The Root-nodules of *Ceanothus americanus*.

BY

PROFESSOR W. B. BOTTOMLEY.

With Plate XXVIII.

AS far as can be ascertained, Dr. W. J. Beal of the Agricultural College, Michigan, U.S.A., was the first to call attention to the tubercles occurring on the roots of *Ceanothus*. At the meeting of the American Association for the advancement of Science held at Indianapolis in 1890 he exhibited specimens of what he called 'root-galls' on the larger roots of *Ceanothus americanus*.

Two years later Atkinson described the structure of these root-galls and stated that the gall is inhabited by a fungus which 'when mature forms compact botryoid clusters in the affected parenchymatous cells of the gall, the central portion being composed of a complexly-branched mass of threads bearing at their ends on the periphery of the mass the globose sporangia'. He considered the fungus to be related to the genus *Frankia* of Brunchorst, and proposed for it the name *Frankia Ceanothi*.

No further mention of these structures can be found until 1910, when Kellerman, in a paper on 'Nitrogen Gathering Plants', states that 'nitrogen fixing Bacteria, apparently similar to the Bacteria isolated from the Leguminosae, have been isolated from nodules of *Ceanothus*'. Unfortunately he gives neither references to any research work nor experimental evidence in support of his statements. Kellerman's paper is illustrated by photographs of root-nodules from a number of non-leguminous plants, and on examining these one is struck by the remarkable resemblance between the nodules of *Alnus*, *Elaeagnus*, and *Ceanothus*. As Hiltner had demonstrated in 1896 that the root-nodules of *Alnus* and *Elaeagnus* are concerned with nitrogen assimilation, the resemblance suggested the possibility of the root-nodules of *Ceanothus* having a similar physiological significance.

There was a difficulty at first in obtaining material for investigation. An examination of the roots of *Ceanothus* plants growing at Kew Gardens, Chelsea Physic Gardens, and various nurseries and private gardens failed to reveal a single nodule. The foreman gardener of one large nursery

stated that during his fourteen years' experience of cultivating *Ceanothus*, both the deciduous forms belonging to the *C. azureus* group and the small-leaved varieties such as *C. dentatus* and *C. floribundus*, he had never seen a root-nodule in any *Ceanothus* plant similar to those he was familiar with on *Elaeagnus* roots. This is perhaps not surprising when one remembers that *Ceanothus* is an imported plant, and presumably the soil is devoid of the requisite nodule-forming Bacteria. A similar case is known in *Soja* bean plants grown in this country, which fail to produce root-nodules unless inoculated with the specific organism from abroad.

Eventually a plentiful supply of root-nodules was obtained from *Ceanothus* plants growing wild in North America, where the genus is indigenous, through the kindness of Dr. Kellerman of the United States Department of Agriculture and Professor F. Clements of the University of Minneapolis.

Nodule-bearing roots of two different species were received—*Ceanothus americanus* (New Jersey tea) and *Ceanothus velutinus* (mountain balm). As a preliminary examination showed that the nodules of both species are practically identical, the following description of *C. americanus* applies equally to *C. velutinus*.

EXTERNAL STRUCTURE OF THE NODULE.

The nodules when young are flesh-coloured, cylindrical outgrowths, from 3 to 8 mm. in length by 1 to 2 mm. broad. They are first visible as tiny lateral swellings on the young roots, and soon attain a breadth of 1 to 2 mm., after which growth is confined to the apex until a length of 6 to 8 mm. is reached. This concludes the growth for the first year. In the following year they continue their growth by branching near the apex, producing two to five branches, and each branch repeats the growth of the primary nodule. This branching is continued in successive years, each year's growth remaining from 1 to 2 mm. in diameter and 4 to 6 mm. in length. Thus the nodules are perennial, and after a few years there is formed a loosely-branched, rotund mass, the size of a small walnut. The number of branches arising each year is variable, two, three, four, or even five branches being produced near together. Hence there is a greater diversity of branching in *Ceanothus* nodules than in other non-leguminous nodules where bi- or trifurcation is characteristic.

INTERNAL MORPHOLOGY.

The material used for the purpose of investigating the internal structure of the nodules was fixed in either Bouin's fixative or alcohol, and microtomed sections were stained with Flemming's triple stain, Heidenhain's iron-haematoxylin, or Kiskalt's amyl gram. A transverse section through

the centre of a young nodule or branch shows that the relation of the different tissue systems differs but little from that of a normal root. There is a central stele surrounded by an abnormal development of cortical cells and a protective corky layer on the outside. The xylem forms a solid mass in the centre of the stele. As all the elements are small it is difficult to distinguish the separate protoxylem groups, but where determined they appear to vary in number from three to five in different nodules. The xylem is surrounded by several layers of parenchymatous cells, the inner layers consisting chiefly of elongated cells (phloem), and the two outer layers of iso-diametric cells (pericycle).

When the nodule branches, secondary thickening occurs in the primary stele, whereby a certain amount of secondary xylem is produced each year, thus forming a strong supporting stalk for the annually increasing cluster of branches. The stele is bounded by a very definite endodermis, which, as in the nodules of *Alnus*, *Elaeagnus*, and *Myrica*, is characterized by the cells being filled with reserve material, chiefly proteid and oil drops.

Outside the endodermis the cortical cells are arranged in two zones—a narrow inner zone of small cells, always free from Bacteria, and an outer broad zone of large cells, most of which are elongated radially, and containing much enlarged cells crowded with Bacteria. This outer zone is enclosed by a layer or layers of suberized cells.

A median longitudinal section shows that the young nodule is surrounded by a protective layer of suberized cells. In certain localized areas these cells occur in relatively large masses. This is seen especially at the apex of the nodule, where they function as a kind of root-cap.

In the older nodules and branches a definite phellogen is present, which produces a few very regular layers of periderm, the cells of which always contain a dark brown substance.

The apex of the young nodule, below the protective layer, is occupied by a group of meristematic cells which covers the end of the stele. Immediately below this meristematic tissue many of the cortical cells are infected with rod-shaped Bacteria, readily seen *in situ* in sections cut from material fixed in alcohol and stained with Kiskalt's amyl gram stain. The organisms occupy at first only a small portion of the cytoplasm of the cell. Here, however, they actively divide, and a little further from the apex of the nodule the infected cells are seen to have increased in size and are filled with Bacteria. Lower down the nodule these cells become still more enlarged and the rod-shaped Bacteria are replaced by large spherical bodies containing either two or four denser portions. These are evidently the 'globose sporangia' of Atkinson.

Towards the base of the nodule the infected cells gradually lose their contents, and are finally seen as dead empty cells.

ORIGIN AND BRANCHING OF THE NODULES.

From the material available it was not possible to trace the earliest stages in the formation of the nodule, but sections of young nodules indicate very clearly that they are modified lateral roots.

They consist of an apical meristematic region below which is a branch from the root-stele surrounded by cortical tissue containing many infected cells, the endodermis of the nodule-stele being continuous with that of the root-stele. Presumably certain cortical cells of the root became infected by Bacteria as in the formation of leguminous nodules, and these stimulate the formation of a lateral root at this point. This lateral root never breaks through the cortex, but as it extends into the infected area the outer cortical cells above this area become meristematic, and by their subsequent growth a long cylindrical nodule is formed instead of a typical lateral root.

The branches of the primary nodule are very evidently endogenous in origin. They arise from an active development of the pericycle cells opposite a protoxylem group. A stelar branch is thus formed, and as this extends into the cortex an apical mass of meristematic cells becomes differentiated which soon produces a visible branch to the nodule.

This method of branching is quite different from that which is characteristic of the nodules of *Alnus*, *Elaeagnus*, and *Cycas* where there is a di- or trichotomy of the apical meristem. In *Ceanothus* as in *Myrica* the branches are primarily due to the formation of stelar outgrowths, the essential difference between the two being that in *Ceanothus* the stele never emerges from the nodule, and the apex remains meristematic, continuing its growth each year, whilst in *Myrica* the stele in its second year pushes through the apex of the nodule and forms a small hair-like rootlet, thus preventing further apical growth.

ISOLATION AND CULTIVATION OF THE BACTERIA.

As described above, two different structures are found in the infected cells, rod-shaped organisms near the apex of the nodule and spherical bodies lower down. The rod-shaped organisms when isolated by the methods described in detail for *Myrica* nodules (Ann. Bot., xxvi, p. 114) appear to be identical with the *Bacillus radicicola* group found in leguminous nodules, giving the characteristic staining reaction with Kiskalt's amyl gram, and typical colonies when grown on nutrient agar. The spherical bodies are very similar in appearance to the 'coccus' forms described by Spratt in *Alnus* and *Elaeagnus* nodules. When grown in ordinary *B. radicicola* culture solution they soon lose their spherical condition and break up into as many typical rod-shaped forms as there are dense portions present. The reverse change from rods to spherical bodies can be seen in old colonies

grown on agar medium. These spherical bodies therefore are not 'sporangia' as Atkinson interpreted them, but are similar to the 'bacteroids' found in leguminous nodules which are known to be a resting and resistant form of the active bacillus produced by unfavourable environmental or nutritive conditions. In *Ceanothus* the alteration in conditions which must necessarily occur in the host cell as it becomes depleted of food and crowded with Bacteria causes the organism to assume the 'bacteroid' form which in this case is spherical instead of the Y- or V-shape of the leguminous nodule.

FIXATION OF ATMOSPHERIC NITROGEN.

To test the possibility of these organisms being concerned with the fixation of atmospheric nitrogen pure cultures from each species of *Ceanothus* were grown in Erlenmeyer flasks in the usual non-nitrogenous culture solution, one set of flasks being autoclaved immediately after inoculation to serve as controls. The flasks were incubated at 26°C. for seven days, during which time the contents of the control flasks remained clear, whilst the non-sterilized flasks became cloudy. Kjeldahl nitrogen determinations of the contents of the flasks gave the following average figures:

	<i>N. in control.</i>	<i>N. in culture.</i>	<i>N. gain per 100 c.c. of culture.</i>
<i>C. americanus</i>	0.24 mg.	2.57 mg.	2.33 mg.
<i>C. velutinus</i>	0.14 "	2.36 "	2.22 "

This gain in nitrogen is very similar in amount to that obtained from cultures of the Bacteria in the root-nodules of *Alnus*, *Elacagnus*, and *Myrica*. It is therefore evident that the root-nodules of *Ceanothus* are definitely concerned with nitrogen assimilation.

SUMMARY.

1. The root-nodules of *Ceanothus americanus* are modified lateral roots.
2. They are perennial and increase in size each year by the formation of endogenous outgrowths (branches) similar in structure to the primary branch.
3. Each primary nodule and branch when fully grown shows four zones: (a) an apical meristematic zone; (b) an infection zone, where the cortical cells are becoming infected with Bacteria; (c) a bacterial zone, containing many radially-elongated enlarged cells filled with Bacteria; (d) a basal zone, almost free from bacterial cells.
4. The younger bacterial cells contain rod-shaped organisms, the older ones spherical bodies. These latter are the 'bacteroid' condition of the active nitrogen-fixing rod-shaped bacillus.

5. The Bacteria, when isolated and grown in pure culture, can fix free atmospheric nitrogen, and from their structure, mode of growth and formation of 'bacteroids' evidently belong to the *Bacillus radicola* group.

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EXPLANATION OF PLATE XXVIII.

- Fig. 1. *Ceanothus velutinus*. Root-nodules. Nat. size.
Fig. 2. *Ceanothus americanus*. Root-nodules. Nat. size.
Fig. 3. *C. americanus*. Transverse section of root-nodule, showing central stele and bacterial zone with radially elongated cells filled with Bacteria. $\times 50$.
Fig. 4. *C. americanus*. Longitudinal section of root-nodule, showing endogenous origin of branch. $\times 30$.
Fig. 5. *C. americanus*. Tangential section of root-nodule, showing the four zones. $\times 50$.

BOTTOMLEY — CEANOTHUS.

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