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BIBLIOGRAPHIC INFORMATION

LOCAL ID
AUTHOR
TITLE Centralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten. 1. Abteilung, Medizinisch-
IMPRINT Jena : G. Fischer, 1902-1945.
ISSN 2698-7031

ARTICLE AUTHOR Conn, H.J.
ARTICLE TITLE The Cholodny technic for the microscopic study of the soil microflora
FORMAT Serial
EDITION
VOLUME 87
NUMBER
DATE 1932
PAGES 233-239

INTERLIBRARY LOAN INFORMATION

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Zentralblatt

für

Bakteriologie, Parasitenkunde und Infektionskrankheiten

Begründet von Oskar Uhlworm

Zweite Abteilung:

Allgemeine, landwirtschaftliche, technische, Nahrungsmittel-Bakteriologie und Mykologie (einschließlich der Gärungsphysiologie und Enzymologie), Protozoologie, Pflanzenkrankheiten und Pflanzenschutz, sowie Tierkrankheiten (ausschließlich der in das Gebiet der Medizin gehörenden)

herausgegeben von

Oberregierungsrat Dr. C. Stapp
Berlin-Dahlem, Königin-Luise-Str. 17/19

87. Band

Mit 70 Abbildungen im Text und 5 Tafeln

Jena
Verlag von Gustav Fischer
1932/33

Mac Whorter, Gr. P., Destruction of mosses by lichens. (Bot. Gaz. Vol. 72. 1921. p. 321—325.) — Palm, B. T., The morphological relationship between lichen-fungi and their algal host-plants (in manuscript). — Philipps, J. F., The influence of Usnea (near barbarta Fr.) upon the supporting tree. (Trans. Roy. Soc. So Africa. Vol. 17. 1929. p. 101—108.) — Porter, Lilian, On the attachment organs of the common corticolous Ramalinae. (Proc. Roy. Irish Acad. Vol. 34. 1917. p. 17—32.) — Richter, A., und Orlowa, K., Quantitative Feststellung der Algen-vegetation in dem Boden bei Saratow. (Journ. f. Landwirtsch. u. Wissensch. Moskau. Bd. 5. 1928. S. 315—323.) — Sparrow, F. K., Jr., Two new species of Pythium parasitic on green Algae. (Ann. of Bot. Vol. 45. 1931 a. p. 257—277.) — Sparrow, F. K., jr., Observations on Pythium dictyosporum. (Mycologia. Vol. 23. 1931 b. p. 191—203.) Waksman, S. A., Principles of soil micro-biology. (Baltimore 1927. pp. 897.)

The Cholodny Technic for the Microscopic Study of the Soil Microflora.

[From the bacteriological laboratories of New York Agricultural Experiment Station, Geneva, N. Y.]

By **H. J. Conn.**

With 4 figures in the text.

Not long ago a paper by Cholodny (1931) appeared, the full significance of which does not yet seem to be appreciated by soil microbiologists. This paper presented a new procedure for the direct microscopic study of the soil microflora and introduced an original and very simple principle of technic. This new idea was to incubate the slide in the soil, instead of transferring the soil organisms to the slide by drying soil infusion on it or otherwise. The method promises to give an entirely new conception of soil microörganisms in their mutual relationships and to offer an interesting method of attack in studying floral changes.

The only reference in the literature yet noticed which shows an appreciation of Cholodny's contribution is by Waksman (1932). In the second edition of the latter's book on Soil Microbiology, the plate illustrating the microscopic appearance of soil organisms has been revised, and all but one of the new illustrations in this plate have been taken from Cholodny. Even Waksman apparenty fails to realize the importance of the procedure, and after giving details of the method, adds a criticism of the microscopic technic in general which is repeated from the first edition of the book. This criticism states that the microscopic procedure is not applicable to quantitative work, altho admitting that it has its value for qualitative purposes. This criticism is hardly fair as applied to the Cholodny method, as this procedure was not intended for quantitative work, and a valuable technic should certainly not be criticised for failing to serve a purpose for which it was not designed by its author.

Historical.

The first suggestion of the direct microscopic study of soil microörganisms seems to have been made by the writer (1917) in a paper before the Society

of American Bacteriologists. This was shortly followed by a more detailed discussion of the procedure and its application (Conn, 1917). The procedure given then has been slightly modified later (see Conn, 1926, 1928). The method is essentially to make an infusion of soil (1 to 9) in dilute gelatine (0.015%), to dry this on a slide and stain with phenolic rose bengal. This dye was selected as giving the desired degree of intensity, that is staining the bacteria sufficiently for easy recognition without coloring the dead organic matter too deeply. The latter papers above mentioned show that other dyes of the same group, such as erythrosin or phloxine (cyanosine) can also be used with practically as good results. The writer continues to prefer rose bengal on account of its shade, but has no objection to erythrosin which has been preferred by Winogradski and Rossi, or to phloxine.

No serious attention was given to the method by other bacteriologists, nor was it developed further by the writer, until Winogradski (1924, 1925), in a series of papers discussed the direct method of studying bacteria in soil. Much of Winogradski's procedure is cultural and should hardly be called a "direct method"; but part of his technic is a modification of the writer's microscopic method. The most important modification consists of the use of a centrifuge to obtain several fractions of the soil infusion, in one of which the desired microörganisms are concentrated. Altho this modification is valuable in concentrating organisms that occur in small numbers only, it makes the method less "direct" than that originally proposed by the writer. The only other significant modification by Winogradski was to employ erythrosin instead of rose bengal, a change which is of little significance, as explained above. Erythrosin is possibly to be preferred to rose bengal in soils of high humus content, but with the ordinary agricultural soils of the United States the writer prefers rose bengal. Winogradski employed this microscopic technic primarily in studying the occurrence of Azotobacter in soils. In his later work in which he has developed methods for studying the distribution of other kinds of bacteria in soil, he has preferred the use of silicate jelly plates rather than the microscopic technic; accordingly, the latter has not been further developed by him.

Meanwhile Winogradski's endorsement of the microscopic procedure has brought it to the attention of various European soil bacteriologists, some of whom even seem to be unaware that it did not originate with him, altho Winogradski makes proper acknowledgment as to its authorship. Several of these later investigators have made important modifications of the procedure. Notable among these are the contributions of Koffman (1928, 1931), Rossi (1928), Rossi and Riccardo (1927), Rossi and Gesuè (1930) and Cholodny (1930).

Koffman's work is intended less to develop a direct method than to adapt the microscopic technic for accurate counting. The usual microscopic technic has, as stated above, been criticised because accurate counts cannot be obtained by it. Koffman, on the other hand, has devised quite an elaborate apparatus for agitating and sampling soil infusion and has worked out methods for fixing and staining an accurately measured portion of the infusion without drying it to the slide. By means of this method none of the soil particles or microörganisms are lost so that a quantitative determination of the different kinds of microörganisms seen can be made.

Also of interest in connection with Koffman's paper is the dye which he employs. He calls the dye "alcohol soluble cyanosin" and very commendably gives its formula so that one can tell just what he is using. The dye is the ethyl ester of cyanosin or phloxine. This is a dye with which the writer has had no experience. It should bear the same relation to phloxine that alcohol soluble eosin does to eosin Y. This dye certainly deserves study as a stain for bacteria in soil.

Rossi's procedure is entirely different and is, beyond question, the most direct method for examining bacteria in the soil that has been proposed. A vertical surface of soil is exposed and a slide held in a special holder is pressed against the surface. The slide is then removed and the contact preparation thus obtained is stained with erythrosin. This method shows the colonies of microörganisms as they actually occur on the soil and brings out many points of interest. It has not as yet been much employed elsewhere, partly because of the difficulty of obtaining a holder for the slides such as described by Rossi.

The modification of the microscopic method which is being considered in the present paper was proposed a few years ago by Cholodny (1930). The procedure as described by its originator is to cut a shallow trench in the soil and then to press a slide against the vertical surface of the undisturbed soil with one end of the slide a little above the top of the soil. The method differs from that of Rossi, however, in that the slide is not removed immediately but the soil is packed in loosely behind it and the slide is allowed to stay in place for some time. When it is finally taken out, the soil is removed carefully behind the slide and the latter pulled sharply away from the undisturbed soil so as to leave the film attached to the slide which has been formed by the microörganisms growing upon it. This slide is dried and stained.

Fig. 1. A tumbler of soil with two slides inserted, for studying the flora by the Cholodny method.

Methods Employed.

The procedure adapted in the present work is that of Cholodny with only very minor modifications. Instead of digging a trench in the field soil for inserting the slide, the soil is placed in jelly tumblers provided with a cover and two slides are inserted. (See figure 1.) Altho soil thus handled is likely to be quite different from that in the field itself, this modification gives the advantage of making it possible to control the conditions and to investigate the effect of various treatments on the soil to much better advantage than can be done in the field.

A second slight modification is in the time of incubation. Cholodny recommended about two weeks before removing the slide, while the writer has ordinarily found more interesting results if the slides are kept in place

only about 5 to 7 days. Actually the time of incubation has varied. Usually one slide is removed in about five days and a new one put in its place. The second slide is removed in about seven days from the time of insertion, and the slide inserted where the first was removed is taken out after being in place five or six days.

The stain employed in the present work has been rose bengal, not erythrosin as employed by Cholodny.

The formula is as follows:

Rose bengal	0 g
Calcium chloride	0.01 g
5% aqueous phenol	100 cc.

Two methods of applying the stain have been tried. One method is to let the slides lie flat on a surface over a boiling water bath and to pour the stain on top of the slide allowing it to stay one minute, replenishing

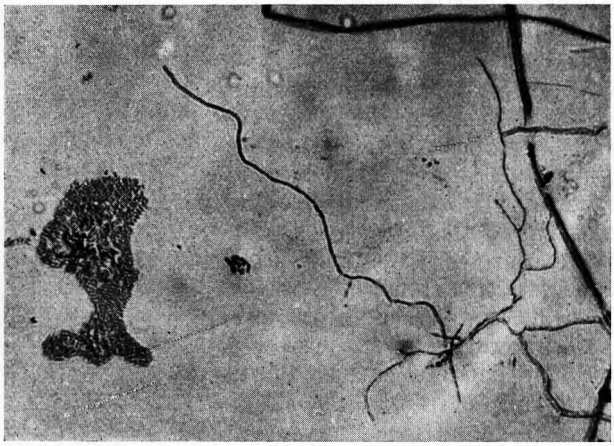

Fig. 2. A colony of bacteria, a small Actinomyces colony, and a fungus filament, as shown by the Cholodny technic.

it occasionally to keep it from drying out. The second method is to keep the stain in a staining jar which sets in boiling water, then to insert the slides after the stain inside the jar begins to steam; the slides are allowed to stay in the hot staining fluid for one minute. The former method seems to give the better stained preparations.

Use of the Procedure.

Cholodny's paper is accompanied by a considerable number of photo-micrographs showing various types of colonies observed in the soil by this method. Practically all of the forms photographed by him have been observed here. One or two illustrations accompany this article.

Figure 2 is interesting as showing a fungus filament crossing the field, an Actinomyces colony and a colony of bacteria.

Figure 3 shows a fungus filament surrounded with bacteria. This phenomenon is shown by Cholodny in his Figures 8 and 9 and has been frequently observed here. It seems to be of some significance in soil but its

exact meaning is not yet determined. The bacteria surrounding these mold filaments might be merely physically attached, but the frequency with which such filaments are found to be devoid of stainable contents suggests that they are dead and that the bacteria are living upon the organic matter of which they are composed. Indications are at hand that this phenomenon is likely to be noticed when a predominately fungal flora is being altered to a bacterial flora in some soil.

The method is being used in the writer's laboratory for three different purposes, for all of which it appears to have considerable promise: First, in determining differences in the flora of different soils; second, in determining the effect of fertilization and moisture changes on the flora; third, studying colony production in pure culture.

The first of these three purposes, that is, studying differences in the flora of different soils, is one of the lines in which this procedure seems most

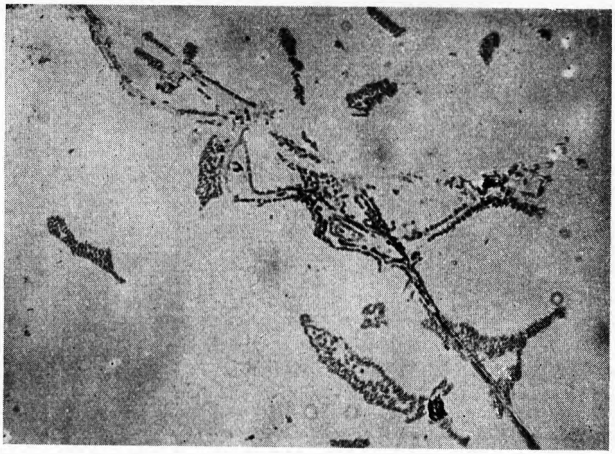

Fig. 3. A decaying fungus filament in soil, surrounded by masses of bacteria.

interesting. This is the case because it is quite difficult by many bacterial methods to bring out the distinct differences between different soils. The Cholodny method, on the other hand, shows very distinct differences. Under some conditions the flora appears to be predominately fungal; under others bacterial. These distinctions show up by this technic even tho plate method and the usual microscopic technic show no appreciable differences. It is still uncertain, to be sure, whether they represent inherent differences between the soils or merely those due to treatment. Altho this point still remains to be determined, the great differences that have been observed are very promising, in view of the lack of variation observed by other methods.

The second application of the method which seems to be important is in studying the effect of soil treatment upon the flora. This is difficult to undertake by the procedure originally described by Cholodny but is easy to accomplish when the method is modified by the use of tumblers as here suggested. It has already proved possible by the addition of mineral salts and by changes in moisture content to alter the flora of a soil from one type to another, and such changes are brought out very distinctly by

this procedure. In the writer's opinion this is at present one of the most promising uses to which the method can be put.

Whether the procedure be employed for studying differences between soils or effects of treatment of a soil, one defect of the method must be taken into account: namely, that there is no way of securing a representative sample to examine. There is evidence that the picture may be distinctly different on two slides a short distance apart. One must, therefore, examine several slides from the same soil under identical conditions before drawing definite conclusions. It still remains to be determined how serious this weakness of the method is in practical use.

Another weakness of the technic at the present time is the inability to judge the species of the different forms observed. This is particularly true in the case of bacteria with a very slight morphological variation. Molds and Actinomycetes, on the other hand, may well prove much

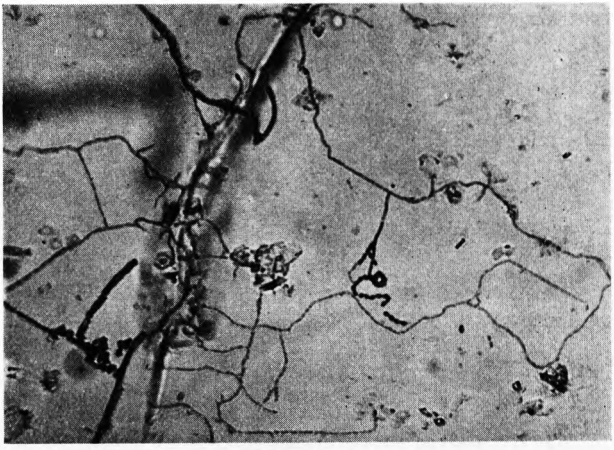

Fig. 4. An Actinomyces colony in soil. The Cholodny method furnishes a picture of conidiophores of both Actinomycetes and fungi in their natural orientation, thus giving some clue as to identity of species.

easier to separate one from another. One of the strong points of the procedure is that the different organisms occur in their natural orientation. In the case of the two groups of organisms just mentioned, it is particularly important because it shows arrangement of the spores and in this way may give an actual clue as to the identity of species. No such definite information can be obtained in the case of bacteria altho it is noticeable that they occur in characteristic colonies. This is the reason for the third application of the method suggested above; namely, the suggestion of colony production in pure culture.

The exact technic for this pure culture work has not yet been perfected. It is, of course, necessary to sterilize the soil, to inoculate it, and to maintain it free from contamination. This can sometimes be done in tumblers by keeping their tops wrapped in cotton. The method is awkward and not always efficient, however, and it is hoped to improve upon it. There seems to be no reason why large test tubes and unusually narrow slides cannot be used for the purpose. Little has as yet been done here in the study of

pure culture by this method and no knowledge of their natural colony forms has been obtained that is at all conclusive. The idea is merely offered here as a suggestion of a possible valuable application of the procedure.

Value of the Method.

The real point to this paper is not to present any results obtained by the means of this procedure, but merely to call attention to the possible value of Cholodny's method. Very little attention has yet been given to the procedure among soil bacteriologists and the writer feels that it should come into general use. The method shows distinct differences between the flora of various soils. It shows so plainly the characteristic colony formations of the organisms and finally brings out so beautifully the occurrence of some soil organisms in association with others, that the wide use of this method may well disclose various secrets of soil microbiology.

Expecially important seems to be the fact that the Cholodny method is satisfactory to indicate a change in the predominating flora of a soil from fungi or Actinomycetes to bacteria or vice versa. This method seems to be the only one so far proposed that can bring out this point, since the plate method tends to count only the spores of the filamentous organisms, while the earlier microscopic methods fail to show these organisms in anything like their true abundance.

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