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Observation of Microorganisms in Soil and Other Natural Habitats[1]

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A procedure is described for visually observing and following the activities and interactions of bacteria, actinomyctes, fungi, protozoa, nematodes, and plant roots in masses of soil. Specific microscope components and objectives are used, and the numerical apertures are adjusted such that light diffraction colors are produced to allow differentiation of the various biological entities and their habitat materials. Strains or other alterations in the organisms and their habitat are not employed, and time-lapse photography can be used to follow the activities of soil microorganisms and plant roots. As a result of the use of this technique, it is apparent that in situ indigenous soil microorganisms differ from similar organisms grown in the laboratory, but that, under the proper conditions, the state of the organism in either habitat can be altered to match that which occurs in the contrasting habitat.

Casida (2) described the use of color infrared photography for the microscopic visualization of nonstained microorganisms in soil and other habitats. Although this procedure allows photographic visualization of the microorganisms, it does not allow either short- or long-term continuous observations of a single cell or group of cells, because differentiation of microorganisms and habitat materials can be made only after development of the film. Another feature of this technique is that use of the thin films of soil required for slide preparations for transmitted light microscopy markedly alters the soil and soil atmosphere conditions both for the initial observation and for further observations during incubation of the soil.

The present study describes a visual microscopic technique, not requiring photography or stains, for the continuous observation of microorganisms residing in a mass of soil or other habitat materials. This method of observation also allows an evaluation of the metabolic state of the organism in its habitat.

MATERIALS AND METHODS

Soil preparation. For observation, soil is placed as a mound on the surface of a no. 1 cover slip, or it is placed in a soil incubation chamber. The latter is fabricated by using epoxy cement to attach approximately a 1-inch cylinder (2.54 cm) cut from an 18-mm (outside diameter) test tube to a no. 1 cover slip (Fig. 1). For both procedures, the soil is moistened, since a moisture bridge is required between the soil and cover slip. For the incubation chamber, either a cotton plug is inserted in the upper portion of the container, or a polyethylene cover is secured with a rubber band. The polyethylene allows continuous incubation with atmospheric interchange but without an appreciable moisture loss. The soil incubation chamber can be autoclaved if aseptic conditions are desired. Also, seeds can be planted in this chamber to allow observations of microbial activity in the rhizoplane or rhizosphere.

Microscopy. Basically, the procedure employs a Zeiss metallurgical reflected-light microscope with an unencumbered floating stage (Fig. 2). The objectives provided with this microscope are removed and replaced by a Zeiss 100× Apochromat, Planapochromat, or Planachromat objective containing a diaphragm which allows numerical apertures (NA) from 0.8 to 1.25 or 1.32, depending on the objective. The apochromatic objectives are preferred; an achromatic objective cannot be used because of its lesser color correction. Lower magnifications are possible by employing a Zeiss 40× apochromatic oil objective with iris diaphragm. All of the above objectives normally are employed for transmitted light and not for reflected light microscopy.

This microscope is used by placing a soil sample on a cover slip or in an incubation chamber on the surface of the microscope stage with an immersion oil bridge between the objective and the underside of the cover slip. The sample can be moved in any direction merely by applying slight pressure to the edges of the floating stage. The aperture diaphragm (in this case, located in the base of the microscope) is closed to its limit, and the objective diaphragm is

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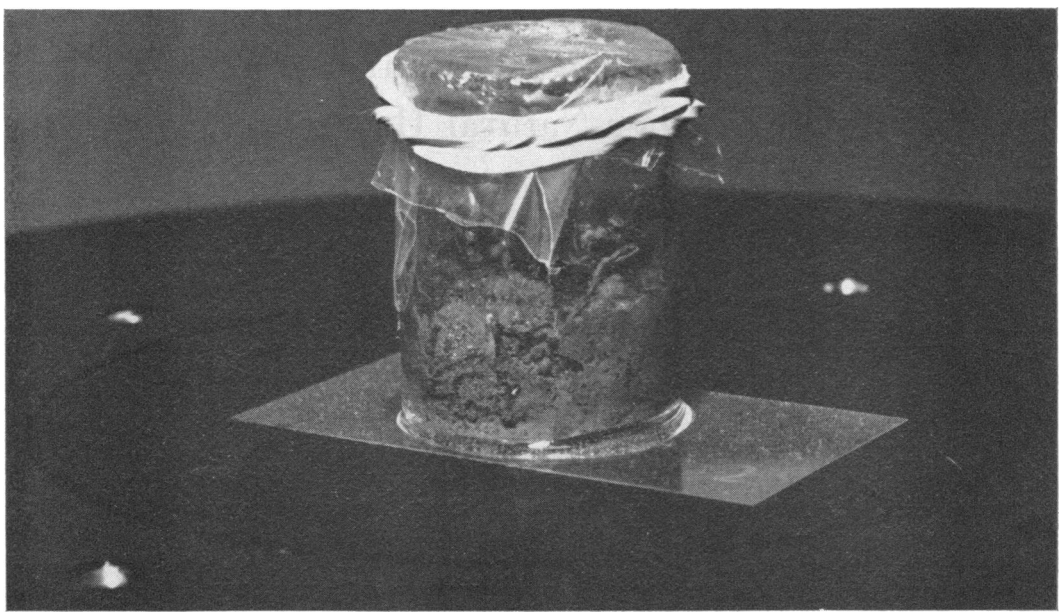

FIG. 1. *Soil incubation chamber on microscope stage. The objective is beneath the stage, and a slight pressure applied at any edge of the floating stage brings a new area of the sample into view for observation.*

set at 0.8 NA. A 12-v light source operating at 5 amp is required so as to obtain sufficient illumination for reflected light microscopy.

For observation of laboratory cultures, an aqueous cell suspension is placed on the surface of a cover slip either with or without a second cover slip or slide placed above. Growth on the surface of agar is observed by cutting out a block of agar and inverting it on a cover slip.

Photography was accomplished by replacing the binocular head with a monocular tube, into which was inserted a Leitz MIKAS with attached Leica MI camera body. The film was Kodak Panatomic X, Ektachrome X, or High Speed Ektachrome type B. The growth and activities of indigenous soil microorganisms were followed by sequentially photographing fields of interest at time intervals ranging from 15 min to 2 days.

RESULTS

When soil or marine mud is observed by reflected light with this microscope, living and dead in situ soil microorganisms on soil minerals and organic materials, or suspended in soil water, diffract light in a manner such that they appear to the eye as green, orange, yellow, red, or sometimes blue (Fig. 3). The particular diffraction color seems to be specific for each individual microbial cell but, nevertheless, contrasts with soil minerals which usually appear gray and translucent, and with the brown to black of soil organic materials. Protozoa appear translucent and colorless, but colored ingested bacteria sometimes can be observed within them. Soil nematodes also appear colorless and translucent, and the same is true for small roots and root hairs of growing plants (Fig. 4). For fungi, the light diffraction coloration is associated with a central area of the mycelium occupying approximately one-fifth of the diameter of the mycelium (Fig. 5) but ceasing just before the growing tip. The light diffraction colors for both bacteria and fungi are observed by focusing at the midplane of the cells and do not appear to be associated with either the upper or lower surfaces.

The portion of the light spectrum responsible for the light diffraction colors of in situ soil microorganisms corresponds approximately with the green light wavelengths of 490 to 500 nm. This was determined by inserting photographic filters of various colors into the light train above the aperture diaphragm while observing for losses of specific coloration of individual microorganisms.

Small fragments of mineral or crystalline materials which diffract light in a manner somewhat resembling that for microorganisms at times are observed in soil and certain other natural habitats. These can be detected by focusing on the artifact, followed by adjusting the objective diaphragm to a NA of 1.25 or 1.32, depending upon the objective being used. As

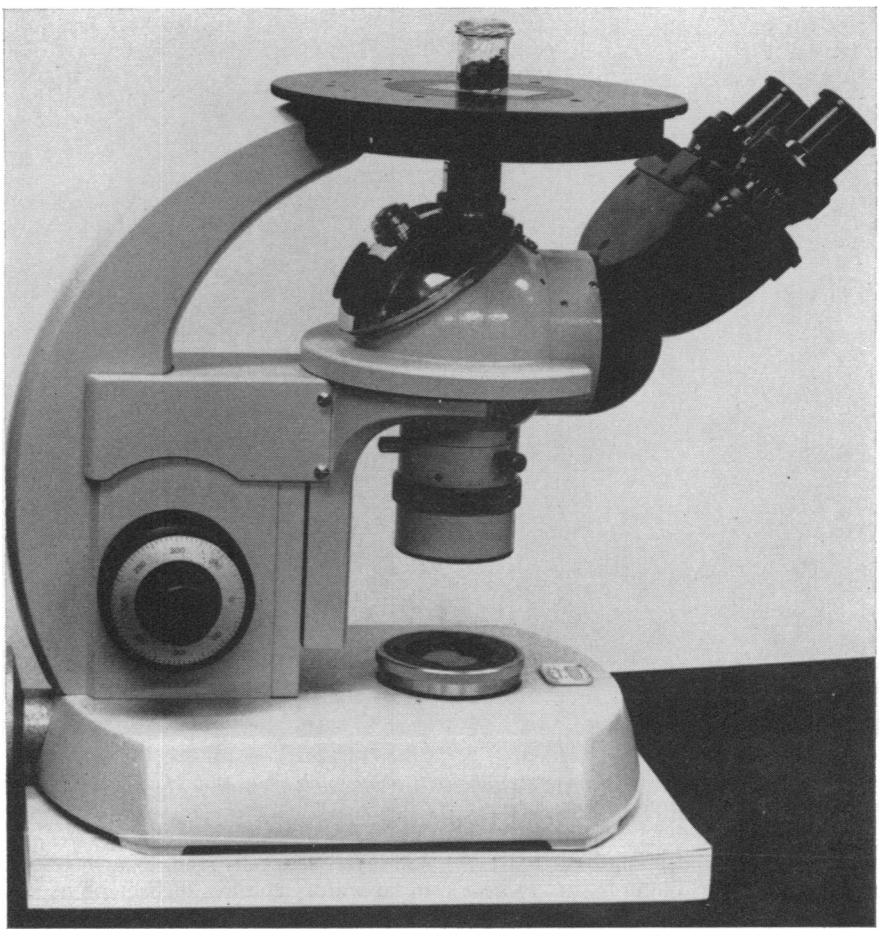

FIG. 2. *Modified metallurgical microscope for observation of microorganisms in their natural habitat. For photography, the binocular head is replaced by a monocular tube with photographic device.*

the numerical aperture is changed, the light diffraction picture for the artifact also changes to yield a bright multiple-diffraction pattern with a marked deterioration of image quality. Under similar conditions, the light diffraction picture for microorganisms does not change, or it changes only slightly if the organism is embedded in or coated with habitat materials.

The occurrence of light diffraction colors for microorganisms in a natural habitat apparently is associated with a state of low metabolic activity in the habitat. This becomes more apparent when one observes laboratory-grown cultures of bacteria or fungi with this microscope. Thus, cultures grown in the laboratory and observed either as wet mounts or on the surfaces of agar blocks in most instances do not show the brilliant light diffraction colors associated with in situ soil microorganisms. Instead, their appearance resembles that encountered when the cells are observed by phase microscopy. Various treatments of laboratory-grown cultures, such as lyophilization, starvation, and contact with heat, solvents, and salt solutions, have not yielded the brilliant light diffraction colors associated with in situ soil organisms. However, bacteria subjected to antibiotic stress in the laboratory do present light diffraction colors. This is true for bacteria growing within inhibition zones surrounding antibiotic discs containing chloramphenicol, dihydrostreptomycin, erythromycin, novobiocin, and tetracycline, but not for penicillin, lincomycin, or neomycin. Light diffraction colors also are observed for bacteria taken from within inhibition zones on crowded plates produced by plating low soil dilutions.

Several unusual cell groupings observed for cells in soil have now been observed for labora-

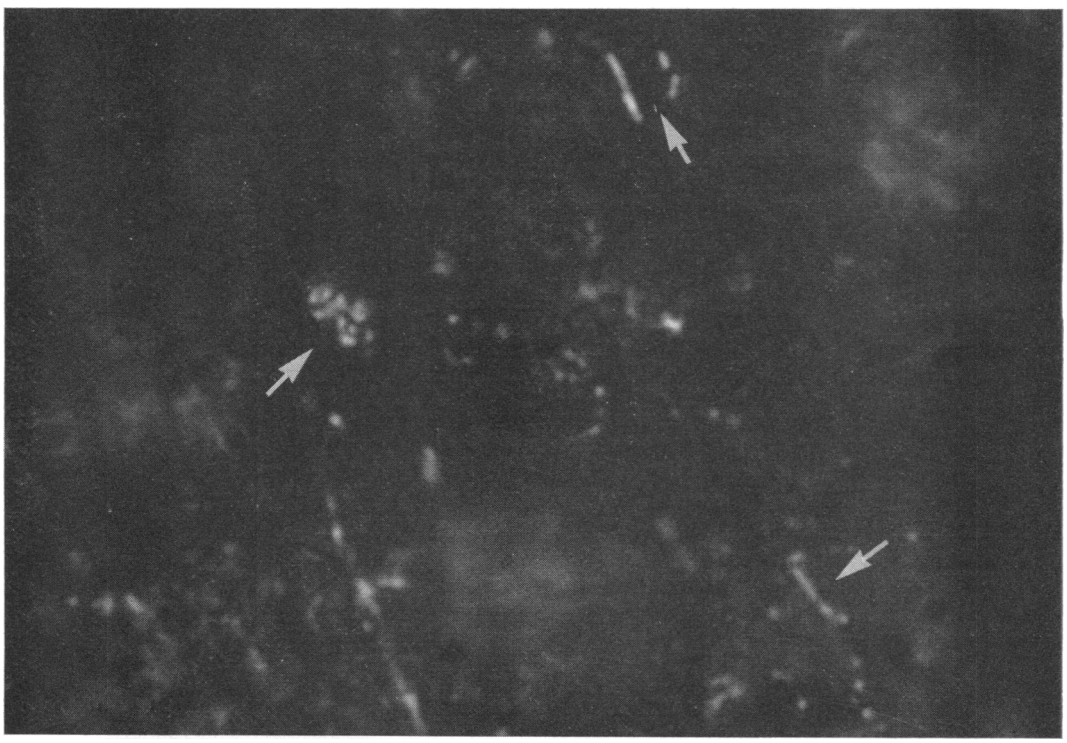

FIG. 3. *Soil bacteria residing on soil minerals. The cells photograph white with black-and-white film, but to the eye appear colored in contrast to the gray minerals.* ×*1,700.*

tory cultures growing under antibiotic stress. However, stable L-form cultures of *Proteus rettgeri* and *Staphylococcus aureus* (kindly supplied by C. W. Godzeski, Lilly Laboratory for Clinical Research, Indianapolis, Ind.) have not yielded the colors associated with antibiotic stress on an organism.

The phase microscopy-like appearance of a laboratory culture of *Arthrobacter globiformis* was changed to that of in situ soil bacteria by adding a washed cell suspension of this organism to soil. The cells did not immediately diffract light as did the indigenous soil bacteria. However, during a period of 1 to 2 weeks of incubation of the soil, those cells which did not lyse eventually took on an appearance resembling that for the in situ soil microorganisms.

From an opposing viewpoint, at least a portion of the in situ soil microorganisms can be made to lose their light diffraction coloration by (i) the presence of active root hairs of a growing plant; (ii) addition of an agar block containing a carbon source such as glucose, sucrose, starch, sodium acetate, or gelatin, but not of a nitrogen source such as NH_4NO_3 or DL-alanine; and (iii) layering of autoclaved soil over a lower natural soil layer. The cells (Fig. 6) then resemble those in laboratory cultures of bacteria as viewed with this microscope. As the available soil nutrients, added as above, become exhausted, the appearance of the bacterial cells reverts in a step-wise manner to that of organisms in unaltered soil. Thus, first the central portion of the cell takes on light diffraction color, then the outer boundary of the cell becomes difficult to detect.

Although this outer boundary of the bacterial cell residing in soil is difficult to detect, it can sometimes be seen by adjusting the NA of the objective to 1.32. In contrast, the outer boundary for cells of laboratory cultures under antibiotic stress can easily be observed at 1.32 NA, and, in this instance, it is obvious that the light diffraction colors are associated with that portion of the cell which does not include the cell wall.

Laboratory cultures of fungi do not demonstrate a central core of light diffraction color. Also, segments of both septate and nonseptate mycelium in soil sometimes lack this characteristic, and, as mentioned previously, this also applies to the growing tip. The outer boundary for mycelia in soil is clearly visible, however, regardless of its internal light diffraction charac-

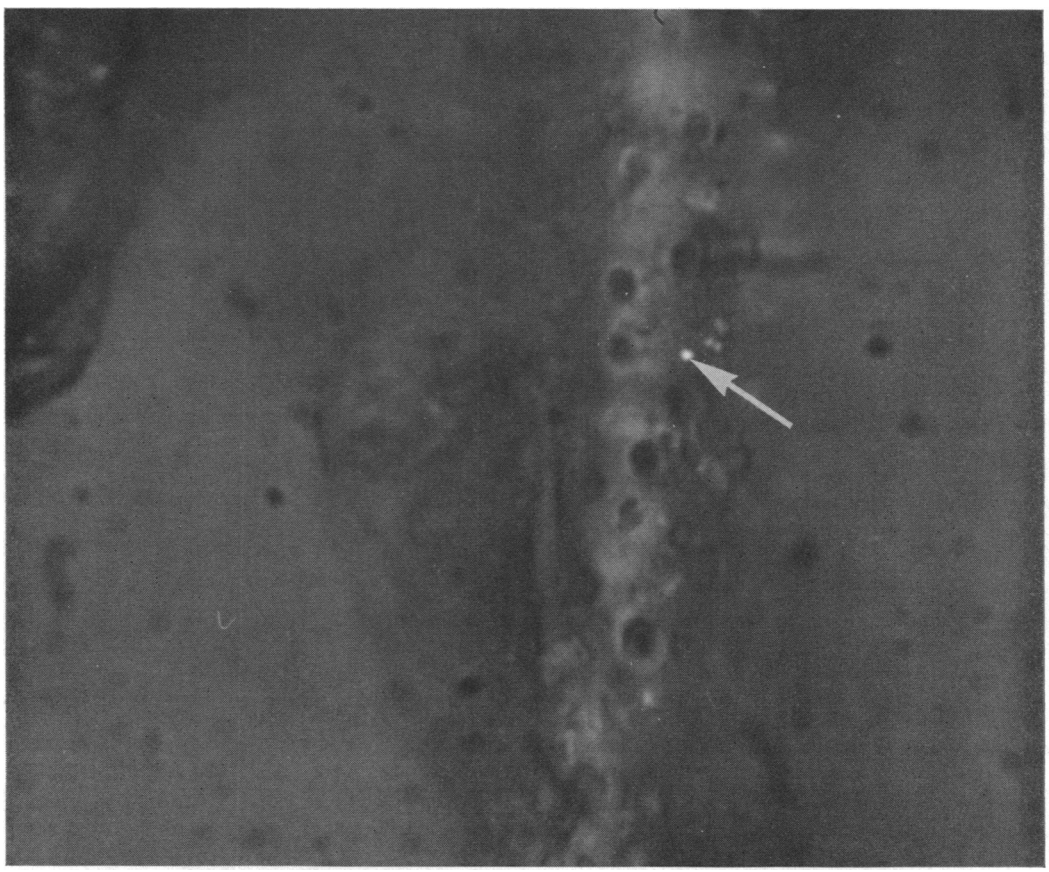

FIG. 4. *Bacteria on the root surface of clover growing in a soil incubation chamber. The bacteria are colored by diffracted light, whereas the root surface is colorless.* × *2,000.*

teristics. The effects of antibiotics on fungi in relation to light diffraction colors have not been studied.

DISCUSSION

The microscopic technique described allows the examination and continuous observation of the growth, activities, and interactions in soil of bacteria, actinomycetes, fungi, nematodes, protozoa, and plant roots without the use of stains or other alterations in the microorganisms of their habitat and environment. Also, by use of the soil incubation chamber described, or of various possible modifications of it, observations of microorganisms and roots should be possible while changing any combination of the soil atmosphere, temperature, and available nutrient level, and during addition of microbial or plant growth inhibitors or stimulators. Specific examples of such studies might be (i) determining the effects of added pesticides on the activities of the soil microflora, and (ii) following under various conditions the rhizosphere activities and infection processes for pathogens of growing plant roots.

This microscope allows a three-dimensional observation of the microorganisms and their habitat, with the depth of the soil which can be visually penetrated being approximately equivalent to the working distance of the objective used. Obviously, the 40× objective, with its greater working distance, allows a greater visual penetration into the soil than does the 100× objective. Regardless of its lower magnification, this objective still allows observation of the various living forms in soil, including bacteria.

The three-dimensional viewing aspect at times causes difficulty in time-lapse photography. Thus, growing plant roots may push soil particles around, or soil particles may move or rotate slightly due to other stresses in soil, with the result that sequential photography of particular

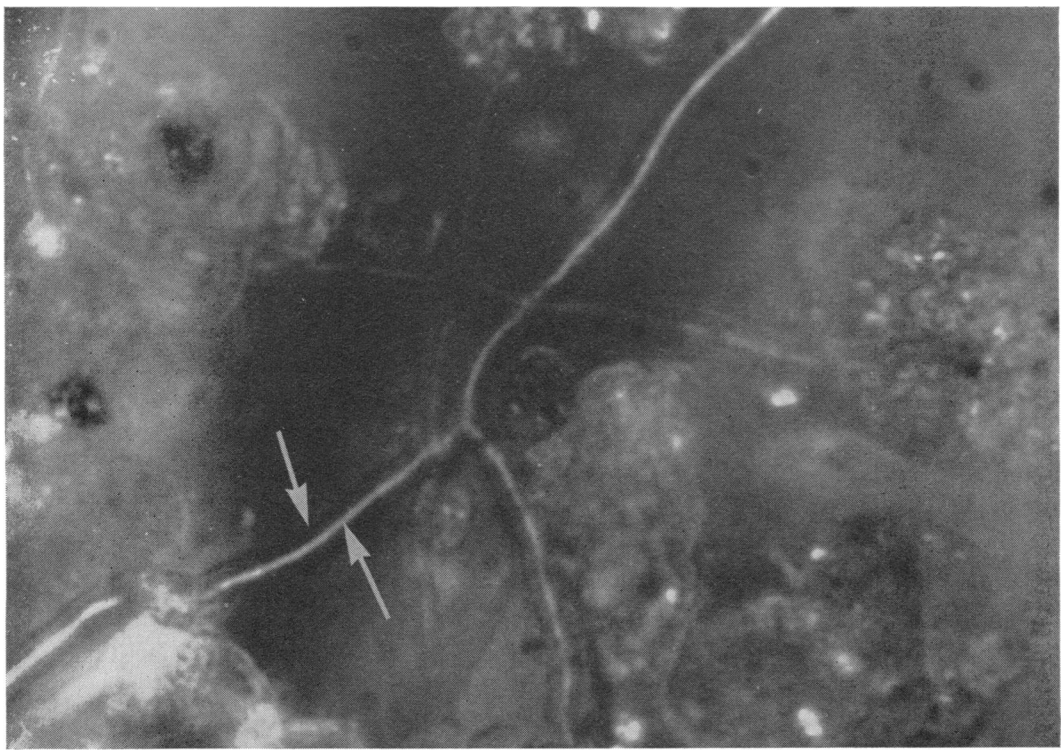

FIG. 5. *Fungal hyphae in soil in an incubation chamber containing decomposing pea seeds. Note the colored light diffraction area running through the central portion of the mycelium, and the colorless outer area of the mycelium.* × *1,700.*

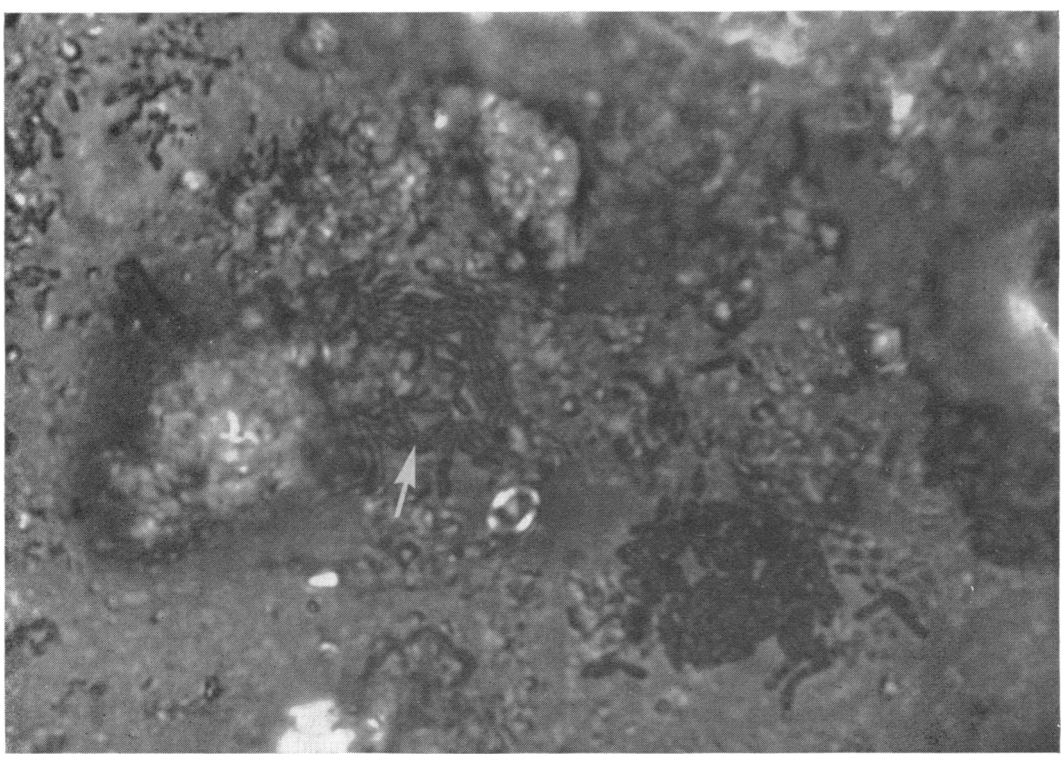

FIG. 6. *Bacterial microcolonies in natural soil with overlay of autoclaved soil. The bacteria are colorless and resemble laboratory-type growth, but a few of the cells have started to take on light diffraction colors.* × *1,700.*

cells at a given plane within the soil may have to be terminated before it is desired to do so.

As a result of these studies, it is apparent that indigenous soil microorganisms undergo considerable change when the nutritional status of their environment is altered, or when they are isolated and grown in the laboratory. It is not known whether this reflects merely a response of the organism to altered nutrient availability, or whether it indicates a release from a soil stasis caused by antibiotics or other possible factors in the soil environment. These observations, nevertheless, tend to corroborate the conclusions of Gledhill and Casida (3) that soil microorganisms can occur in soil in a state different from that which they take on during adaptation to and growth on laboratory media.

The relationship of the optical phenomena observed for microorganisms in soil and the chemical and physical states of their cell constituents is not clear at present. It is known, however, that various of the antibiotics which induce light diffraction colors for laboratory cultures cause changes in cell nucleic acids which are visible be light and electron microscopy [*see* Brock (1)].

It would appear that use of the particular combination of microscope components and objectives, as described in this study, is a requirement for obtaining light diffraction colors of microorganisms residing in masses of soil, and for viewing the relationships of these organisms to the roots of growing plants. Other combinations of equipment and objectives do not provide the specific light path through the microscope and sample, nor do they allow the required adjustments of NA.

It also should be noted that the light diffraction colors observed for in situ soil microorganisms are not to be confused with the false red color observed when photographing soil bacteria with Kodak Ektachrome Infrared film and transmitted light (2). The false red color for the infrared photography occurs only at the lower surface of the cell, the surface closest to the light source, and it is caused by a portion of the light spectrum different from that for the visually observed light diffraction colors. Thus, use of color infrared film has not been successful with the reflected light microscopy described in this study.

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