II.—STUDIES ON SOIL ACTINOMYCES. PART II.
Their Mode of Occurrence in the Soil.

By V. Subrahmanyan.

The investigations of Hiltner (*Jahrb. Ver. angew. Bot.*, 1907, **5**, 200), Conn (*Jour. Amer. Soc. Agron.*, 1913, **5**, 218), Krainsky (*Zentr. Bakt.*, II, 1914, **41**, 649), Waksman and Curtis (*Soil Sci.*, 1916, **1**, 99) and others have shown that the numbers of soil *Actinomyces* appearing on plates can vary according to the conditions obtaining in the soil. Although not indicating whether the organisms at the time of counting occurred as filaments or as conidia their results suggest that the *Actinomyces* must have existed in the vegetative condition for a brief period at least.

Conn (*Soil Sci.*, 1922, **14**, 149) by his direct staining method was able to demonstrate the presence of filamentous forms in soil in which young grass was growing and, associating this observation with his previous plate-counts of *Actinomyces* from the sodden soil, inferred that these filaments belonged to the group of ray fungi. But he did not prove the absence of fungal filaments which, in many cases, appear identical with those of *Actinomyces*, and which might have accounted for all the stained hyphae noticed by him. Waksman, by his direct inoculation method (*Soil Sci.*, 1922, **14**, 153) demonstrated the presence of vegetative fungi in the soil, but not the freely occurring hyphae of *Actinomyces*.

The determination of the exact condition in which *Actinomyces* occur in the soil is of great importance in assessing the value of the part played by the organisms in various phenomena. Thus, if a certain form of readily decomposable matter be added to the soil, the *Actinomyces* if present in the vegetative condition, will compete with the other organisms and utilise it for effecting the different changes characteristic of them but, if present exclusively as spores, the bacteria and other forms would use up the material present before the *Actinomyces* could germinate, while if present partly in the vegetative and partly in the spore condition, they will play a part intermediate between the two cases described above. The object of the present investigation was to define the exact condition in which *Actinomyces* occur in the soil.

EXPERIMENTAL.

A preliminary series of trials was carried out, with pure strains of *Actinomyces*, to determine whether the hyphae and the conidia could be identified as such when occurring either free or mixed with the soil.

Two common soil forms, *A. chromogenus* (Gasperini)[1] and *A. reticuli* (Waksman and Curtis), were used throughout the following series. The strains had been raised on starch-agar (Waksman, *Soil Sci.*, 1919, 8, 71) making satisfactory growth, and the cultures were about a month old at the time of usage. Growth-circlets of about 2 mm. diameter were removed with a sterile platinum needle and transferred to stoppered conical flasks each containing 200 c.c. of sterile saline (5 gms. of sodium chloride and 1 gm. of hydrated magnesium sulphate dissolved together in 1 litre of distilled water). The flasks were shaken vigorously to ensure even distribution of the broken hyphae and the conidia: the contents were then halved and while one portion, in each case, was left undisturbed, to each of the others soil (10 gms.) was added and the flasks again shaken to secure uniform dispersion.

The soil used was a surface specimen from low-lying paddy land at Dacca, Bengal, and was chosen because of its extreme uniformity and general freedom from plant residues and sundry forms of organic matter. The soil was air-dried and passed through a millimeter-mesh sieve before usage. Loopfuls of the suspensions, with and without the added soil, were examined directly under the microscope. It was observed that even with pure suspensions the conidia, which were small, short, rod-like forms as distinguished from the big, round or oval spores of fungi, were more prominent than the hyphae which, in spite of the shaking, tended to cohere and could not be readily observed. In the soil suspension neither the hyphae nor the conidia could be distinguished from the other forms of micro-organisms.

Conn's direct staining method (*loc. cit.*) was next tried. In addition to Loeffler's alkaline methylene blue, saturated aqueous solutions of six different commercial preparations of methylene blue and erythrosin were used to stain the organisms after fixing them on the slide. It was observed that the hyphae took the stains readily though they could not be obtained as evenly dispersed as their numbers would warrant. The process was repeated several times, but always with the same result. Similar trials were carried out with suspensions of two fungi, *Aspergillus niger* and *Penicillium glaucum*, but in these cases

[1] The author desires to take this opportunity of thanking the Curator of the National Collection of Type Cultures, Lister Institute, London, for his courtesy in supplying the cultures.

the stained hyphae were found to lie evenly dispersed and could, therefore, be readily distinguished from the other microflora.

Further observations showed that the actinomycetic mycelia, which were very much more interwoven than those of the fungi, could not be dispersed unless they were teased out with the needle. Such a procedure would not, of course, be possible when attempting to detect their presence in the soil. Since, it was also noticed that the actinomycetic hyphae, while generally thinner (1·6–2·3 μ), could not be readily distinguished from those of the fungi (2·1–3·8 μ) in the stained preparations, it may be inferred that Conn's method cannot be adopted satisfactorily for the detection of *Actinomyces* if they occur vegetatively in the soil. The conidia measuring 1·8–2·5 $\mu \times$ 0·8–1·1 μ did not in any case take the stains. When mixed with the soil suspension they could not be distinguished from the bacterial forms occurring therein.

Before trying Waksman's direct inoculation method (*loc. cit.*) it was felt necessary to determine (*a*) the maximum period required for the vegetative hyphae to multiply and form colonies of visible size, (*b*) the minimum period required for the spores to germinate, so that a period intermediate between the two could be chosen to distinguish the colonies originating from the hyphae from those derived from the spores.

Suspensions of *A. chromogenus* and *A. reticuli* in sterile saline with and without admixture of sterile soil were prepared in the same way as in the previous trials with the difference that the cultures were only ten days old and that the conidia were suspended separately from the mycelia. The separation was effected by removing gently, from each colony, the white, woolly portion in the case of *A. reticuli*, and the greyish, powdery crests in the case of *A. chromogenus* which contained most of the conidia, with a sterile platinum needle and washing the residual portion with sterile water until traces of the whitish or grey crests disappeared completely. The portions first removed with the needle, and the residues after washing, were suspended separately in sterile saline. Microscopic examination of the suspensions showed that the former were composed entirely of conidia, and the latter, almost completely, of fragments of mycelia. Each loopful contained many conidia in the one case and many bits of hyphae in the other; there were no blanks. Attempts to free the suspensions of hyphae from the small numbers of conidia that appeared to cling to them were not successful. Subsequent observations showed, however, that complete separation was not necessary for the germination studies.

Inoculations of suspensions were made by taking out loopfuls and depositing them on isolated spots on sterile starch-agar. Ten such

inoculations were made on each petri-dish and using three plates for each specimen, thirty trials were thus carried out in each case. The plates were incubated at 25–30° and observations on the growths of colonies made at frequent intervals. The hyphae germinated quickly, though not under twenty-four hours as the fungal hyphae were observed to do (Waksman, *loc. cit.*). Very small spot-colonies were visible after two days, but it was not until the beginning of the fourth day that the growths became distinctly visible.

Among the thirty sowings, hyphae of *A. chromogenus* were observed to have germinated in nineteen and twenty-one cases when inoculated from sterile saline and from soil suspensions, respectively. Similar counts for *A. reticuli* were sixteen and fifteen respectively. Since germinations of the same set on individual plates tended to vary from each other by one or two colonies, it may be inferred that the hyphae of both strains germinated nearly to the same extent both in saline and soil suspensions. It is, however, noteworthy that though the cultures were young, under two-thirds of the total number of inoculated hyphae could germinate. From Orskov (Investigations into the Morphology of Ray Fungi, 1923), it may be inferred that fragments from older mycelia would, under similar conditions, germinate in very much smaller numbers.

The plates inoculated with suspensions of the conidia showed no signs of germination when colonies began to appear on the other sets of plates. The first colony of *A. chromogenus* became visible only on the sixth day and that of *A. reticuli* on the seventh. It was further noticed that at the end of a fortnight, less than half the number of conidia came up on the plates, and that many had not germinated even at the end of one month, as seen from Table I.

TABLE I.

Suspending medium	*A. chromogenus*		*A. reticuli*	
	Days			
	14	30	14	30
Sterile Saline	14	14	11	13
,, ,, + Soil	12	13	11	11

The foregoing observations showed that the hyphae germinated much more quickly than the conidia on suitable media; and that following direct inoculation, colonies appearing on the plates on the fourth day suggest the presence of *Actinomyces* in the vegetative condition in the soil. In view, however, of the observation that all the bits of mycelia that were present could not come out equally well, it may be argued that the germination test will not provide a conclusive proof of the absence of hyphae in the soil: but it will show whether they are present in the active condition and can readily respond to soil treatment.

Persistence of Hyphae in the Soil.

To ascertain whether the vegetative mycelia of *Actinomyces* can persist in the active condition in the soil for any great length of time, trials were carried out by inoculating the mycelia of each of the two strains into four different types of soil and observing their conditions from time to time. The four soils were from different provinces of India and represented the alluvial, laterite, black cotton and peaty types respectively. Air-dry specimens ot these soils, after being powdered and passed through the millimeter-mesh sieve, were uniformly spread into petri-dishes in portions of 10 gms. After sterilising the dishes and their contents by autoclaving at 20 lbs. for 15 minutes, the soils were moistened with sterile tap water in quantities just sufficient to cause the particles to adhere together and to the plates, thus forming media [1] containing only the natural soils, but comparable with any artificial medium set in gelatin or agar with regard to ease of handling.

In the middle of each 'soil-plate' circlets of half-inch diameter were marked with Indian ink and sown thickly but evenly with actinomycetic mycelia. Specimens were taken out from day to day with a sterile platinum needle and examined microscopically for their respective condition. It was noticed that even at the end of three days the hyphae were observable only sparsely: on the seventh day they were hardly visible on examination either directly or after staining.

To ascertain whether the hyphae had merely split into invisible fragments, but remained viable, direct inoculations were made on the seventh day from the soil-plates into starch-agar as already described, and the growths obtained after incubation for three days noted. It

[1] Similar 'soil-medium plates' have been used by the author for various examinations of soils, and have always proved very satisfactory. Their use can be safely recommended for such chemical and biological trials as cannot be conveniently carried out with large and heterogeneous masses of soils.

was observed that on nineteen out of the twenty-four plates examined there were no colonies at all: the remaining plates carried only one to three apiece, the average per plate being less than 0·3 of a colony. Although the direct examination threw no light on the changes undergone by the inoculated hyphae, yet the germination studies showed clearly that the bits of mycelium, even if they existed as such, could no longer germinate as they would have done when transferred from one nutrient medium to another.

To determine whether the organisms remained viable in the form of conidia, the plates were incubated for a fortnight and the growths appearing at the end of that period noted. It was observed that there were more colonies on the plates than at the end of three days. The average was 5·0 colonies per plate, that for *A. chromogenus* (5·3) being slightly higher than that for *A. reticuli* (4·7). The results while indicating that the new colonies were probably derived from conidia, did not show whether the latter were (1) newly formed from the mycelia, or (2) merely those already present with the bits of mycelia, or (3) partly (1) and partly (2). The fact that the colonies appeared on only five out of every ten inoculated spots suggested however, that their numbers could not have been large.

To decide whether the hyphae passed into the conidial form and, if so, to estimate them quantitatively, an attempt was made to count the numbers of conidia present at different stages with the haemocytometer. In view of the difficulties attending direct microscope examination of the soil, acid-washed quartz sand (passing the 30-mesh sieve) was used in its place. The growths of the two *Actinomyces* on starch-agar were removed at the end of ten days, shaken with sterile water (10 c.c.), aliquot parts (3 c.c.) were pipetted aseptically on sterile quartz (25 gms.) in 250 c.c. conical flasks and incubated at 37°. The contents of the flasks were moistened from time to time with sterile water to prevent their drying. At four days' intervals the flasks were taken out, the volumes of suspensions made up to 25 c.c. with sterile saline, loopfuls removed and the number of conidia counted.

In the earlier stages some difficulty was experienced in counting, owing to the small size of the conidia and their general similarity to the shorter lengths of hyphae: but, later, it was found possible to distinguish them readily from the latter by their (1) oval shape and perfectly round edges, (2) greater breadth and thicker membrane and (3) comparatively greater opalescence; all these features rendered them much more prominent than the hyphae in the counting chamber.

The periodic countings were repeated later with two other strains of *Actinomyces*, *A. scabies* (Thaxter) Gussow and *A. albosporeus Kr.* (Waksman and Curtis) after four days' growths on potato plugs. The combined results are presented in Table II.

TABLE II.

Strain	No. of conidia per c.c. × 10,000		
	0 days	4th day	8th day
A. chromogenus	214	126	84
A. reticuli	132	74	38
A. albosporeus	175	56	33
A. scabies	158	125	80

With the marked decrease in the numbers of conidia it was also found that the hyphae observable in quite large numbers initially became later more and more scarce. In the case of *A. albosporeus* they were not to be seen at all on the eighth day.

It may be inferred that (1) even the conidia did not entirely persist, under conditions of starvation in presence of moisture, (2) there was no appreciable conversion of the vegetative mycelium into conidia or, if there were, it was more than counterbalanced by the death of the conidia themselves. The degradation of the mycelia and the conidia could not have been due to plasmolysis or autolysis of the cells because the added water was never so excessive as to flood the sand. The only inference to be drawn is that the cells, under such conditions, underwent some auto-transformation in presence of air, which led to their rapid destruction. This transformation and its attendant biochemical phenomena are now being studied, and will form the subject of a future communication.

It should be noted that sand, with its (1) readiness to become wet or dry, and (2) utter lack of colloidal, organic or other mineral matter helping to sustain life, does not adequately reproduce the conditions in a normal soil. The destruction of mycelia and conidia would not have proceeded so rapidly in soil as on sand, which, no doubt, accounts for the fact that *Actinomyces*, as observed by a number of workers, are present in practically all types of soil though in comparatively smaller numbers than the bacteria. From the foregoing observations it may be inferred that while the vegetative mycelium

neither persisted nor changed into the conidial form after standing for some time with the soil, the conidia remained to some extent as such and in the viable condition, thereby helping to perpetuate the species.

Extended Examination of Soils.

In order to confirm the evidence from the previous observations, fifty specimens representing surface and sub-soils from different parts of India and Ceylon were examined by the direct inoculation method (Waksman, *loc. cit.*).

The localities of the soils varied from equatorial to mild temperate regions. They received rainfalls varying from over 200 inches per annum to practically nil, and represented most of the commonly known types of soils, namely, peat, clay, loam (alluvial), sand, laterite and black cotton. Their reactions varied from marked acidity to high alkalinity. They carried vegetation representative of the different types of crops found in India, namely, tea, paddy, wheat, cotton, coconut, etc. Eight of the specimens were in the wet and the rest in the air-dried condition. Further details regarding the chemical composition and other characteristics of these soils in relation to their actinomycetic flora will be presented in a later communication.

The inoculations were made by placing, aseptically, lumps of soil, each about 1 cm. in diameter, on petri-dishes containing solidified starch-agar. Five such lumps were placed on each petri-dish and since two plates were allotted for each specimen of soil, ten samples were thus examined in each case. The plates after inoculation were incubated at 25–30° and examined on the fourth day for the presence of *Actinomyces*. It was observed that there were in every case dense growths of fungi round the soil lumps, overgrowing all other types of microflora and rendering the examination exceedingly difficult. It was also noticed that the lumps of soil were inconveniently large and often tended to break up readily into fragments which rolled over the plates and caused the rapid spread of fungi. To obviate the latter difficulty and to obtain large numbers of representative growths, the inoculations were made with smaller quantities of soil in a later series.

Air-dried specimens were powdered, passed through a millimeter-mesh sieve and inoculated into starch-agar plates with a platinum loop of slightly less than 1 mm. diameter. Care was taken that the inoculation was confined to selected areas of the plates. Ten such inoculations were made on each plate and two dishes were allotted for each specimen of soil. The plates were incubated at 25–30° and the growths examined on the fourth day. It was observed that though on most

plates there was no overlapping of colonies, bacteria and fungi alone grew round the soil particles. Only ten plates inoculated with five soils carried colonies of *Actinomyces*. Eight of these had only one or two colonies apiece; but on the remaining two, inoculated with specimens of a surface soil from a paddy cultivated area from Chirakkal, South India, colonies of *Actinomyces* appeared on every one of the inoculated spots and outgrew the contemporary bacteria and fungi.

Apart from the exceptional results given by this specimen, the observations confirmed the conclusions derived from the previous studies, namely, that *Actinomyces* normally do not persist in the vegetative condition in the soil. In view of the general experience that actinomycetic colonies usually appear on the plates only after about one week of incubation and the previous observation that only the conidia began germinating at that time, it may be inferred that the organisms are normally present almost exclusively in the conidial form in the soil.

Abnormal Vegetation of Actinomyces in the Chirakkal Soil.

The early germination of *Actinomyces* from the inoculations of the Chirakkal soil suggested that the organisms must have been present in the vegetative condition in that soil. To ascertain whether abnormality of the soil led to the exceptional results, specimens were examined under the microscope with and without staining. Even superficial observation with the naked eye showed that the specimen contained large amounts of undecomposed plant residues: microscopic examination also revealed the existence of such material in a finely divided condition. The stained preparations showed the presence of certain filamentous forms, but did not, as was expected, provide any evidence identifying the organisms.

The occurrence of plant residues in large amount suggested either that (*a*) the land might have been just harvested with the result that the plant roots, straw, etc., had not had sufficient time to be decomposed, or (*b*) the soil might have been given a dressing of some organic manure, just a few days before the specimen was collected.

In view of the previous observation that the hyphae could not persist in the soil, it was essential to determine how the organisms existed in the vegetative state even after the soil had been kept for several months in the air-dried state. Since, however, the specimen contained large amounts of plant residues, it appeared probable that the hyphæ occurred on the latter. To verify this, a portion of the soil was freed from the plant residues, as far as possible, with the aid of a

hand lens, and loopfuls of it inoculated into starch-agar, on ten dishes, in the manner already described. It was observed that the counts taken at the end of three days, though varying among themselves, were distinctly lower than those obtained for the portion containing the organic matter. Only one plate carried five colonies while others contained either none, or only one to three apiece, the average being 1·9 colonies per plate.

From the above observation it may be inferred that even in the soil which gave rather abnormal evidence of *Actinomyces* existing vegetatively, the organisms did not occur in the soil itself, but on the plant residues and other forms of undecomposed organic matter which were really foreign to the soil.

Effect of Different Substances on the Condition of Actinomyces.

The previous observations while showing that in the normal soil *Actinomyces* occur mostly in the conidial form, did not prove that this would persist under soil-conditions of all types. To determine whether specific nutrients or stimulants could quicken the germination of the conidia and help the organisms to function as they would if occurring vegetatively, trials were conducted by the direct inoculation method already described, adding different types of substances to the Dacca soil used in an earlier experiment. Portions of the soil (10 gms.) were weighed out into sterile petri-dishes and the following substances (0·1 gm.) added each being dissolved, suspended, or merely shaken with sterile water (3 c.c.) according to their respective properties :—

Carbohydrates.—Xylose, arabinose, dextrose, laevulose, sucrose, maltose, lactose, raffinose, maltodextrin, starch (soluble) and glycogen ; also glycerol.

Organic Nitrogen Compounds.—Asparagine, glycine, cystine, tyrosine, indole, caffeine, strychnine, peptone, casein, gelatin.

Minerals.—Ammonium sulphate, potassium nitrate, potassium sulphate, calcium chloride, calcium sulphate, calcium dihydrogen phosphate, magnesium sulphate, potassium cyanide, silver nitrate, mercuric chloride.

Plant Materials.—Dried leaf of *Lantana camara*, Linn., rice-bran, ragi-straw, powdered paddy-husk.

Organic Disinfectants, Volatile Antiseptics, etc.—Phenol, camphor, toluene, ether, chloroform, acetone, amyl alcohol, carbon disulphide.

The added materials were distributed by dropping them evenly and allowing the soil to settle down and adhere to the dishes so as to form soil-plates of the type described already. After incubating for three days at 25–30°, inoculations were made from them into starch-agar plates; these were incubated for three days and the types of growth formed on the inoculated spots noted.

It was observed that the carbohydrates and the organic nitrogen compounds brought out predominantly certain types of bacteria, and the minerals excepting mercuric chloride, bacteria together with fungi. Inoculations from the mercuric chloride plate showed the presence of only stray bacterial colonies. The plant materials caused an increased prominence of bacteria and fungi; the volatile antiseptics and disinfectants excepting camphor, of specific bacterial types only. Camphor alone quickened the vegetation of *Actinomyces*, which appeared on seven out of the ten inoculated spots. Since, however, this substance rarely finds its way to the soil, it may be inferred that the commoner mineral nutrients, and stimulants, do not excite any unexpectedly quick response from *Actinomyces*.

The foregoing observations did not, however, preclude the possibility of the conidia germinating after about one week and then competing with the other organisms for their nutrition. They may play a very important part when materials decompose slowly, as happens in the manure heap, the rotting of which generally takes several months.

SUMMARY AND CONCLUSIONS.

1. The vegetative mycelia of *Actinomyces* perished completely or passed into some non-reproductive condition after remaining for some time in the soil. They did not appreciably pass into the conidial form.

2. Though the conidia numerically decreased considerably on standing in moist sand, there was no evidence to show that they perished completely in the soil.

3. Examination of soils in large number and variety showed that their *Actinomyces* occurred almost exclusively in the form of conidia. In an exceptional case where they were observed to be present in the vegetative condition, they were so found not on the soil itself, but on the undecomposed plant residues mixed with it.

4. Different types of microbial nutrients, stimulants, etc., did not generally quicken the vegetation of *Actinomyces* when added to the soil.

In conclusion it is the pleasant duty of the author to record his sincere thanks to Prof. R. V. Norris for his keen interest in the progress of the work and suggestive criticism.

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