Contributed and Selected

ACTINOMYCES MYRICARUM (YOUNGKEN), THE CAUSE OF MYRICA AND COMPTONIA TUBERCLES.*

HEBER W. YOUNGKEN, PH. G., A. M., PH. D.

Within the past thirty years various investigations have been carried on by Brunchorst, Moller, Shibata, Chevalier, Harshberger, and Arzberger in respect to an endophytic organism living in the tissues of the Myricas and forming tubercles.

Brunchorst[13] was the first to mention the tubercles on Myrica Gale and named the fungus producing them—Frankia Subtilies—because he considered this organism similar to that in the tubercles of Alnus.

Moller[14] later found the organism to differ considerably from that infesting Alnus, and named it—Frankia Brunchorstii.

Shibata[15] investigated the tubercles found on Myrica rubra, his observations being on both fresh and preserved material. He described the morphology of the tubercle, showing that the fungus confines itself to a ring of from one to three layers of cells beneath the cork, thus differing from the condition found in Alnus. He also pointed out that infection takes place acropetally by means of fungal threads. He traced these threads into the already differentiated meristomatic cells where they grew rapidly to form a dense thready reticulum, then branched into radiate threads whose free ends became swollen in clavate fashion. He assigned to the fungus a position in the genus Actinomyces.

Chevalier[7] (p. 124-139) examined the tubercles on the roots of Myrica Gale (Gale pabustris), Myrica cerifera, Myrica Caroliniensis (M. Pennsylvanica), and Myrica sapida var. longifolia. He found them on main roots, adventitious roots of subterranean branches, and on subterranean stems. He described at length their general gross structure and histology, the occurrence of gummy lignin in the cells attacked, and called the infesting organism, Frankia Brunchorstii, previously observed by Moller.

Harshberger[16] observed the tubercles on the adventitious roots of Myrica cerifera. He studied the structure of the mature tubercles from dry material only which had been boiled in water and afterwards treated with alcohol. He called the tubercles mycodomatia and claimed for the infesting fungus a position closely related to the Comycetes.

Arzberger[17] investigated the root tubercles of Myrica cerifera, Myrica Gale, and Comptonia asplenifolia (Myrica asplenifolia) and stated, like Harshberger,

* Abstract from The Comparative Morphology, Taxonomy, and Distribution of the Myricaceae of the Eastern United States, a thesis presented to the faculty of the Graduate School, U. of P., May, 1915, by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

that these structures appear on adventitious roots which grow out from the lower part of the stem or from branches or stems which have been covered over with leaf mold or soil for several years. He described the morphology and cytology of the tubercles, but his illustrations do not show the true nature of the radiating clavate branches. He favored the opinion of Shibata in placing the fungus in the genus Actinomyces.

During the last three years the writer has collected and examined abundant tubercle material of M. cerifera, M. Caroliniensis, M. Macfarlanei (M. cerifera and M. Caroliniensis) Youngken, and Comptonia asplenifolia at Palermo and Tuckahoe, N. J.; of M. Caroliniensis and Comptonia asplenifolia at Clementon and Albion, N. J.; of Comptonia asplenifolia near Mainville, Pa.; and of M. cerifera, M. Caroliniensis, and M. Macfarlanei at Wildwood and Rio Grande, N. J. He has raised M. cerifera seedlings bearing tubercles from seeds which he planted in sandy soil in the University of Pennsylvania greenhouse. He has furthermore examined tubercle material of M. Gale collected by Dr. John M. Macfarlane along Trefethan Bay in Chebeague Island of Casco Bay, and on the south eastern part of Peak's Island in Casco Bay, Maine.

METHODS.

Material from many plants of each species was macroscopically and microscopically examined both in its fresh and preserved condition. All of the preserved material was fixed in weak, medium, and strong Flemming's on the ground immediately after the position and nature of the tubercles on the plants had been ascertained. Samples of each lot were then dehydrated in gradually increasing strengths of alcohol, cleared in cedar oil and xylol and imbedded in paraffine. Transverse, tangential, longitudinal radial, and longitudinal tangential sections were then cut 6-10 microns thick and subsequently stained in several ways. The best results were obtained with the Methylene Blue and Acid Fuchsin combination, although satisfactory results were also obtained with a combination of Safranin and Gentian Violet.

The writer employed the following technique in isolating the endophyte which produces the tubercles on M. cerifera, M. Caroliniensis, M. Macfarlanei, M. Gale, Comptonia asplenifolia and probably most, if not all, of these lesions on other plants of the Myricaceæ.

A tubercle cluster from a root of one of the seedlings grown in the University of Pennsylvania Greenhouse was washed thoroughly with clean water to remove all traces of adhering soil. It was then introduced into a test tube containing 1 : 1000 corrosive sublimate solution for twenty seconds in order to destroy any surface organisms. From this it was transferred with sterile forceps to a test tube containing distilled water which had previously been sterilized in the autoclave. Into this was introduced a sterile scalpel and two of the tubercles were cut into small fragments. These fragments were next transferred to five tubes of sterile slant agar by means of a sterile platinum loop. The tubes containing the culture were then stored in a dark closet at ordinary room temperature for several weeks. All five cultures when examined revealed the presence of Actinomyces rosettes, non-septate thin filaments, and rods of different sizes as well as coccus forms, all of which stained well by Gram's method. The coccus forms are prob-

ably for the most part products of the degeneration of the filament. Jordan supports this view in regard to similar forms of Actinomyces found in cattle, sheep, hogs, and man. The Actinomyces rosettes were found to be present in the depth of the agar. This shows the anærobic nature of the organism. From two of the above cultures, the writer has recently successfully grown pure sub-cultures on coagulated horse serum in sealed tubes, kept at the temperature of 37.5° C.

Five seedlings of M. cerifera, which the writer had previously grown from seed in the University of Pennsylvania Greenhouse, were then removed from the soil and their root systems loosened from adhering sand by gently washing in clean water. With the aid of Mr. Lambert, of the University Gardens, who, like the writer, took special care to insure against sources of infection by other organisms, the root systems were one by one quickly dipped into 1 : 1000 corrosive sublimate solution and then washed in sterile distilled water. While Mr. Lambert, with sterile hands, held each seedling so treated, the writer, by means of a long needle previously sterilized by passing through the Bunsen flame, removed a small portion of the Actinomyces culture from one of the tubes and pricked it into the root of four seedlings, marking the place of inoculation by tying a sterilized piece of cord just above the puncture. The last seedling was treated similarly to the first four, with the exception that it was merely pricked with a sterile needle. This served as a control. Each seedling was then planted in a sterile pot containing sterile sand. Both pot and sand were previously sterilized in the hot air oven at a temperature of 210° C. for eight hours. The potted seedlings were then placed in a special case in the Greenhouse and daily watered with sterile Knop's solution. At the expiration of nine weeks, the seedlings were carefully removed, washed in clean water and their roots examined for the presence of tubercles. These were found in a primitive state at the points of inoculation on all but two, including the control, which was pricked with a sterile needle only. (This merely developed the usual healthy suberous scar tissue.) Thin hand sections of one of the tubercles revealed the presence of Actinomyces in the same condition as observed in the cells of the tubercles of the M. cerifera seedling, as well as of the tubercles on the other species above noted. The appearance of the infesting Actinomyces within the cells of the host plants will be treated under the caption dealing with the histology of the tubercles.

GROSS STRUCTURE OF TUBERCLES.

The writer has found tubercles on the M. cerifera, M. Caroliniensis, and M. Macfarlanei seedling primary roots of five to six months' growth, and from thence onward on the secondary roots inserted on the hypocotyl axis, on nearly all the adventitious roots of subterranean branches and on the subterranean branches of M. cerifera, M. Caroliniensis, M. Gale, M. Macfarlanei, and Comptonia asplenifolia..

The tubercles occur either singly, as is frequently the case on subterranean branches, in small groups the size of a pea, or in larger coralloid loose or compact clusters which frequently attain the size of a large black walnut. Each tubercle is a short cylindrical blunt ended root-like structure which branches di or trichotomously after attaining a certain length. The branches frequently rebranch at their tips which grow out into long thread-like structures from 1-3 cm. in length,

which may also branch and become entwined about the roots of other plants. The maximum length of a tubercle is five mm. The average length of the branches is from 2-3 mm. The color of the youngest tubercles is a pinkish gray brown. As the tubercles become older their color changes to brown, dark brown, and even black.

HISTOLOGY.

The tubercles when studied microscopically exhibit the following structural detail:

A cork constituted of from 2 to 4 layers of suberous cells, whose outer ones are dead, filled with gummy lignin, and in the process of exfoliation, forms the external bounding layer. The cork tissue is derived from the outer layer of pericambium of the host root which functions as a phellogen during the development of the tubercle. Beneath the cork lies a very broad cortex which, instead of being formed as in normal roots of 5-12 layers of cells separated by large intercellular air spaces, is constituted of from 15-24 layers of very closely united parenchyma cells. The outer 3-5 layers of this region are composed of rounded to tangentially elongated cells, some of which contain starch grains, others tannin, a few gummy lignin. Underneath this lies a zone usually 2 to 3 cells broad of radially elongated cells and a few smaller rounded cells which are separated by small air spaces. The radially elongated cells are hypertrophied and contain the Actinomyces parasite, which may or may not be enveloped by gummy lignin. Many of the abutting smaller cells are rich in tannin and show no evidence of the parasite. Beneath this zone of infested cells is found usually a broader zone of smaller isodiametric cells intermingled with a few oblong cells. In M. Caroliniensis as noted by Chevalier, in M. Macfarlanei, M. Gale and Comptonia asplenifolia as noted by the writer, it frequently happens that other cells scattered without order throughout the cortex are also infested by Actinomyces. These, like those of the infested radially elongated zone, are also hypertrophied. All of the infested cells are united by means of Actinomyces threads which run through the cell walls from cell to cell as well as the intercellular air spaces. The endodermis or innermost layer of the cortex is composed of small oval thick-walled cells which contain a yellowish brown substance (gummy lignin). The walls of these cells become suberized very early. Underneath the endodermis is found the vascular cylinder, which is quite reduced in size as compared with that of the normal root. In the young tubercle it is constituted of a radial tetrarch fibrovascular bundle which surrounds a small pith. The phloem elements of the bundle become inactive very early. The xylem is composed mostly of wood fibres intermingled with a few tracheæ. Secondary development is of very short duration.

In the younger tubercles the vascular cylinder extends only part way into the apex, while in older ones the cylinder with some cortical parenchyma cells surrounding it grows out into a slender thread from which lateral branches are then cut off.

Actinomyces living in the tubercles is best observed in its various relations, in a radial longitudinal section. There the youngest stages may be observed in the meristematic region of the apex, while the older stages may be traced back toward

the base of the tubercle. As observed by Shibata in M. rubra tubercles, the writer has likewise noted in the case of the tubercles of M. cerifera, M. Caroliniensis, M. Macfarlanei, M. Gale, and Comptonia asplenifolia that the differentiation of the ring of infested cells starts in the meristem near the growing apex, thus indicating that infection takes place acropetally. Since tubercles are found on the seedling roots of 5-6 months' growth, it would indicate that infection takes place very early in the life of the young seedlings.

These infested meristematic cells become radially elongated and are found to contain several extremely fine non-septate and branched thread-like structures. The Actinomyces (Streptothrix) threads extend through the transverse and longitudinal walls of these cells, aided evidently by the secretion of a ferment which dissolves the cell wall in the line of the organism's progress. They then invade neighboring cells where they run between the starch grains toward the nucleus around which the organism seems to derive its greatest benefit. Shortly after the appearance of parasite threads within the invaded cells, the starch grains become dissolved, and in this form are appropriated as food by the organism. The nucleus becomes hypertrophied and finally perishes. The endophyte by this time has grown very rapidly into a dense thready reticulum. Gummy lignin appears at first of a clear yellow color, but later becoming yellowish brown. The dense thready web of the organism sends out clusters of radial thread branches forming an Actinomyces rosette, which in many cells completely fills up the whole cell lumen. These threads frequently become club-shaped at their extremities. Some of the threads after piercing through the wall of a cell develop clavate ends. In due course of time, as is evidenced in many older infested cells, the fungal threads become shrunken together and impregnated with gummy lignin, forming a good-sized lump of degenerating material within the cell, which remains connected with similar lumps, or mycelial webs of adjacent cells by means of threads which penetrate the cell wall. Some of the cells containing the rosettes also show coccus-like forms, while other cells, especially in the older basal portion of the tubercle, are almost completely filled with these. These cocci are probably products of the disintegration of the filament. They may be involution forms of the Actinomyces organism which appear in cells whose contents are poorly adapted to the trophic needs of the endophyte. Their presence in such large number on artificial culture media would support this hypothesis. While Actinomyces is the primary infecting agent responsible for the tubercles on Comptonia asplenifolia, there frequently later appears in the cells and intercellular air spaces of some of the tubercles a mycelium producing fungus with unseptate hyphæ belonging probably to the Comycetes, as Harshberger suggested. The hyphæ of this fungus are several times as thick as those of Actinomyces. They penetrate through the cell walls of the tubercle, passing from cell to cell, and often coil up into a mycelial mass in many of the cells invaded.

Since Actinomyces is frequently a virulent pathogenic organism in cattle, and other domestic animals up to man, because the swellings it produces on plants are analagous to those on animals, since the forms of the organism as shown by Jordan[18] in the infested lesions of animals are similar to those which the writer has described in the lesions of Myrica, and since the cultural characteristics of

the organism isolated from the lesions of animals by Wright,[19] Wolff and Israel[20] are in many respects similar to those isolated from the Myricas and described by the writer, he would regard the organism as a parasite and suggest its possible pathogenic relation to such animals.

The Actinomyces not only confines itself to the cortex of the tubercular roots, it later works its way into the tracheæ of these structures, passes into the pitted vessels of the main roots, thence into those of the stems, and, conveyed by the transpiration stream gradually upward, is carried through the axes of catkins so as finally to reach the flowers, bracts, and fruits. In these it confines its existence to the parts corresponding to the mediocortex of the root tubercles, namely, the mesophyll and outer mesocarp regions respectively.

The writer having isolated the organism in pure culture from the lesion produced by it on the seedling tubercles, hereby assigns to it the name Actinomyces Myricarum.

Actinomyces Myricarum has been observed by the writer in its most luxuriant form in the cells of the middle fruit wall of the various species studied. Here it can be recognized best in thin hand sections stained with safranin and methyl-green in the form of rosettes almost filling the cell lumina. When the fruits fall to the ground and subsequently break open their walls, the organism probably makes its way from the infected cells into the soil where it spreads through wide areas infecting the roots and stems of other Myricas and producing characteristic lesions.

LITERATURE CONSULTED.

7. p. 124-139, Chevalier: Monograph des Myricacees, Memoires de la Societe Nationale des Sciences Naturelles et Mathematiques de Cherbourg.
13. Brunchorst: Die Structure d. Inhaltskörper in d. Zellen einiger Wurzelanschwellungen, Bergens Museums, Arber, p. 233, and plate 21.
14. Moller: Beitrag zur Kenntnis der Frankia subtilis Brunchorst, Berichte der deutschen Bot. Gesellschaft, p. 215-224 (1890).
15. Shibata: Die Wurzelanschwellungen von Alnus und Myrica in Cytologische Studien über die endotrophen Mykorrhizen, Jahrbücher, für wissenschaftliche Botanik, Bd. 37, p. 668-670.
16. Harshberger: The form and structure of the mycodomatia of M. cerifera, Proc. Acad. Nat. Sci of Phila., 55: 352-361.
17. Arzberger: The fungus root tubercles of Ceanothus Americana, Elaeagnus Argentea and Myrica cerifera, Missouri Botanical Garden Report, p. 82 (1910).
18. Jordan: General Bacteriology, p. 404-413 (1908).
19. Wright: Pub. Mass General Hosp., Boston, 1905; Journal Med. Res., 8, p. 349 (1905).
20. Wolff and Isreal: Arch. f. path. Anat., 126, p. 11. (1891).

THE AVERAGE COST OF PRESCRIPTIONS.

The following schedule was prepared by F. W. Nitardy and is compiled from answers to *Question* 15, of the Colorado Pharmaceutical Association, presenting the following interrogatories: "Have you ever taken the time to calculate the average cost of prescriptions and the average price received? Can you furnish us with figures, giving:

A—Cost of materials used in filling 1,000 consecutive prescriptions?

B—Estimate of the number of hours of time required to fill them?

C—Cost of containers, labels, corks and other incidentals necessary?