The Morphology of the Root Tubercles of Alnus and Elaeagnus, and the Polymorphism of the Organism causing their Formation.

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With Plates XIII and XIV.

AMONGST the earlier investigators in the domain of the utilization of atmospheric nitrogen by plants, Hiltner stands out prominently, and in his work of 1899 the roots of *Alnus* and *Elaeagnus*, two non-leguminous plants, were shown to possess root tubercles which were associated with the assimilation of atmospheric nitrogen. These observations have been amplified more recently by Bottomley, who isolated a bacillus from the internal tissue of these nodules, and with a pure culture of this organism inoculated some *Vicia sativa* seedlings, upon the roots of which characteristic tubercles containing *Pseudomonas radicicola* were subsequently produced. From this experiment it was inferred that the tubercles of *Alnus* and *Elaeagnus* contained *Pseudomonas radicicola*, and infection of the roots by this organism was regarded as the probable cause of their formation. The literature concerning the tubercles of both these plants has greatly increased during recent years, and the various authors all find within the nodules a spore-producing hyphal-fungus, which has been placed in the group Hyphomycetes, but they do not appear to agree with one another in the detailed structure of this parasite, and its effect upon the host plant. The present communication is an account of some investigations which entirely support the original view, put forward by Hiltner and upheld by Bottomley, that there is a symbiotic relationship between these two plants and the organisms causing the tubercle formation.

Nodules have been obtained, at various seasons of the year, from plants of *Alnus incana*, *Elaeagnus edulis*, and *Elaeagnus rhamnoides*, growing at Chelsea, Sevenoaks, and North Wales. The material used for sectionizing was fixed in either Flemming's or Bouin's fixative or absolute alcohol, the latter having the advantage that only in this case is the oil which is present in large quantities in the tubercles of *Elaeagnus* removed by

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subsequent treatment with xylol. Microtomed sections were almost exclusively employed, as the details could not be satisfactorily determined with certainty from the freehand sections which have been recommended by some writers. A large variety of stains were used, the principal being Flemming's triple, Heidenhain's iron haematoxylin, Ehrlich's haematoxylin, Löffler's blue, methyl green and fuchsin, methyl violet and fuchsin, carbol fuchsin, and Kiskalt's amyl gram. The last named proved the most satisfactory, since it is a good nuclear stain and also one by means of which *Pseudomonas radicicola* can be differentiated.

In order to ascertain whether these root tubercles were connected with the presence of organisms possessing the power of assimilating atmospheric nitrogen, an attempt was made to isolate any such Bacteria from their internal tissues. For this purpose a medium of the following composition was utilized :

1 grm. saccharose,
0·5 grm. acid potassium phosphate,
0·02 grm. magnesium sulphate,
100 c.c. distilled water.

Into each of three Erlenmeyer flasks, 300 c.c. capacity, 50 c.c. of such a medium was placed, and then submitted to a temperature of 140° C. and a pressure of two and a half atmospheres for ten minutes in an autoclave. They were then neutralized with sodium hydrate and allowed to cool. Some of the tubercles were removed from the roots of both *Alnus* and *Elaeagnus*, thoroughly washed, and then placed in a sterilizing fluid composed of :

2·5 c.c. concentrated hydrochloric acid,
1 grm. mercuric chloride,
500 c.c. distilled water.

They were allowed to remain in this solution for two minutes, when they were removed with sterile forceps and washed in distilled water contained in a sterile vessel. These nodules were then crushed with previously sterilized instruments, and the *Alnus* nodules were put into one of the above flasks, the *Elaeagnus* into another, and the third was left untouched. All were then incubated at a temperature of 25° C.

After two days the solutions into which the nodules had been placed were quite cloudy, whilst the other one had remained unchanged. A drop of the cloudy liquid was placed on a slide, air-dried, then stained with carbol fuchsin, and examined microscopically. In both cases it was a pure culture of rod-shaped organisms, many of which contained two or three small round densely staining bodies, and were apparently very characteristic *Pseudomonas radicicola* (Pl. XIV, Fig. 9). Another slide was prepared, and aniline gentian violet employed as a staining reagent, and this was found to be immediately

withdrawn from the organisms by absolute ethyl alcohol, but was retained by them when amyl alcohol was used as a dehydrating agent; which reactions clearly indicated that they were *Pseudomonas radicicola*, according to the investigations of Harrison and Barlow.

Exactly the same results were obtained when maltose or glucose replaced saccharose in the above culture medium. Solid media were also prepared by adding 2 per cent. agar-agar to the above media, boiling, and then pouring into sterile Petri dishes and allowing to cool. These media were inoculated with very dilute crushed nodular tissue, prepared as above, by means of a sterile platinum loop, and incubated at 25° C. Very characteristic colonies were produced on the agar media, which were ovoid to circular in shape, with an entire margin, slightly raised above the surface, translucent, shining, and mucilaginous. They increased in size from 0·5 mm. diameter in one day to 1·5 mm. diameter in three days.

Streak and stab methods of cultivation produced equally typical growths. The streak produced an abundant, translucent, shining, mucilaginous growth, along the line of infection, with an entire margin, increasing in opacity, size, and viscosity from day to day. The stab induced the formation of a large typical colony on the surface of the agar, and a filiform growth along the stab in the medium.

The colonies embedded in the agar medium were very small, translucent, and disc-shaped or lenticular.

All the above cultures, after a period of incubation lasting four days or upwards, revealed on microscopic examination the presence of some larger organisms, tending to become ovoid in shape, amongst the characteristic rod-shaped *Pseudomonas radicicola* (Fig. 10). These larger organisms also contain the densely stained round bodies which were present in the rods, their larger size being caused by an increase in the enveloping capsule. Division takes place so that one organism contains only one of the small bodies, which then grows until it fills the surrounding capsule, and the whole structure becomes spherical in shape and appears to have a very definite cell-wall (Fig. 11).

When the larger bodies are present in the colonies on the solid media, a white opaque appearance is assumed. The agar media were very favourable to the production of the larger spherical form, whilst in liquid cultures their appearance seems to be correlated with depletion of the medium of its carbohydrate, their source of energy, and probably also with the accumulation of the products of their own metabolic activities. That they are better able to resist the influence of the external environment than are the actively motile bacilli was demonstrated by boiling two cultures for ten minutes, one of which contained only the small rods, whilst the other contained only the larger spherical bodies, and subsequently inoculating freshly prepared culture media with them, and incubating

for two days, at the end of which period a normal healthy culture had been produced only in the medium inoculated from the culture containing the larger spherical organisms. These bodies appear to be analogous with the so-called 'bacteroid' forms produced by other species of *Pseudomonas radicicola*, since by inoculating freshly prepared media with them the small typical bacilli are formed, which subsequently give rise to them again.

The above data indicate that the organisms living inside the root tubercles of these two non-leguminous plants are morphologically identical with *Pseudomonas radicicola*, which inhabits the tubercles of leguminous plants. An attempt was made to determine also their physiological capabilities with regard to the assimilation of atmospheric nitrogen in the following experiments:

Into each of two Erlenmeyer flasks, 400 c.c. capacity, was placed 100 c.c. distilled water, 1 grm. saccharose, 0·5 grm. potassium hydrogen phosphate, 0·02 grm. magnesium sulphate. To one of these flasks, the control, was added 2 c.c. of a culture obtained from *Alnus* as described above. Both were then autoclaved and subsequently neutralized with sodium hydrate. The other flask, after cooling, was inoculated with 2 c.c. of the same culture as the control, and both were incubated at 25° C. for ten days. During this period the control remained apparently unaltered, but in the one containing the living organisms there was a visible change as described above.

The nitrogen content of both flasks was determined by the Kjeldahl method of analysis.

The following results have been obtained with cultures from *Alnus* and *Elaeagnus* respectively:

	Nitrogen founa in control.	*Nitrogen found in culture.*	*Gain in Nitrogen due to organisms.*
Alnus	0·47 mg.	3·96 mg.	3·49 mg.
	0·62 ,,	3·85 ,,	3·23 ,,
Elaeagnus	0·45 ,,	3·07 ,,	2·62 ,,
	0·82 ,,	3·32 ,,	2·5 ,,

The increased nitrogen content can only have been produced by the growth of the living Bacteria introduced, and must have been derived by them from the atmosphere. Every precaution having been taken to exclude all other organisms except those derived from the tubercles, these experiments demonstrate that the root tubercles of *Alnus* and *Elaeagnus*, like those of leguminous plants, assist in the assimilation of atmospheric nitrogen, and this explains how, as Hiltner observed, the plant benefits from the presence of these tubercles on its roots.

The root tubercles of *Alnus* and *Elaeagnus* are usually present in clusters (Pl. XIII, Figs. 1 and 2), which may in *Alnus* attain a diameter of three inches or even more, but in *Elaeagnus* they are rarely more than an inch

and a half across. The tubercles are enabled to grow in clusters of such dimensions by their perennial habit and the repeated bifurcation which occurs when they are from a quarter to half an inch in length and about an eighth of an inch in diameter. In some cases, particularly when the tubercles occur in isolated positions on the roots, trifurcation occurs.

Transverse sections of the tubercles of both plants (Figs. 3 and 13) immediately reveal the fact that they are modified lateral roots, each possessing a well-defined central stele, and the increased diameter is caused by the enlargement of the cortical cells owing to their infection with the Bacteria. The organisms penetrated the root-hair, and entering the cortical cells caused them to hypertrophy, and this arrested the growth in length of the stele, so that a short swollen structure is produced instead of a typical lateral root. The tip of the nodule is occupied by a meristematic zone (Figs. 7 and 16), by means of which it grows, not only during one season, but also from year to year, growth being renewed each spring, when both meristematic apex and central stele branch (Figs. 7 and 16). The vascular tissue in the tubercles of *Elaeagnus* is in the form of a triarch stele (Fig. 13), possessing a relatively large amount of phloem, two or three layers of pericycle, and a distinct endodermis which is always closely packed with reserve food material, usually in the form of oil, but in some of the tubercles gathered in the spring there was a large proportion of starch. The xylem is composed of annular and spiral vessels and parenchyma. Towards the base of the older nodules a considerable amount of secondary vascular tissue is produced, and amongst this xylem there are scalariform vessels. In the tubercles of *Alnus* the central stele varies from triarch to hexarch (Figs. 3, 4, and 5). In the older regions there is a solid central mass of xylem (Fig. 5), whilst at the tip the centre of the stele is occupied by parenchyma, and the protoxylems are arranged round this (Fig. 4). Here also secondary thickening occurs, a considerable quantity of phloem is present, surrounded by two or three layers of pericycle and an endodermis, the cells of which are filled with reserve food material in the form of definite structures usually somewhat spherical in shape and giving very definite proteid reactions.

In the roots the phellogen has its origin in the pericycle, and forms a few layers of periderm externally, and several layers of secondary cortex on the inside. The tubercles are always surrounded by a few layers of periderm (Figs. 3, 7, 13, 16), which are formed by the continued growth of the original phellogen of the root to keep pace with the growth of the tubercle. In the spring the periderm near the tips of the tubercles in *Elaeagnus* frequently becomes very irregular, large masses being split off at various places, giving rise to small excrescences on the surface which are sometimes visible to the naked eye. This is caused by the inability of the phellogen to divide sufficiently rapidly to keep pace with the

rapid expansion and growth of the nodular tissue when reawakened to activity.

In the tubercles of *Elaeagnus* the cortical cells situated immediately behind the meristematic zone appear to contain a protoplasmic network, while those a little further back are often somewhat abnormally enlarged and are filled with densely staining spherical bodies. This appearance suggests the idea which has been held by Woronin, Brunchorst, Shröter, and others, that the tubercles are produced by one of the Plasmodiophoraceae. When, however, the sections are stained with Kiskalt's amyl gram it becomes evident that the cortical cells contain the nitrogen-fixing organism *Pseudomonas radicicola* (Fig. 8).

The apparent network is then seen to be a zoogloea thread in which are embedded the small rod-shaped Bacteria (Fig. 8, *b*, *z*). This is further demonstrated by applying a little ethyl alcohol, when the stain is instantly removed from the network, remaining only in the nuclei of the host cells. This condition always prevails in the youngest cells inhabited by the Bacteria, and is undoubtedly the form in which the organism passes from cell to cell, being exactly comparable with the so-called 'infection threads' produced in leguminous tubercles, and, as in them, seems to be attracted by the host nucleus around which it twines. The contents of the slightly older, enlarged cells are also very clearly defined by Kiskalt's amyl-gram stain, as distinct spherical bodies, having a definite wall and a strong resemblance to the organisms obtained in the old cultures and on the plates (Fig. 8, *c*). Detailed examination of a large number of sections of nodules of various sizes and ages, at different seasons of the year, showed that these spherical organisms are produced from the zoogloea after it has attained certain dimensions with regard to the host cell, and consequently has reached a particular stage in its development. In addition to the cells containing only the small rods in the zoogloea, others were examined in which there were a number of larger structures in the slime thread, and amongst these some very definite spherical bodies, the number of which evidently increases in proportion, until the host cell contains only these, the network being at first masked, but afterwards entirely used up as the bacilli assume the coccoid form (Fig. 8). The nuclei of the host cells under the influence of the zoogloea frequently assume a somewhat amoeboid form, become very vacuolate, may have several nucleoli, and in some cases appear to disintegrate (Fig. 8).

In order to ascertain the connexion between the two structures described above, the following methods of cultivation were tried. Part of the internal nodular tissue was crushed and put into a hanging drop so that it could be watched under the microscope, or incubated as desired. Some of these preparations were subsequently dried and stained. Thin hand sections were treated in the same way. The most satisfactory method

of cultivation, however, was to take a nodule, the outside of which had been sterilized, and cut it into three or four pieces, in order to expose some of the cortical cells directly to the influence of external agencies, and then place them in liquid culture media and incubate them at 25° C. The media used were those described above for the cultivation of the isolated organisms. The influence of adding a trace of ammonium phosphate or asparagin was also investigated. The pieces of nodule were incubated for periods varying from 12 hours to 6 days, when they were removed from the culture medium, fixed in Bouin's fixative, embedded and microtomed, and the sections stained with amyl gram or carbol fuchsin. In every case there was a steady increase from day to day in the proportion of bacilli present, and a corresponding decrease in the spherical bodies until after the fourth day, when all the latter forms had disappeared. The coccus forms were seen to divide, leaving a clear area across the centre, which always remained unstained (Fig. 14). Each half then divided again (Fig. 14), and the resulting bodies each produced a typical bacillus. In some cases there appeared to be more than four rod-shaped organisms produced from the one coccus, but the spherical body very soon loses its identity, especially when they are not too crowded together. All these stages are very evident in freshly gathered nodules, especially in the spring. These observations, like those in connexion with the isolated organisms, point to the conclusion that the amount of available carbohydrate present is one of the factors determining the production of the coccus form. It is extremely probable that the twining of the zoogloea round the nucleus of the host cell, as well as the activities of the Bacteria in these cells, impairs to a large extent the metabolic processes of the latter, and consequently renders it more difficult for the Bacteria to obtain the carbohydrate, their source of energy, and they consequently undergo a morphological change which renders them more resistant to the influence of their environment, and in this condition they rest. In the tubercles themselves, they become much more abundant in the autumn, in the winter appear to be the only form of organism present, and in the spring, when food material is once more available and fresh cortical cells are being formed in the nodule, they divide and once more assume the active bacillus form. The greater resistant power of the cocci was illustrated by moving some of the pieces of tubercle which contained none of this form from one culture medium to another, when it was found that in 12 hours the formation of a large number had been induced by this sudden change in the constitution of the environment. The two forms are clearly polymorphic of the same organism, which is a species of *Pseudomonas radicicola.*

The tissue at the base of the tubercles contains an abundant supply of reserve food material, which consists mostly of oil, but in the spring a good deal of starch is present. In the older nodules this tissue becomes some-

what compressed laterally, and here and there are little colonies of Bacteria (Fig. 15) which have not migrated to the younger tissue nearer the tip of the tubercle, although they assume the rod-shaped form in the spring.

The cortical cells of the tubercles of *Alnus* also clearly contain organisms of two forms—small bacilli, undoubted *Pseudomonas radicicola*, and larger coccus forms (Fig. 6). These forms are evidently analogous to the same forms in *Elaeagnus*. Cells are frequently met with also, which contain a mixture of these two forms in varying proportions (Fig. 6), and in some tubercles the spherical bodies are seen to be in a state of division, so that they clearly produce rods again (Fig. 6, *d*). *Alnus* tubercles were subjected to the cultural methods described above for *Elaeagnus*, which revealed the same polymorphic nature of the organism inhabiting it, the only difference being that the *Alnus* bacillus does not form such an evident zoogloea in the cells of its host. Probably correlated with this, the nuclei of the cortical cells do not appear to undergo any definite changes. In *Alnus*, too, the bacteroidal cells traverse the complete length of the nodule, and between them, in zones more or less concentric with the endodermis, are cells containing chiefly reserve food material in the form of oil and proteid globules.

The entire absence in all the tubercles examined of any filaments or hyphae in *Alnus*, and only the very narrow zoogloea threads being present in *Elaeagnus*, together with the inability of the spherical bodies, which appear to have been called 'spores' by some authors, to germinate after the manner of a spore, seems to preclude the possibility of the tubercles being produced by the parasitism of a spore-producing hyphal-fungus. The present series of investigations entirely support the idea that the root tubercles of *Alnus* and *Elaeagnus*, although morphologically different, are physiologically analogous to the root tubercles of the Leguminoseae, which have been shown to be actively concerned in the fixation of atmospheric nitrogen, thus rendering this vast store available to the leguminous plant. This is done for *Alnus* and *Elaeagnus* by a species of *Pseudomonas radicicola*, which, in one of its forms, is morphologically identical with the organism found in leguminous nodules, but like the latter it is polymorphic and assumes a comparatively large spherical form, which does not usually occur in other species.

In conclusion, my heartiest thanks and gratitude are due to Professor W. B. Bottomley, in whose laboratory the investigations have been pursued, for his kindness, sympathy, and advice.

Summary.

1. The root tubercles of *Alnus* and *Elaeagnus* are modified lateral roots. They are perennial, dichotomously or trichotomously branched structures.

2. The tubercles are produced by the infection of the root with a species of the nitrogen-fixing organism *Pseudomonas radicicola.*

3. The bacillus enters the root and afterwards propagates itself in the cortex of the nodule as a rod-shaped organism. In *Elaeagnus* it produces a very definite zoogloea.

4. The further development of the organism, in both cases, gives rise to relatively large spherical bodies, which increase in numerical proportion until they fill the entire cell.

5. Under certain conditions the larger bodies divide into two, and then each divides again, and possibly even further, until they lose their identity and a group of bacilli remain in their place.

6. *Pseudomonas radicicola* is a polymorphic organism, the bacillus and coccus being different forms of one and the same organism.

7. In *Elaeagnus* the nuclei of the host cells appear to undergo some change under the influence of the zoogloea.

8. In *Elaeagnus* the Bacteria are found mainly in the region immediately behind the growing point, whilst in *Alnus* the bacteroidal tissue traverses the entire length of the nodule.

9. In *Elaeagnus* the food storage cells are found towards the base of the tubercle, in *Alnus* there are zones of tissue concentric with the endodermis, and in both the endodermis performs this function.

10. In *Elaeagnus* isolated groups of bacilli occur in the basal region.

11. The coccus form appears to be correlated with scarcity of available carbohydrate and change of environment. It is much more resistant to the influence of external agencies than the rod-shaped form.

12. The organism is capable of fixing free atmospheric nitrogen when isolated from the tubercles, and its presence is undoubtedly beneficial to the plant.

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DESCRIPTION OF PLATES XIII AND XIV.

Illustrating Miss Spratt's paper on the Root Tubercles of *Alnus* and *Elaeagnus*.

In Figs. 3, 4, 5, 7, 13, and 16, x = xylem; ph = phloem; p = pericycle; e = endodermis; c = cork; b = bacteroidal cells; f = reserve food material; m = meristematic zone.

In Figs. 6, 8, 14, 15, b = bacillus form; c = coccus form; d = coccus form dividing; z = zoogloea; n = nucleus.

Fig. 1. Root tubercles of *Alnus incana*. Natural size.

Fig. 2. Root tubercles of *Elaeagnus edulis*. Natural size.

Fig. 3. Transverse section of root tubercle of *Alnus*. × 70.

Fig. 4. Transverse section of stele at tip of tubercle of *Alnus*. × 70. s = parenchyma.

Fig. 5. Transverse section of stele near base of tubercle of *Alnus*. × 70.

Fig. 6. Cortical cells of *Alnus incana*. × 325. g = proteid globules.

Fig. 7. Longitudinal section of tubercle of *Alnus*. × 37.

Fig. 8. Cortical cells of *Elaeagnus*, showing bacillus and coccus forms, zoogloea, and host nucleii. × 325.

Fig. 9. *Pseudomonas radicicola* isolated, rod-shaped form. × 1,180.

Fig. 10. *Pseudomonas radicicola* isolated, rod-shaped form changing to coccus. × 1,180.

Fig. 11. *Pseudomonas radicicola* isolated, coccus form. × 1,180.

Fig. 12. *Pseudomonas radicicola* isolated, coccus form changing to bacillus. × 1,180.

Fig. 13. Transverse section of tubercle of *Elaeagnus*. × 70. $s.x.$ = secondary xylem.

Fig. 14. Cortical cells of *Elaeagnus*, showing coccus forms changing to bacillus. × 325.

Fig. 15. Cortical cells from basal region of *Elaeagnus* tubercle showing Bacteria, and reserve food material as oil at *O*. × 325.

Fig. 16. Longitudinal section of root tubercle of *Elaeagnus*. × 37.

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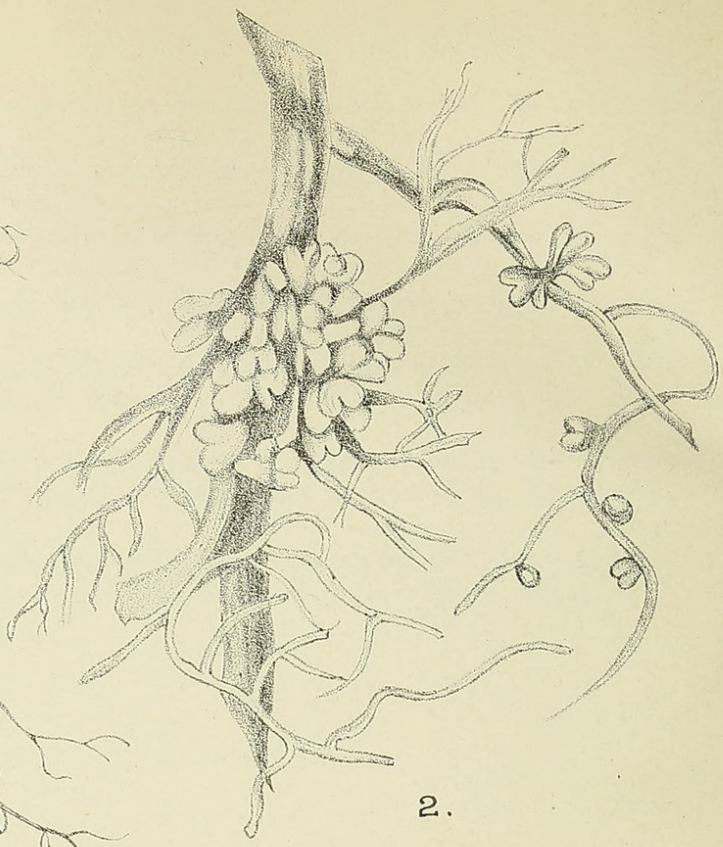

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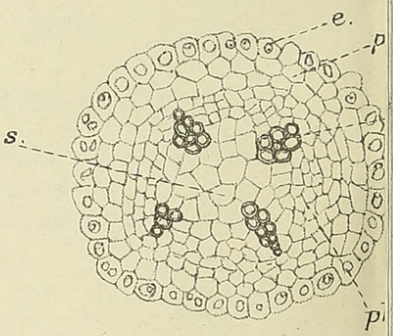

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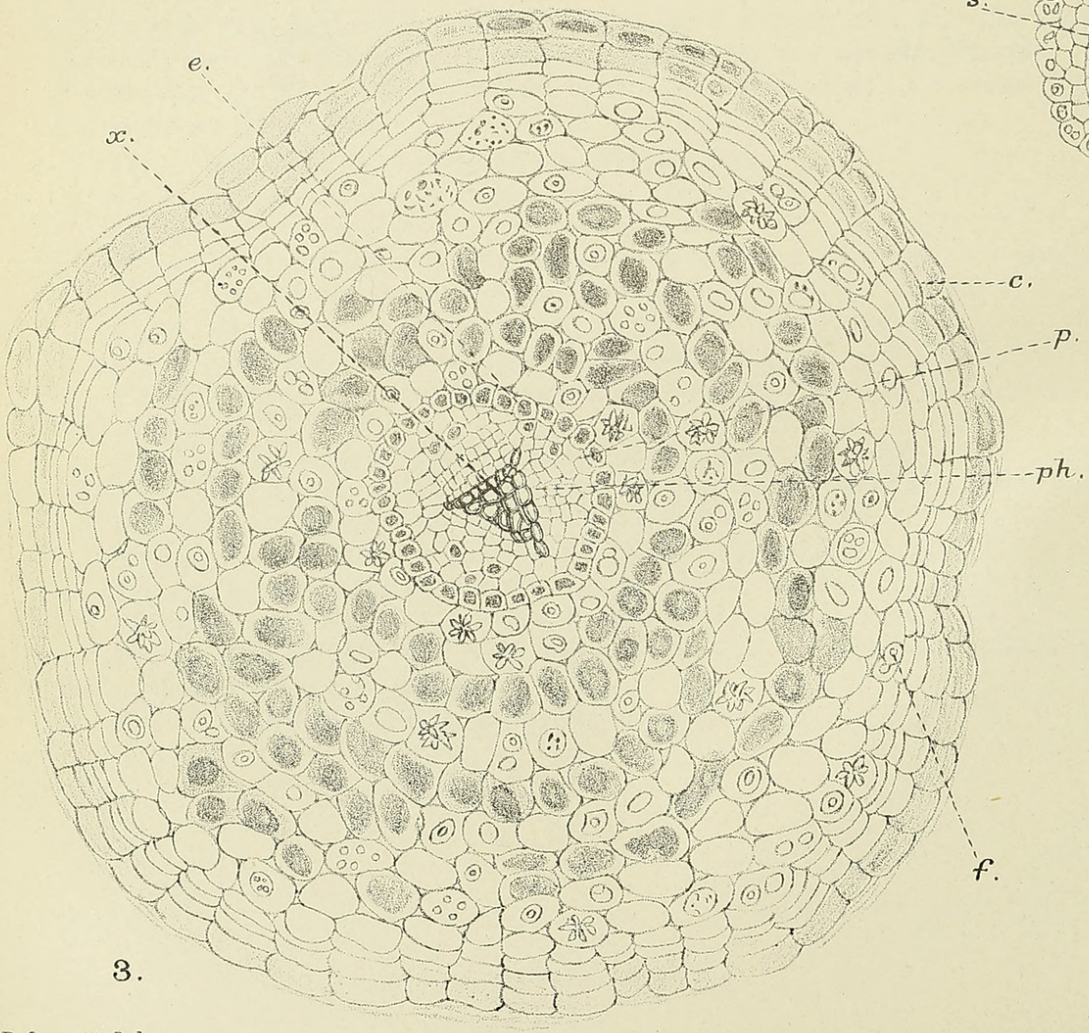

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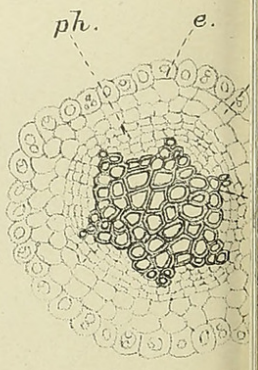

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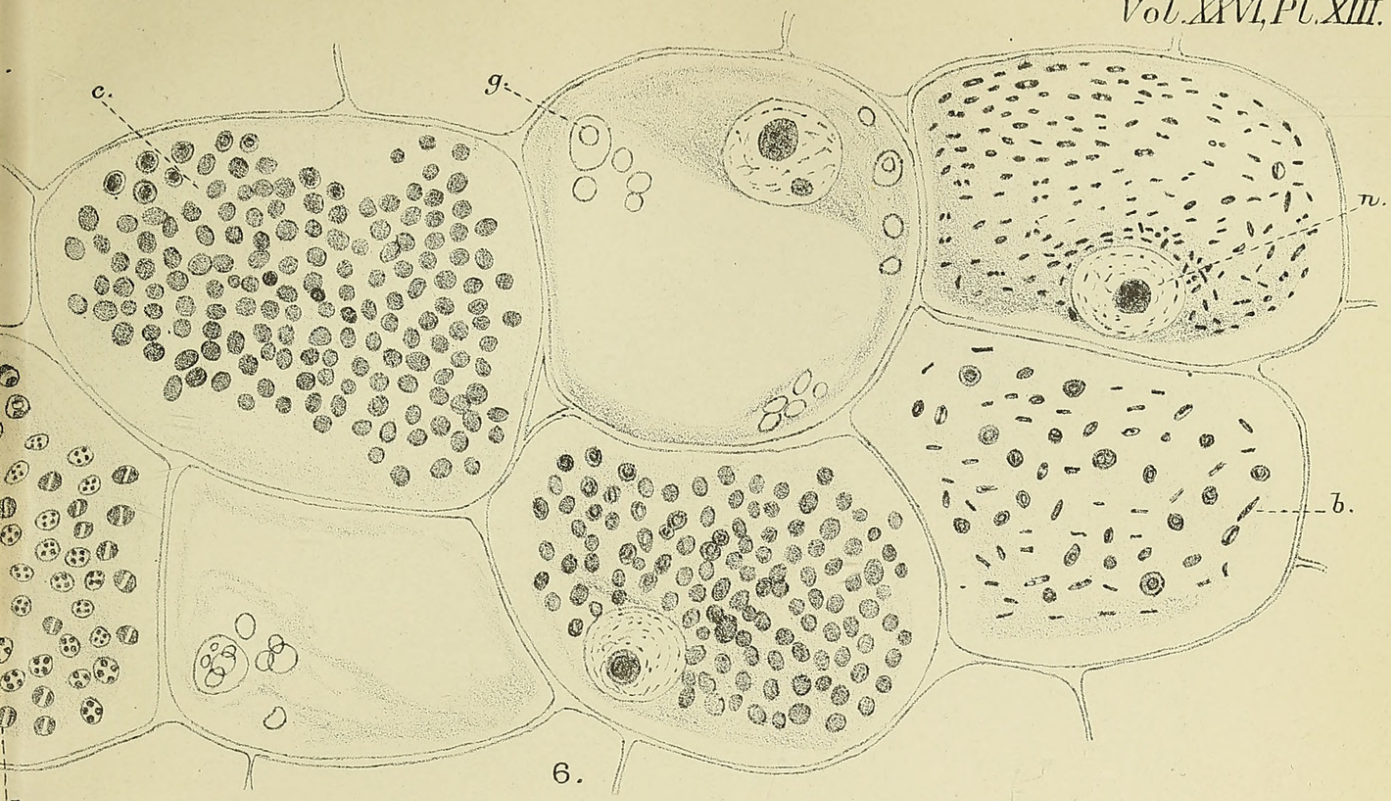
SPRATT — ROOT-TUBERCLES OF ALNUS AND ELÆAGNUS.



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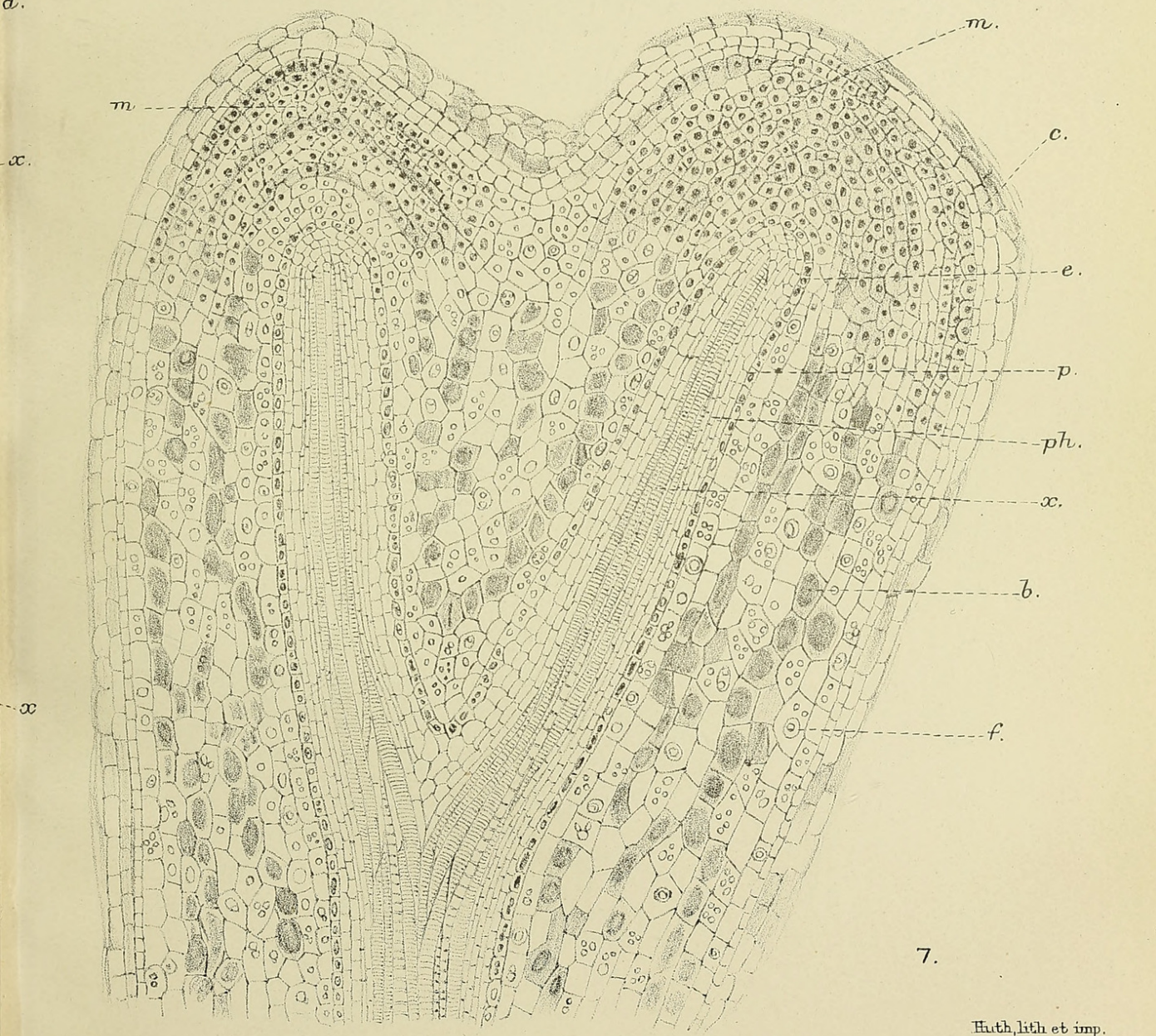

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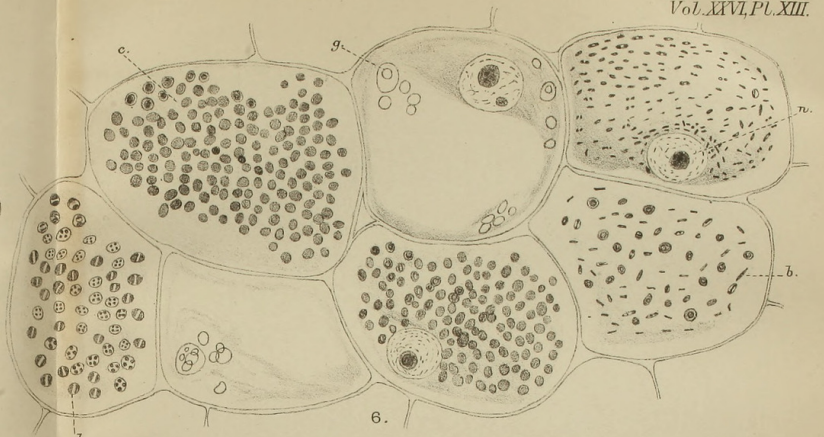

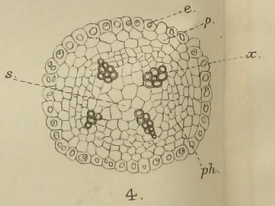

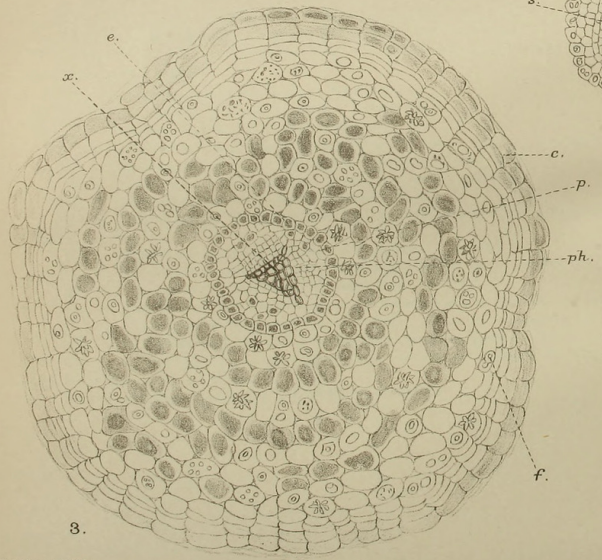

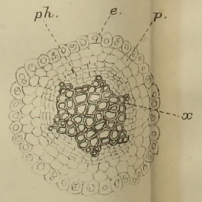

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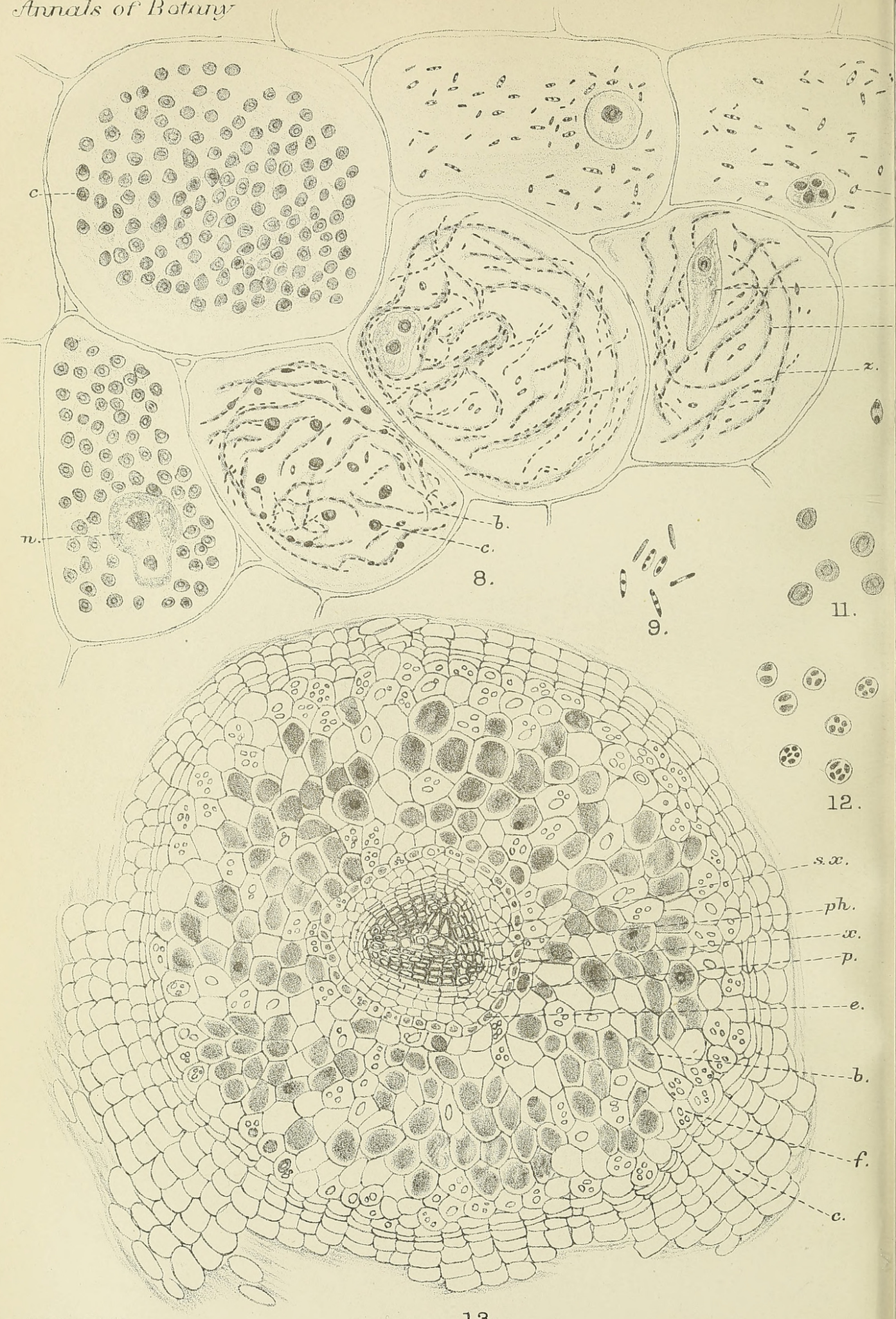
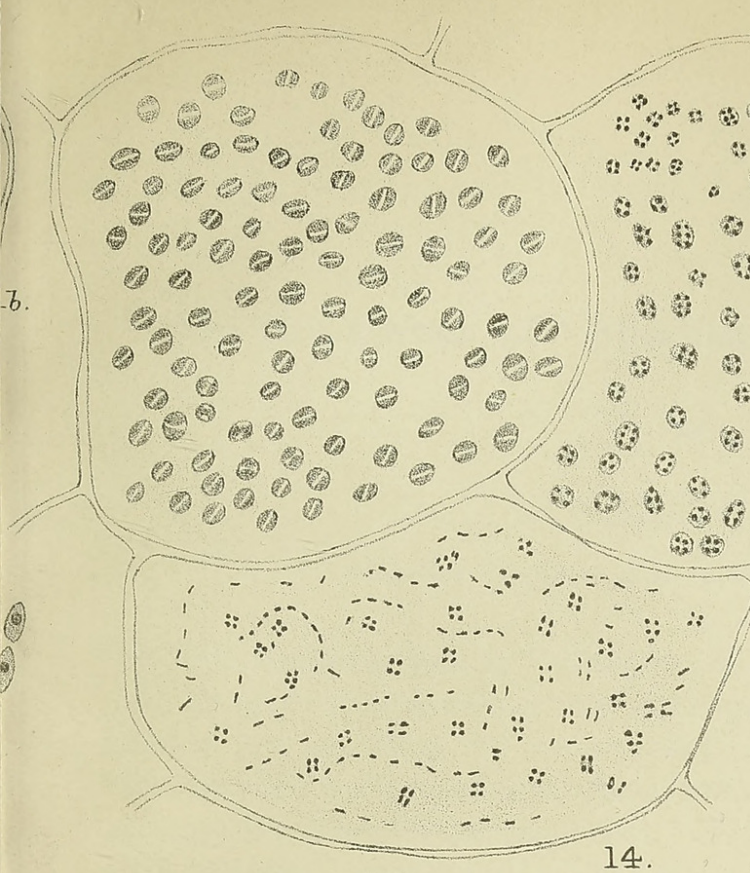
SPRATT — ROOT-TUBERCLES OF ALNUS AND ELÆAGNUS.



E.R. Spratt del.

SPRATT—ROOT-TUBERCLES OF ALNUS AND ELÆAGNUS.



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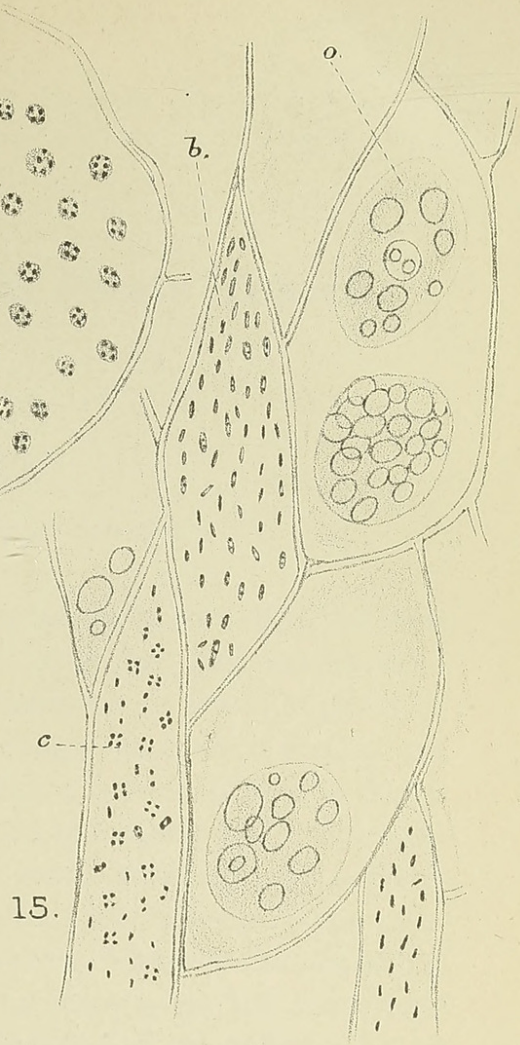

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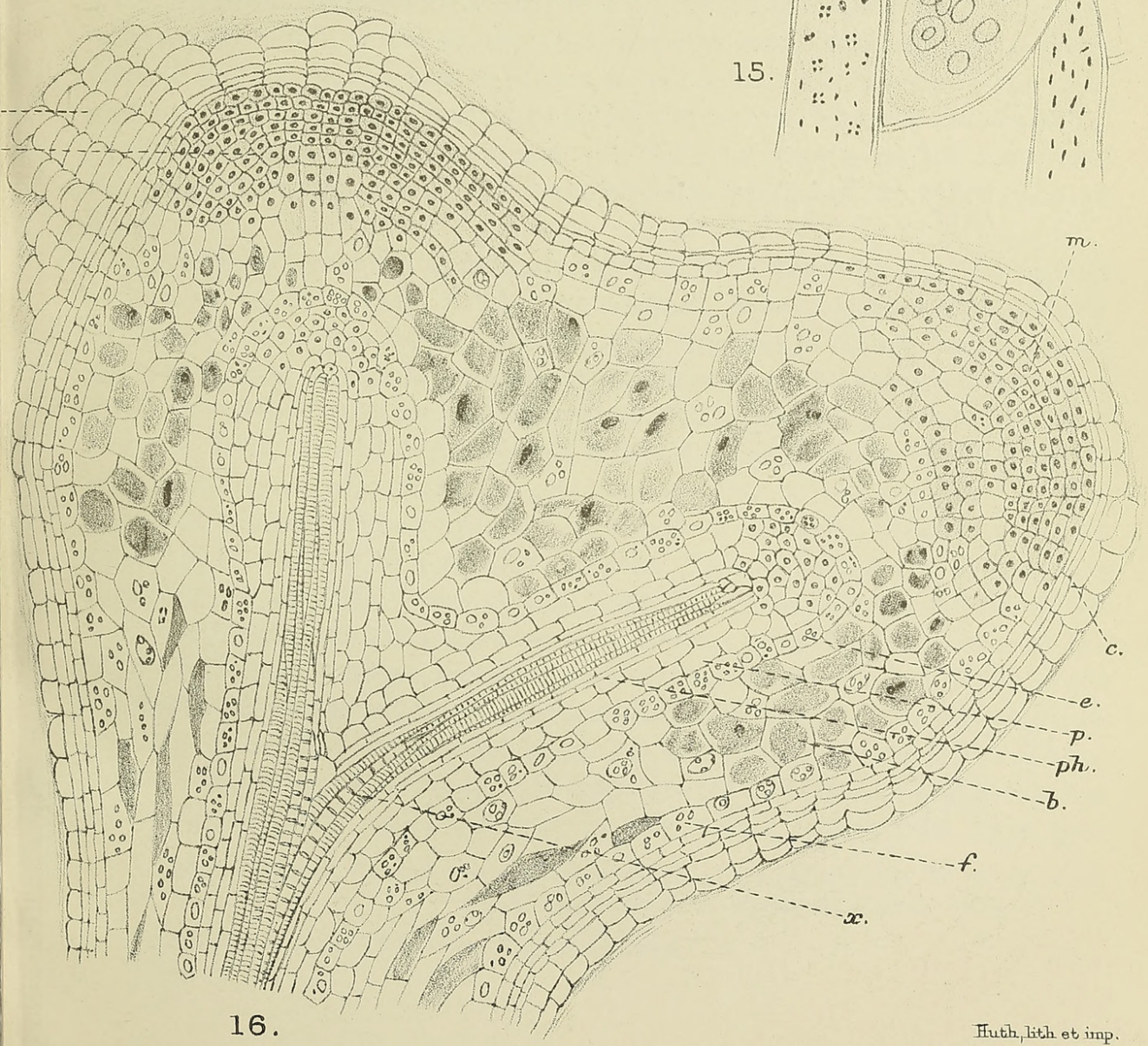
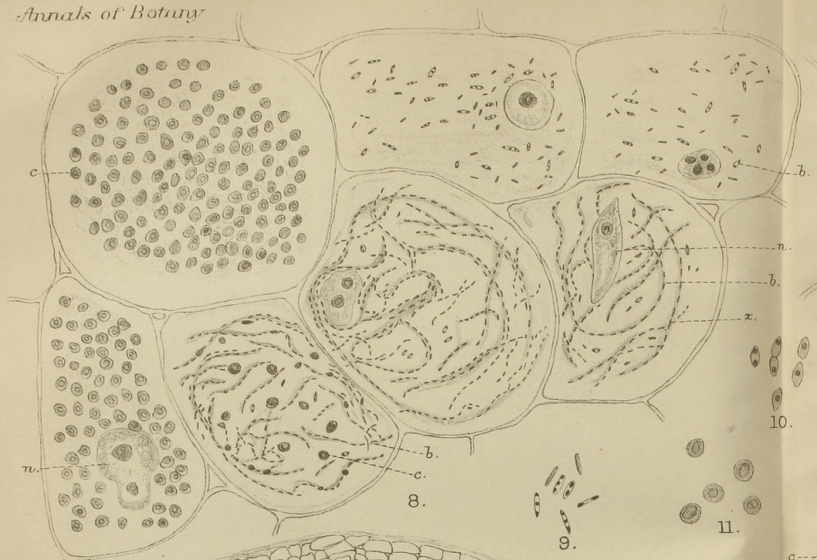


16.

Huth, lith. et imp.



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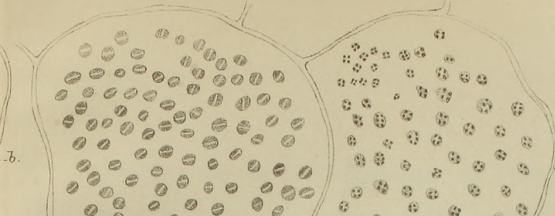

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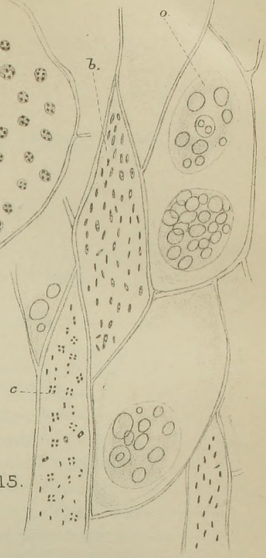

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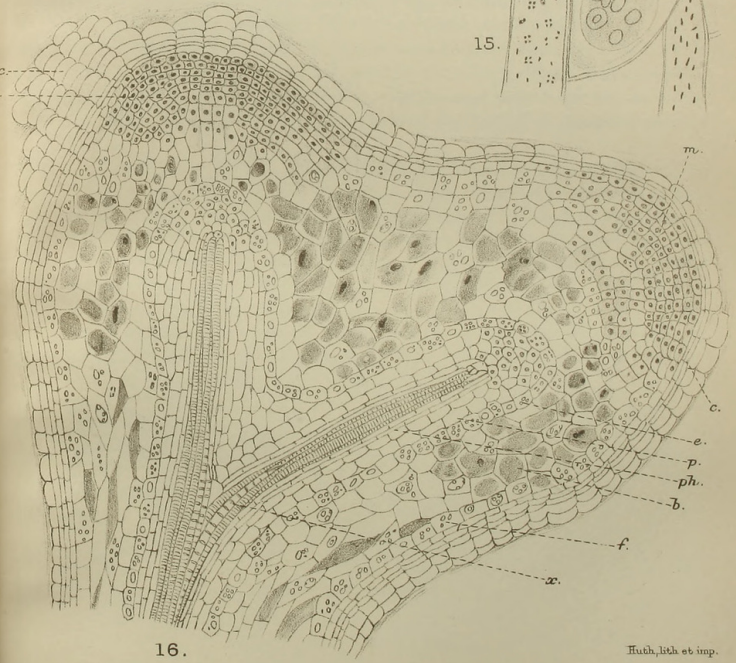

16.

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Huth, lith et imp.

SPRATT—ROOT-TUBERCLES OF ALNUS AND ELÆAGNUS.