tubercles. In a bulletin on "Alfalfa in New York" J. L. Stone gives a discussion on this subject. In speaking of the successful growth of alfalfa in New York on sandy soils which are frequently poor in mineral plant food, he speaks of inoculating the soil with alfalfa bacteria. This inoculat-

ing consists in securing the bacteria (which will be discussed later) of the tubercles in some form and distributing them over the field. A simple means is to take soil from a field where the tubercles grow and distribute it in the field to be inoculated. In those places of New York where alfalfa has been grown successfully, artificial inoculation was unnecessary. Hopkins, of whom the bulletin says nothing more, has shown that the bacteria working on the roots of the weed known as 'sweet' or Bokhora clover are identical with alfalfa bacteria. The observation of the Experiment Station of New York confirms this view; for in localities where this weed does not grow, inoculation takes place very slowly if at all, while in localities where the sweet clover does grow, inoculation takes place very rapidly. The appearance of the field will show to some extent if inoculation, either natural or artificial, has taken place. A healthy green growth is usually inoculated, while a pale sickly growth is not. In the experience of the station, about one hundred pounds of the soil from a field that has been inoculated, if scattered over a new field, brings about inoculation. Preferably the material should be worked in with the soil of the new field. The bulletin mentions that the United States Department of Agriculture, Washington, D. C. has succeeded in separating and propagating the active form of bacteria of various legumes, and putting them up in convenient forms for transportation to any part of the country. The instructions for the use of the materials in each package are simple and easily carried out. In the experience of the

department this mode of artificial inoculation has been very successful.

The tubercles are quite prominent lateral outgrowths of the roots of the genera leguminosae. They generally appear on the smaller roots and root hairs. They vary in length from four millimeters to seven millimeters, and are either simple or forked di- or tri-chotomously. As a rule they stand nearly at right angles to the root, however no law governs their position. On some plants they are crowded on the roots near the union with the stem, while on others they are widely distributed over the root system. In my experience in collecting the tubercles, which has been mostly from alfalfa, I have found them at a depth of from two to twelve inches. However, tubercles have been found as deep as three or four feet. When young and growing rapidly the tubercles are of a flesh color, but when they have attained some size and age the older portion presents a light brown color.

In an article found in the "Botanical Gazette", Prof. Geo. T. Atkinson gives a brief review of earlier investigations of the causes of the tubercles on leguminous plants, which he calls a "Historical Resume". He divides it into three periods of investigation, viz: Early period of investigation, middle period of investigation and recent period of investigation. The different investigations of the subject during these three periods each produced theories, and no two seemed to agree. The principal investigators of the earlier period were Bivona and Jessen;

of the middle period were Erickson, De Vries, Schindler, King and B. Frank; and of the recent were Ward, Beyerinck, (from whom the bacteria received the name 'Bacillus Radicicola') Vuillemin and Prazmowski.

In 1888 Beyerinck extended his study from peas, on which most of the previous work had been done, to a large number of leguminous plants and found that the formation of tubercles was very general in the family. It was further found that there were bacteria associated with the tubercles and that the bacteria from different species of the plants carried certain constant characteristics. He gave the organism the name "Bacillus Radicicola". He regarded the bacteroids, suggested by previous investigators, and discussed later, as degenerate forms of the organism. After isolating the organism and growing it in pure culture for some time, he was able, at will, to inoculate soils and produce tubercles from the pure culture of the organism. In 1890 Prazmowski stated conclusively that if proper precautions were taken to sterilize the soil no tubercles would be produced, but that they could be produced by the addition of soil infusion as described above. The results indicated that the micro-organisms are present in abundance where leguminous plants are grown but not so elsewhere.

In making a microscopic study, Prazmowski found that the hyphae were abundant in the young tubercles and that bacteroids in the older ones, as previous observers had found. He prefers to call the organism a "Bacterium", instead of a bacillus since it appears as a short rod and never in the form of a slender thread. He also found

several varieties of the organism associated with the different species of the legumes. In artificial cultures the organism always remained a typical bacterium, never assuming the bacteroid form found in the tubercle. From this he concluded that the degenerated forms appear only under the influence of the root cells of the host plants.

The study of the tubercles of leguminosae involved several fields of investigation. One concerned with the economic phases of the subject, that is obtaining the nitrogen of the air by plants and then enriching the soil with their decay, or by the decay of tubercles that obtain the nitrogen by means of organisms within the tubercle. Another field is the biology of the subject. This deals with the facts relating to the morphology, development and kinship of the lowly organisms to the structure of the tubercles and the relation of the symbionts.

At the youngest stage the tubercles present only a small convex protuberance on one side of the root, and the root still possesses some normal root hairs, some of which occupy the surface of the young tubercle. In many instances this root hair presents a peculiar form, being sometimes in the form of a crook. The organism usually enters at the end of the root and must exert a peculiar and powerful influence in order to bring about this change in form. The protoplasm of the root hair is a suitable nutrient so the organism grows down the root hair in search of the cortical parenchyma of the root. The contents of the infecting hyphae are homogenous and easily distinguished from the contents of the root hair. When the organism has

entered the cortical parenchyma it has come into its own arena of real activity. It just forms itself into a disc-like shape over the cell wall, and as the hyphae being very slender reaches through the cell wall again broadens out into a disc. Frequently the hypha enlarges within the cell very near the epidermis, and this occurs again further within the tissue of the tubercle. This growth produces the number of protuberances in the nature of buds. The buds present various forms being either spherical, oval, pyriform, clovate, cylindrical or forked. Soon after entering the root the hyphae branches. Some of these branches continue toward the center of the root and others take a lateral course. Many times the hyphae approach the nucleus and surround it. The nucleus thus approached seems to be stimulated and becomes larger than nuclei not approached. The previous description is according to Prof. Atkinson. According to Prazmowski's investigation the development and growth of the tubercles are as follows:- *Bacterium Radicicola* lives normally in the earth and collects in numbers on the outside of the roots of various legumes. Some of the organisms succeed in forcing their way into the root tissue of the younger roots, sometimes remaining there for a time as free bacteria, but the plant plasm seems to exert an injurious influence on them and hence the development of a sheath or pouch around them. Prazmowski thinks that this pouch is a development of themselves. Bacteria which do not succeed in getting into this pouch, degenerate into bacteroids which appear later in great numbers. The bulk of bacteria are enclosed within the pouch where they

continue to develop in great numbers and with much vigor. The pouches begin to grow into thread like masses and these threads are often branched, and also these threads permeate the roots, looking very much like the mycelium of a mold. The development of this mass of threads soon forms a swelling on the root which develops into the tubercle. The cells of the plant continue to develop a corky like layer around these bacteria, which seems to be injurious to the bacteria thread and hence holds them within its limits. Further development goes on until it seems that the membrane inclosing the bacteria bursts, letting the organism into the cellular tissue of the root; at all events there appears the bacteroid tissue probably through the influence of the plant plasm. The bacteria continue to grow only so long as enclosed within the protecting membrane.

The principal structure of the tubercle is thus developed from the root tissue while the inside, composed of bacteroids is what gives the tubercle the flesh red color. Some of these central cells are so completely filled with them that nothing else can be seen, while in others a nucleus can be distinguished. The bacteroids are afterwards absorbed by the plant leaving the tubercle as an empty pouch. This practically ends the life history of the tubercle.

Prof. Atkinson gives a good method of obtaining pure cultures. He found in his experiments that to isolate the organism, young but fair size tubercles were preferable. These were washed carefully, then rinsed in distilled water and allowed to drain until dry. Then to prevent contam-

ination the razor used in cutting the tubercle was heated. When cutting, the razor was passed over rapidly to prevent killing the organisms by heat, and a little was cut from the end of the tubercle. Then by the use of the platinum wire little of the inside tissue including organisms, was pulled out and a puncture was made in tubes of agar. In the first transplanting twelve tubes were inoculated, some of which were found to be contaminated with common forms of bacillus, micrococcus and bacterium. Others showed a growth in eight or ten days consisting of a whitish pearly glistening color at the point of inoculation. The growth in about a week had spread over the surface but not very much down into the media. The growth was very slow but finally a colony of sufficient size had grown so that an examination could be made of the organism. The individuals were held firmly together by a viscial secretion so they appeared stringy when lifted by the needle. They were very small ranging from two tenths microns to two or three microns, (one micron is equal to a thousandth of a millimeter) yet they were easily distinguishable from other organisms and had quite a characteristic form. They resembled some yeast yet were smaller than any known yeast organisms. In form they were triangular, oval, elliptical, oblong and amoeboid. A nearly homogenous protoplasm occupied the greater part of the central portion, while the granular protoplasm was located at the growing part of the individual. In from six weeks to two months the organism was transplanted to fresh culture media to prevent death from lack of nutrition.

Inoculation Experiment.- Prof. Atkinson.

During the month of April, 1892, an experiment was put into operation to determine the relation of this organism to the orientation of the tubercle. Vetch seeds which had been gathered the previous year were planted in two different vessels. The vessels contained sand from which the soil had been washed and the sand was then saturated with distilled water, to which was added a small quantity of Kainite and acid phosphate, and the vessels were then steam sterilized two hours per day for three consecutive days. The sand was so saturated with water that it was covered with a thin layer, which was necessary in order that the dry vetch would germinate. After the plants had attained the height of five to ten centimeters they were inoculated with organisms from a culture on nutrient agar. This inoculation was made in vessels #1 and ~~#2~~, #2 being held as a check. During the first month the growth was slow. At the end of this time the plants in #1 were found to possess several small tubercles each, while not a single tubercle was found in #2.

The function of the root tubercles has been as much discussed as their nature and structure. They were at first regarded as purely parasitic and therefore injurious rather than beneficial to the plant. It was soon found, however, that they contain large amounts of nitrogenous matter and were for a time supposed to be reservoirs of compounds of nitrogen. It was later discovered that the plants possessing the tubercles contained more nitrogen than those without, and it was thought that in some way the tubercles enabled the plants to secure the nitrogen from the soil. It is now

rather definitely proved that they have some connection with the fixation of the free nitrogen of the air. It is only within recent years that the plants have been known to acquire nitrogen from the air.

In 1881 and 1882, Atwater instituted a series of experiments which gave positive evidence that peas acquired large amounts of nitrogen from the air.

The relation of root tubercles to the acquisition of atmospheric nitrogen was first suggested by Hellriegel who was aided in his work by Wilfarth. Hellriegel found that when peas were grown in pure quartz and sand freed from nitrogen compounds and fed with materials containing no nitrogen, flourished and showed a gain of nitrogen. In the growth of these plants in nitrogen-free soil he found two stages. The development was rapid at first and continued until the reserve material in the seed was exhausted. Then there occurred a somewhat cessation of growth something like a 'starvation period'. In the case of legumes, however, the plants soon recovered, its leaves turned green again, the growth went on and final analysis showed that nitrogen had been accumulated. Considerable variation in the growth of plants side by side was noticed. Some of the plants grew vigorously and were found to possess tubercles on the roots, while those stunted did not. The recovery of the plants from the first starvation period was found to be closely connected with the growth of tubercles. Experiments were carried on with peas in which some of the plants were watered with an infusion from

a fertile field and others without the infusion. All of the peas watered with the infusion showed vigorous growth; while those not, varied from vigorous recovery to complete failure to recover from the starvation period. It was also finally proved that if the infusion was sterilized by heat it was powerless to produce any effect. From this it was concluded that in some way the leguminous plants acquire the nitrogen from the atmosphere, but this power does not lie within the plants themselves.

Two other points of significance were brought out by Hellriegel: (1) If plants were grown in soils containing nitrogen they could assimilate this equally well whether root tubercles were present or not, thus indicating that the root tubercles are not necessary for the assimilation of nitrogen. (2) Different species of legumes seemed to have different species of tubercle micro-organisms associated with them, for it was found that while soil in which peas had been growing might be well adapted to produce the necessary tubercles in other pea plants, it might not produce them in other species of legumes.

W. O. Atwater and C. D. Wood conducted eighty nine experiments divided into five series with peas, alfalfa and oats. The results of these experiments and other similar ones by these authors and others are thus summarized:-

(1) Peas, alfalfa, serradella, lupine, clover in all probability and apparently leguminous plants in general are able to acquire large quantities of nitrogen from the air during their period of growth.

(2) There is scarcely room to doubt that the free nitrogen of

the air is thus acquired by the plants.

(3) That there is a connection between root tubercles and this acquisition of nitrogen is clearly demonstrated.

What this connection is, what is the relation of the micro-organisms to the tubercle and the acquisition of nitrogen, and in general how the nitrogen is obtained are questions still to be solved.

(4) The cereals with which experiments have been completed have not manifested this power of acquiring nitrogen nor do they have such tubercles as are found on roots of legumes.

(5) In experiments reported, the addition of soil infusions did not seem necessary for the production of root tubercles. A plausible supposition is that the micro-organisms or their spores were floating in the air and were deposited in the soil where the plants grew.

(6) As a rule the greater the abundance of root tubercles, in these experiments, the larger and more vigorous the plants and the greater was the gain of nitrogen from the air.

(7) In a number of these experiments, as in similar ones similarly reported, there was a loss of nitrogen instead of a gain. The loss occurred where there were no tubercles, especially large with oat plants and largest where they had the most nitrogen at their disposal in form of nitrates. As the gain of nitrogen explains why the legumes act as 'renovating crop', and the loss, in case of the oat, why it is an exhaustive crop.

Frank's hypothesis that all green plants have the power to fix the free nitrogen of the air and that probably

the leaves are the chief agents in this work is not accepted by recent authors, at least as far as the leguminous plants are concerned. In this they are sustained by Kassowitch whose conclusions are given. The root tubercles are considered by the authors as parts of leguminous plants where the nitrogen is assimilated. The direct agents of assimilation are bacteroids and not the bacteria themselves. As defined previously the bacteroids are formed by the checking of the fission of bacteria or if this is not checked to the retention of numerous individuals within a common sheath for a considerable length of time being in this way protected from any injury by the host plant. The exact nature of their formation is still unknown. They vary with different species of plants but one of the more common forms is rod shaped but very much larger than individual bacteria. These bacteria probably gain access to the plant through the root hairs from the soil.

The main question of the ability of fixing the free nitrogen by the tubercle, is the next for discussion. Some investigators speak of a sort of symbiosis between the bacteria and the plant, that they stand in living relation toward each other, to the mutual advantage of each. It has been stated by recent authors that the bacteria directly store up free nitrogen. The authors think this view untenable. Experiments were conducted by Tharrand with peas and species of robinia. Pea plants were inoculated with organisms from pure cultures of pea tubercles, but not being supplied with sufficient soil nitrogen, the demand for growth was not satisfied to the evident injury of the plant. A microscopical examination of the tubercles showed that the

bacteria were not changed to bacteroids. However, in soil containing nitrogen there was bacteria formation and similar effects were noted on the tubercles. In another experiment with robinia there were produced in the nitrogen-free soil tubercles as large as peas while in nitrogen soil they were as small as rape seed. Upon examining with the microscope it was found that the increase in the tubercles was begun at the same time as the change of bacteria into bacteroids. The conclusions of the authors are as follows:-

- (1.) Nitrogen assimilation by the leguminosae is not through bacteria but through bacteroids.
- (2.) Bacteria infests the plant at the time of its greatest growth, increasing at this time rapidly in number, and the small tubercles also to the ultimate growth of the plant.
- (3.) When inoculated the weaker the plant the sooner the bacteroids are formed and the more tubercles thus strengthening the plant. It still remains to be shown how the plant collects the free nitrogen. The availability of the tubercles for this purpose is evident from their swollen forms and the net work of cells. The water laden with the nitrogen it has washed from the air passes through the cells and the nitrogen is separated and stored up in the tubercles.

The humidity has a certain influence on the growth of the tubercles. E. Gain conducted a series of experiments with peas, lupines, and beans to determine the effect of moisture on the tubercle development. Similar plants were chosen and planted the only difference in condition being the amount of moisture applied. The temperature taken at mid-day showed that the sur-

face of moist soil was seven degrees C. cooler than dry soil. At ten centimeters below the surface the difference was three degrees C. The root systems developed in the various plats varied greatly. Those in moist soil spread widely, were filled with water, became covered with root hairs and presented a large surface of young tissue. In the dry soil the system was less spreading and the epidermis was thicker. In dry soil no tubercles were produced on the superficial roots. In the moist soil the tubercles of peas were scattered all over the roots, five or six times as abundant as those in dry soil, and were about four times as large and ovoid in shape. The results were similar with lupines. On the beans about twenty times as many tubercles were formed in the moist soil as in the dry, and a microscopic examination showed important differences in the abundance of bacteria and in the structure of the tubercle. The experiment showed that humidity of soils favor the growth of tubercles, hence greater assimilation of nitrogen.

In order to see for myself the organism producing the tubercles on leguminous plants I sent to the Department of Agriculture, Washington, D. C. for pure cultures. The department sent me four tubes of agar inoculated with *Bs. Radicicola* from red clover, common bean, common pea, and lupines. I inoculated agar, gelatine, bouillon and potato the first day. The media was kept in an incubator three days and then the growth had developed enough for description. The growth on the agar was thin, whitish glistening and on the surface. On the potato the growth was similar or perhaps slightly darker.

After the growth on the agar was sufficiently large I ex-

amined the organism under the microscope. The first slide I made I went through the regular process of staining with carbal fuchsin. I found that they stained very easily and the first slide was stained too heavily. The organisms on the slide were very numerous. The next slide I made I simply put a drop of sterile water on the coverglass, inoculated it with the organism from the common pea and then without staining mounted it on the slide. The organisms were very small but could be easily seen and appeared singly or close together and each organism was free. There was a rolling motion. The shape of the organism was a little oblong.

We have now made a short sketch of what is known about these organisms that have a power, possessed by no other organism, that is, in fixing the free atmospheric nitrogen. Their economic value perhaps is only just beginning to be realized. The day is undoubtedly not far distant when the agriculturist will use them indirectly in a commercial way, that is, to produce the nitrogen compounds for his crops which he will sell.