The Root-nodules of Myrica Gale.

BY

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With Plates XI and XII.

The discovery by Hellriegel and Wilfarth in 1886, that the root-nodules of leguminous plants are able to assimilate atmospheric nitrogen by means of the Bacteria present in the cortical cells, directed attention to the possibility of certain non-leguminous root-nodules having a similar function. The earlier observers failing to demonstrate the presence of Bacteria in the nodules they investigated, but finding the nodules, especially the older ones, filled with hypha-like threads, considered them instances of 'Wurzelsymbiosen' caused by mycorrhizal filaments.

In 1896 Hiltner demonstrated the presence of Bacteria in the root-nodules of Alder and Elaeagnus, and showed that young Alder plants without nodules would not thrive in a nitrogen-free soil. When, however, these starved plants were inoculated with organisms from Alder tubercles, nodules were produced and growth was normal. He also demonstrated that the poorer the soil in available nitrogen, the greater is the number of root-nodules developed on the Alder roots, provided the necessary organisms are in the soil. Hence under conditions of nitrogen deficiency the number of nodules on the roots becomes a measure of the nitrogen assimilating activities of the plant.

In 1899 Nobbe and Hiltner reported that the root-nodules of Podocarpus were active agents in the assimilation of nitrogen. A Podocarpus plant possessing root-nodules was grown for five years in nitrogen-free sand, thriving well during the whole period.

In 1907 the author demonstrated the presence of nitrogen-fixing organisms in the root-nodules of Cycas.

Hitherto, these four groups of non-leguminous plants—Alder, Elaeagnaceae, Podocarpaceae, and Cycadaceae—have been the only ones recognized as possessing root-nodules concerned with the assimilation of atmospheric nitrogen.

The peculiar nodule formations on the roots of *Myrica Gale* were first described and figured by Brunchorst in 1886. He states that the nodules are caused by an endotrophic fungus with septate hyphae and terminal spores, and considered them simply disease formations.

Möller in 1889 confirmed Brunchorst's observations, and, without adequate reasons, placed the fungus in the group *Frankia*, naming it *Frankia Brunchorstii*, considering it to be closely related to a similar fungus said to occur in Alder nodules.

Marshall Ward in 1889 mentions *Myrica* nodules as possible instances of symbiosis as yet unexplained.

Tubeuf in 1896 speaks of the Mycodomatia of *Myrica*. He says the nodules 'may increase to very large tubers with surfaces resembling a bunch of grapes. In the large cells of the middle layers of the primary root-cortex of these growths, coils of very fine fungus-threads are sheltered; these extend year after year into the younger parts of the enlarging tubercles, and gradually disappear in the older parts. What may be the significance of these structures for plants possessing chlorophyll and furnished with normal roots is as yet unknown.'

In 1902 Shibata stated that the inhabiting fungus is found exclusively in a peripheral sub-cork layer of parenchymatous tissue one to three cells thick, whilst the remaining parenchymatous cells of the rind are filled with starch bodies. Because of the peculiar ray branching of the fungus and its club-shaped spores he considered it to belong to the group *Actinomyces*.

Peklo in 1910, working on material from plants grown in a greenhouse, supported Shibata's views.

**External Structure of the Nodules.**

For the purpose of this investigation root-nodules of *Myrica Gale* were obtained from plants growing wild in Wales, Ireland, and the North of England, and from cultivated plants in the Chelsea Physic Gardens. The roots of all the plants were found to possess nodules of varying sizes. The young nodules (Pl. XI, Fig. 1) are visible first as tiny swellings on the sides of the roots. These grow until they are from 2–3 mm. long and 0.8–1 mm. broad. In this stage they resemble the single nodules found on *Vicia sativa*. This primary nodule then ceases to elongate, but from the distal end a thin hair-like rootlet grows out, and around this rootlet, from the body of the primary nodule, three secondary nodules arise. These secondary nodules grow to the size of the primary nodule and then each again gives rise to a rootlet and three tertiary nodules. These again repeat the process until by repeated branching the characteristic 'cluster' nodules (Fig. 2), with their fringe of radiating rootlets, are formed. When the branches become
closely crowded together sometimes only two branches arise from the end of the nodule, instead of the normal three, owing to lack of space for development.

The branching is apparently associated with the outgrowth of lateral roots, and is not due to dichotomy of the apical meristem of the nodule as in the case of the branched nodules of Alder, Elaeagnus, and Cycas.

**Internal Structure of the Nodule.**

A transverse section of a single nodule (Fig. 3) shows a central tetrarch stele like that of the normal root, and indicates that the nodule itself is a modified root. The stele is surrounded by an endodermis characterized by neither radial dot nor thickened walls, but by the cells being filled with oil drops. Outside the endodermis are several layers of cortical parenchymatous cells covered on the outside by a definite small-celled cork layer. In mature nodules this cortical tissue is characterized by the presence of (1) somewhat enlarged cells filled with Bacteria, (2) cells filled with oil drops. In sections cut from material fixed in alcohol and stained with Kiskalt's amyl-gram stain the Bacteria can be seen in situ in the enlarged cells as small rods. In transverse sections cut near the apex of the nodules zoogloea threads of Bacteria are seen passing from cell to cell. These are comparable to the 'infection threads' seen in leguminous nodules.

In a median longitudinal section of a nodule (Pl. XII, Fig. 6) four fairly well defined areas can be distinguished:

1. **Meristem zone.** An apical mass of meristematic cells beneath the outer protective cork layer. This crowns the end of the stele and extends some distance down the sides (Figs. 4, 5, 6, m.).

2. **Infection zone.** A zone of cells in which the infection threads are seen passing from cell to cell. In a young nodule some of the cells contain starch grains (Fig. 8).

3. **Bacterial zone.** This area includes the bulk of the cortical tissue of the nodule and consists of parenchymatous cells. The majority of the cells are enlarged and crowded with Bacteria. Amongst these are scattered cells containing reserve food material in the form of oil drops (Fig. 3, 4, 5, 6, b. and a.).

4. **The basal zone.** In this region the bacterial cells are few in number, and in fully matured nodules are quite absent, but the oil-containing cells are numerous.

As the individual nodule matures and branches, the seat of bacterial activity is transferred to the branch nodules, and the Bacteria gradually disappear from the bacterial zone.

True fungal hyphae are often found ramifying through the cells of the basal zone, and in the old nodules they may sometimes be seen filling the majority of the cortical cells. These fungal hyphae were considered by the
earlier investigators of *Myrica* to be the cause of the formation of the nodules. This view, though now seen to be erroneous, was quite natural before the bacterial nature of the nodule was demonstrated, and it is possible that these hyphae, whilst not the cause of nodule formation, may be of a mycorrhizal nature and benefit the *Myrica* plant.

**Origin of the Nodules.**

The nodules evidently arise as modifications of lateral roots. The method of infection has not yet been observed, but it is presumably similar to that of leguminous plants. The Bacteria enter a root-hair and pass into the cortical cells as an ‘infection thread’. It is possible that the presence of these Bacteria in the cortical cells stimulates the stele to produce a lateral branch at this point, for every nodule possesses a well-defined tetrarch stele. The cortical cells become enlarged by the growth of the infecting Bacteria, and a short swollen nodule is formed instead of a typical lateral root.

The tip of the nodule is rounded and does not possess a root-cap, for the stele of the lateral root does not break through the surrounding cortex until later.

When the nodule has reached its mature size further expansion ceases, but the stele, which up to this stage has remained completely within the nodule, ending a short distance below the apex, now grows on and out through the apex, and forms a thin hair-like rootlet (Fig. 6, r.) with a typical tetrarch stele (Fig. 7) surrounded by a few layers of cortical cells, but possessing no definite root-cap.

Around the rootlet three secondary nodules or branches arise from the primary nodule in a similar manner to the formation of the primary nodule from the root. These secondary nodules repeat the growth and branching of the primary nodule, a rootlet and three tertiary nodules arising from each. By repeated branching in this manner the peculiar ‘cluster’ nodules are produced.

**Bacteria—Isolation and Cultivation.**

Small nodules were removed from the roots and sterilized by being placed for two minutes in the sterilizing fluid used by Harrison and Barlow in their investigations on leguminous nodules. This fluid consists of hydrochloric acid 2.5 c.c., mercuric chloride crystals 1 grm., distilled water 500 c.c. After removal and washing in distilled water one of the nodules was crushed on a slide. The exuded matter adhering to the slide was then air-dried under sterile conditions and stained with Ziel’s carbol fuchsin. On microscopic examination numerous small rod-shaped Bacteria were seen. These were evidently similar to *Pseudomonas radicicola*, the organism found in the root-nodules of the Leguminosae. They further showed the characteristic staining reaction with Kiskalt’s amyl-gram stain, the aniline gentian
violet being removed by ethyl alcohol, but retained when treated with amyl alcohol. Harrison and Barlow consider this a special differentiating stain for *Pseudomonas radicicola*.

Pure cultures were also obtained by plating out some of the expressed matter from a sterilized nodule on to nutrient agar plates.

The nutrient material consisted of maltose 1 grm., potassium phosphate 0.5 grm., magnesium sulphate 0.02 grm., ammonium phosphate 0.5 grm., agar 1 grm., and 100 c.c. distilled water.

The inoculated plates, after incubation for two days at 28°C, showed numerous colonies which were round to lenticular in shape, raised, entire, viscous, and 0.75 to 1 mm. in diameter. These colonies corresponded in all respects to the characteristic colonies of *Pseudomonas radicicola*. Microscopic examination of the colonies showed the typical rod-shaped Bacteria.

**Estimation of Nitrogen Fixation by the Bacteria.**

Cultures of the Bacteria were made in Erlenmeyer flasks of 300 c.c. capacity containing a nutrient solution consisting of 100 c.c. distilled water, 1 grm. maltose, 0.5 grm. potassium phosphate, and 0.02 grm. magnesium sulphate. Some of the flasks were inoculated with a loop from the colonies on a nutrient agar plate; others were left uninoculated and formed controls. All the flasks were incubated at 25°C for seven days. During this time the contents of the uninoculated flasks remained clear, but the inoculated flasks became cloudy.

Kjeldahl nitrogen determinations of the contents of the flasks gave the following average results:

- **Control flasks** . . . 0.53 mg. N per 100 c.c.
- **Inoculated flasks** . . . 2.58 " , "

showing a fixation of nitrogen of 2.05 mg. per 100 c.c. of culture.

**Effect of Nodules on Growth of Myrica Plants.**

To ascertain the effect of the presence of nodules on the growth of *Myrica* plants attempts were first made to strike *Myrica* cuttings in sand watered with nutrient solution, but all ended in failure.

Excellent results were, however, obtained with young *Myrica* plants procured from Heysham Moss last spring. Some of these plants possessed small nodules on their roots; others appeared to be quite devoid of nodules. Six of each were planted out in a greenhouse, in pots containing sterilized soil deficient in nitrogen. All the plants with nodules flourished well. Of the six plants without nodules, two made no growth for some time, but afterwards began to shoot. On examining their roots a few nodules were found. Evidently these were already infected with Bacteria, but the nodules had been too small to see, when they were planted out. The remaining
four made no fresh growth, and after a time showed evident signs of starvation. At this stage two of these starved plants were watered twice, at an interval of seven days, with a liquid culture of *Myrica* nodule organisms. Soon these plants commenced active growth, and caught up and surpassed the two plants possessing root-nodules. By the end of the summer they were larger than any of the *Myrica* plants in the greenhouse. The two starved plants, which were not inoculated, lingered on for a time and were quite dead by the end of June.

It is evident from these results that the root-nodules of *Myrica* are definitely concerned with nitrogen assimilation, and the Bacteria in the bacterial zone of the nodule are the active agents in nitrogen fixation.

**Summary.**

1. The root-nodules of *Myrica Gale* are modified lateral roots.
2. The young primary nodules give rise by branching to the characteristic 'cluster' nodules, surrounded by rootlets which grow out through the end of each branch.
3. Three branches or secondary nodules arise from the end of each primary nodule, and, like it, are modified lateral roots. After the formation of these branches the stele of the primary nodule elongates and grows through the apex of the nodule, giving rise to the hair-like rootlet.
4. In each mature nodule four zones can be distinguished:
   (a) The apical meristem;
   (b) The 'infection thread' area;
   (c) The 'bacterial' zone, which includes most of the cortical tissue of the nodule, and consists chiefly of the enlarged cells containing Bacteria;
   (d) The basal zone. The lower end of the nodule, devoid of bacterial cells, but containing numerous cells filled with oil drops.
5. After the nodules have branched and reached their full size the Bacteria disappear from the cells of the bacterial zone, and the basal zone gradually encroaches upon and finally replaces all the other zones.
6. In old nodules, and sometimes in the basal zone of younger nodules, mycorrhiza filaments are found.
7. Pure cultures of the Bacteria from the 'bacterial' cells were made, and on examination were found to be identical in structure and growth with *Pseudomonas radicicola*, the organism of the root-nodules of the Leguminosae.
8. Nitrogen determinations of liquid cultures, incubated for 7 days at 25° C., showed a fixation of 2.05 mg. of nitrogen per 100 c.c.
LITERATURE CITED.


EXPLANATION OF FIGURES IN PLATES XI AND XII.

Illustrating Prof. Bottomley’s paper on the Root-nodules of Myrica Gale.

Fig. 1. Photograph of root and nodules of Myrica. Natural size.

Fig. 2. Photograph of ‘cluster’ nodule. × 5.

In the following figures c. denotes cork; b., bacterial cells; o., oil-drops; e., endodermis; p., pericycle; ph., phloem; x., xylem; m., meristematic zone.

Fig. 3. Transverse section of nodule, showing the tetrarch main stele. × 70.

Fig. 4. Transverse section of nodule, showing the stele producing three branches. p.x. denotes protoxylem; br., branch of stele. × 70.

Fig. 5. Transverse section of nodule near the apex, showing the main vascular bundle (m.b.), which is continued into the emerging root, and the three branches of the bundle (br.b.) which supplies the branches of the nodule. × 70.

Fig. 6. Longitudinal section of the root-nodule, showing the branched vascular tissue and the emerging rootlet (r.) passing through the broken tip of the nodule (t.). × 70.

Fig. 7. Transverse section of the rootlet which has emerged from the tubercle. × 140.

Fig. 8. Section of the cortical cells of the nodule, showing the Bacteria (b.r.) and the infection threads (i.t.). n. denotes nucleus of host cell. × 325.
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