STUDIES ON THE MICROFUNGI IN THE FOREST FLOOR OF SUBARCTIC PINE FORESTS

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Preface

Microbiology in humus soils has received unfortunately little attention. The few publications that have dealt with problems in this field have been limited in scope, and directed mainly toward the composition and quantity of a microbe population or its subpopulations; largely lacking are studies on population changes, and especially the factors responsible for these changes. This project has been carried out to partly satisfy the need for more information on factors underlying population trends and changes.

The field work dates from 1958 and 1959 and has been carried out near the Arctic Circle, in the vicinity of Rovaniemi.

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Introduction

This project was designed for the study of the microfungal population in the forest floor[1] of some firm-land pine forests in the subarctic zone. Data have been compiled on the density, its changes, and general composition of the population; in addition, an attempt has been made to find the reasons for the changes. In studying the reaction of the microfungi to environmental changes, the determination of the effects of moisture content and temperature of the forest floor has been an important problem. The observations on these effects may throw light on general activity also of other groups of soil microbes, under conditions similar to those prevailing in this project.

Material and methods

General procedure

The dilution plate method and the soil-plate method of WARCUP (1950) were used in the study. BURGES (1958), WARCUP (1960), and JENSEN (1962, 1963 a) have given an excellent account of these methods so that only a few comments concerning methoddology need be added here.

The dilution plate method can be used with some reservations in both quantitative and qualitative studies. The method is not quite accurate for the purposes of this project, since it only indicates the number of microfungal fragments, not the actual number of microfungi, and is selective for microfungus species (VARTIOVAARA 1935). Despite its limitations, the dilution plate method is well adapted for general work and is very reliable as a quantitative technique.

Warcup's method is a simple modification of the one above. It is better than the dilution plate method in limiting the spreading of abundantly spore-forming microfungi in the culture plates (ALEXANDER 1961). This makes the soil-plate method well suited for the qualitative studies of microfungi with variable growth rates. The method, however, is not suitable for quantitative studies.

The dilution plate method was used in calculating the microfungal density in

[1] Forest floor, as here used, comprises the surface accumulation of organic detritus resting on mineral soil. In the conventional sense, in a mor humus type, it assumes the presence of a litter (L) layer, a fermentation (F) layer, and a genuine humus (H) layer.

the forest floor; in the qualitative identification of this population, the soil-plate method was used for isolating cellulose-decomposing microfungi. The modifications necessary from the normal procedure are desribed in the chapter on cultures.

Sampling areas and samples

The material was collected from five almost virgin pine stands through the year. The samples were taken from unfrozen soil approximately once a month, and from frozen soil approximately once every two months. The sample plots were located near the field laboratory; thus it was possible to start working on the unfrozen samples within an hour from the time of sampling. The frozen samples were transported in that condition to Helsinki where they were similarly given a laboratory examination after thawing them.

The most important data from the sample plots are given in the following table:

Sample plot No.	Forest type	Age of stand years	Thickness of the forest floor mm	Mineral soil texture
1	EMT	110	20 ± 6	sandy soil
2	»	130	21 ± 8	— » —
3	ErClT	25	8 ± 4	sand
4	VT	140	13 ± 5	fine sandy soil
5	»	90	17 ± 4	— » —

The sample plot size was only 10 by 10 meters. In connection with sampling, the moisture content, the temperature, and the pH of the forest floor were measured. These data are given in table 1.

Each sample of forest floor consisted of twenty randomly selected subsamples taken with a sampling tube, composited by mixing carefully and thoroughly in a sterilized glass container equipped with a lid.

Cultures

The sample taken from the mixed material for the dilution plate cultures was diluted to give a volume ratio of the sample to the suspension spread on the culture medium of 1 : 10000. The weight ratio was from 1 : 25000 to 1 : 40000, depending on the volume weight of the sample. A 0.1 % peptone solution, wich has a considerably lower surface tension than distilled or tap water and makes it therefore easier to get a microbiologically even suspension (Schalin 1964),

Table 1. Moisture content in fresh samples, average temperature, and pH in the forest floor.

Date Date of sampling	Moisture, %					Temperature, °C					pH				
	Sample plot					Sample plot					Sample plot				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
18. VI. 58	60.1	54.0	—	48.2	42.4	5.1	6.0	—	7.8	4.9	4.7	4.7	—	4.8	4.8
8. VII. 58	46.2	51.2	29.3	43.7	54.2	9.9	7.7	12.4	10.7	9.6	4.4	4.7	4.6	4.6	4.5
2. VIII. 58	24.4	28.6	7.9	24.8	24.9	17.8	16.7	20.4	19.8	16.2	4.4	4.4	4.5	4.5	4.5
20. VIII. 58	42.9	39.4	39.7	44.5	34.3	13.5	13.4	16.4	15.4	14.2	4.7	4.8	4.6	4.7	4.7
9. IX. 58	61.2	55.0	50.4	—	—	10.6	10.0	11.4	—	—	4.7	4.9	4.7	—	—
21. X. 58	65.8	52.9	44.6	52.9	51.2	2.2	1.9	1.8	1.6	1.6	4.7	4.8	4.8	4.8	4.7
7. XI. 58	60.4	56.6	51.4	57.4	45.5	4.3	4.0	4.3	2.4	2.6	4.7	4.7	4.7	4.8	4.6
10. XII. 58	70.1	72.9	61.5	77.1	69.6	−2.3	−3.2	−4.1	−3.7	−2.6	4.6	4.6	4.6	4.7	4.6
19. I. 59	56.8	67.7	47.6	56.5	53.5	−2.2	−3.0	−4.3	−2.8	−2.1	4.4	4.3	4.1	4.4	4.5
16. III. 59	58.4	66.9	48.9	55.6	60.1	−1.7	−2.6	−4.3	−1.6	−1.8	4.8	4.7	4.5	4.8	4.9
28. V. 59	—	58.1	33.3	50.7	52.9	—	1.5	4.8	3.9	2.3	—	4.9	4.9	4.8	5.0
25. VI. 59	—	50.4	44.8	53.6	46.6	—	6.4	10.7	6.1	7.6	—	4.7	4.6	4.7	4.8
20. VII. 59	—	41.0	32.4	35.8	36.4	—	7.2	12.0	10.7	9.8	—	4.7	4.5	4.7	4.9
14. IX. 59	—	47.6	49.3	42.1	43.3	—	6.2	6.7	7.1	6.7	—	4.9	4.7	4.8	4.9

was used in the dilution. The temperature of the suspension and the composite sample was maintained at the level existing in the forest floor at the time of sampling to the time of culturing by either the dilution plate or soil-plate method.

The reason for choosing volume rather than weight for measurement is partly the better indication that the former gives of the forest-floor microfungal density. A more important reason, however, was the considerable decrease of the error for the thin-organic-layer, which had consistently smaller organic-matter content and larger proportion of mineral soil than in the thick-organic-layer samples. Sample plot 3 had on an average 24.5 % organic matter of the total sample dry weight; sample plot 1, on the contrary, had only about 68.0 %. The organic layer in the former was the thinnest, in the latter the thickest. Since the quantity of the microfungi depends on the organic-matter content of the forest floor (Romell 1932, Alexander 1961), the example above rather clearly suggests that sample weight should not be used in the determinations of the forest-floor microfungal density.

It may be further noted that in the determination of the organic-matter and mineral-soil contents of the samples on a weight basis, not one sample exceeding 10 % was found; the maximum difference found between any two samples was smaller than this percentage. Thus a much more reliable figure for the microfungal density is reached by determining this per unit volume and not weight.

The medium used in the dilution plate cultures was Martin's medium for fungi (Martin 1950), henceforth, for simplicity, refered to as rose bengal agar. With the help of a dye (Smith and Dawson 1944) and streptomycine added to the

medium (JOHNSON 1957, PAPAVIZAS and DAVEY 1959), the invasion of profusely spore-forming microfungi and bacteria in the plates was almost completely controlled. The suspension in these cultures was spread on the bottom of the plate-dishes.

The composition of the medium (cellulose-agar) in the soil-plate cultures was the following:

$(NH_4)_2SO_4$	1.0 g
K_2HPO_4	0.3 »
KH_2PO_4	0.7 »
$MgSO_4.7H_2O$	0.5 »
NaCl	0.2 »
$FeSO_4.7H_2O$	0.4 »
Washed agar	10.0 »
Aqua dist.	1000.0 ml

This medium proved to be specific enough to render the use of inhibitors unnecessary. A filter paper (cellulose content 98 %) was spread on the agar solidified in the dishes; particles from the composite sample were then distributed on the paper surface.

The rose bengal agar cultures were incubated in + 25° C for an average of 7—9 days, a period somewhat longer than the normal incubation time (WAKSMAN 1922), but was made necessary by the relatively small amount of nutrients (LOCHHEAD and BURTON 1956) and the inhibitors (PAPAVIZAS and DAVEY 1959) in the medium. The optimum incubation time for the cellulose cultures was found to be 14—24 days, with the temperature remaining below, rather than over, + 15° C. In a temperature as low as this, the profusely spore-forming microfungi did not cause much difficulty to the growth of the other types on the plates.

Results

The microfungal density

The microfungal density (the number of viable microfungus units, originated from spores and mycelial fragments, per cm^3) have been given relative values. These have been determined by comparing the sum of the microfungal colonies from each of the six replicate dishes for any given sample, to that for the first sample from sample plot 1, giving this sample the value 100 (table 2).

The thicker the organic layer, the more microfungi were found. Nevertheless the microfungal density can not be fully explained by the accumulated amount of organic matter (see ROMELL 1932), since at the dilution stage an attempt to standardize this effect had already been made. The factors responsible for the

Table 2. The microfungal density per cm^3 of the forest floor (rose bengal agar).

Date of sampling	Sample plot 1	2	3	4	5
18. VI. 58	100	143	—	78	91
8. VII. 58	45	64	36	42	59
2. VIII. 58	40	50	41	34	49
20. VIII. 58	48	68	45	57	58
9. IX. 58	135	117	66	—	—
21. X. 58	98	93	46	47	65
7. XI. 58	109	98	53	56	70
10. XII. 58	82	84	35	50	42
19. I. 59	68	66	30	35	40
16. III. 59	47	40	21	28	24
28. V. 59	—	51	32	41	48
25. VI. 59	—	41	45	72	66
20. VII. 59	—	47	43	50	49
14. IX. 59	—	48	56	66	68

observed dicrepancies can be found from the environment of the microfungi; the most important are moisture, temperature, and acidity (MENON and WILLIAMS 1957). The effect of at least the first of these two factors is related to the thickness of the organic layer.

An optimum pH range (4.1—5.0) was maintained for the microfungi. This pH is below the limit of effective competition by bacteria and actinomycetes with microfungi for nutrients.

The effect of moisture and temperature on the activity of microfungi must be considerable even during the growing season, wherever seasonal differences are marked, and when the weather is variable. Spring moisture and temperature were found to have a favorable effect on the microfungi, a long period of summer drought and a cold winter, an unfavorable effect (tables 1 and 2). The effect of the autumn on the microfungi depended most closely on the temperature.

The above observations are quite general in nature and do not express the relative importance of moisture and temperature on the microfungal density, or the possibility of either factor to replace the other to any extent. Statistical methods have been used to determine these relationships. Among several possible methods to determine the dependence of the microfungal density (y) on variables having a simultaneous effect, a regression analysis for two independent variables, moisture (x_1) and temperature (x_2), was believed to provide the most reliable results. The total correlation value from the analysis indicates the combined effect of moisture and temperature; partial correlations were also calculated for the individual effects of these factors on the microfungal densities; these analyses indicated the mutual dependence of the moisture and the temperature.

The following regression equation shows that temperature tends to have a considerable greater effect on population densities but moisture is also important.

$$y = 1.0631\, x_1 + 3.5049\, x_2 - 13.23$$

The correlation coefficients are the following:

$$r_{x_1x_2 \cdot y} = 0.6888,\ r_{x_1 \cdot y} = 0.2560,\ r_{x_2 \cdot y} = 0.2839,\ \text{and}\ r_{x_1 \cdot x_2} = -0.7374.$$

These were tested with Fisher's table (SNEDECOR 1956), the significance was calculated at the 5 % and 1 % levels. With an f-value of 63—3, the correlation was significant at the 1 % level for the first and the last of these coefficients and at the 5 % level for the second and the third of these coefficients.

The microfungal density as related with the moisture content and the temperature of the organic layer reveals certain interesting and somewhat unexpected relationships. The results are in line with the general opinion that microfungi have an ample requirement range for both factors, but this generalisation does not adequately explain the behavior of these organisms at all combinations. Under natural conditions favorable moisture and temperature coincide rarely, and then only for short periods; most often the factors have opposite effects. Thus, moisture and temperature can not be properly examined as separate and

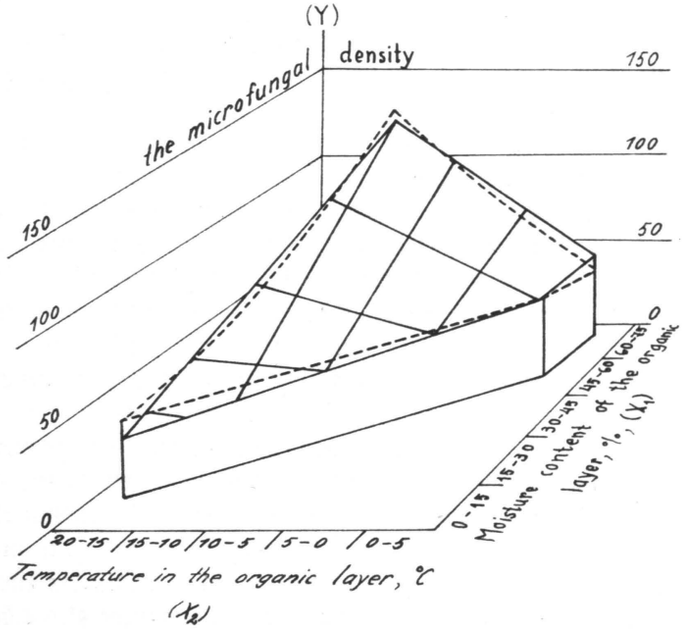

Figure 1. The correlation between the microfungal density and the combined factor of moisture and temperature.

independent factors; they must be examined jointly. The combination of the effects of both factors serves as an indicator of the suitability of the conditions for microfungi. This is indicated by the total correlation between moisture and temperature and the microfungal density, as well as the correlations between the separate factors and the organisms.

The correlation can be illustrated aptly in a threedimensional diagram (figure 1) based on the regression equation values. The curved surface for the actual observations is shown by the dotted lines; this surface is somewhat different from the flat regression-equation surface. For the sake of clarity, the usual method of drawing all co-ordinates through a common point was not followed.

The theoretical possibility exists that the plane indicating the correlation of the microfungal density to the combined factor of moisture -temperature could cross the one formed by the x_1 and x_2 co-ordinates at the line connecting the points $x_1 = 30$, $x_2 = -5$ and $x_1 = 0$, $x_2 = +5$; yet its incompleteness emphasizes the fact that the conditions in which very low temperatures combine with a low moisture content, or high temperatures with a high moisture content apperently never do obtain in the organic layers studied.

The microfungal density increased with the simultaneous increase of the moisture content and the temperature up to the maximum values recorded. For both factors, a limit was found, above which a more favorable degree of one factor could to an extent replace a less favorable degree of the other factor; this was not found below these limits. For moisture, the limit was 35 %, for temperature, between 0° C and + 5° C.

In the subartic zone the content of easily soluble organic matter in the organic layer is the highest in the autumn after leaf-fall, and also in the spring; a simultaneous increase in the activity of microfungi occurs. However, the overriding importance of moisture and temperature, which also were at an optimum for the microfungi in the forest floor of the pine stands under study, is evident. It can be shown that even in the spring and in the autumn, if moisture and temperature conditions were unfavorable, the microfungal density was small (table 2, latter part).

The organic matter content and the pH of the organic layer determined only the composition of the microfungal population in the layer. The density of the population, on the other hand, depended on the moisture content and the temperature of the organic layer and is correlated with the combined effect of these ecological factors.

Composition of the microfungal flora

Fungi growing on rose bengal agar

From the cultures used in the determination of the microfungal density, a sample from each type of colony was transferred to a plate with Hagem agar as the medium. After a few days of incubating until the cultures had developed to the stage at which the spore formation of the microfungi was clearly visible, the colonies were identified according to the method reported by GILMAN (1957).

The precentages of the cultures representing the genera in the microfungal population are shown in table 3.

The microfungal populations from the forest floor were composed of a very small number of species; the same species occurring in all sample plots. The small within species variation as shown in the table indicates the evenness of distribution of the samples taken at the same time from the different plots.

The most important fungi of the microfungal population were the *Mucor* species, which resemble each other closely, two identified representatives of the *Mortierella* genus, *M. vinaceae* and *M. isobellina*, and the *Penicillium velutina*-type brush-microfungi. These species represented almost 90 % of the total population. Much less, but still regularly, non-spore-forming microfungi were seen. The most important of the accidental microfungal species was *Trichoderma* sp.

The *Mucor* and *Penicillium* species were correlated in a way differing from each other and all the others to the moisture-temperature factor-combination (tables 1 and 3). This was seen very clearly during the first growing season, 1958, when the microclimatological conditions were much more extreme than at the later stage of the study. In the spring and in the autumn, when the moisture content of the forest floor was high, the *Mucor* species composed the dominant growth in the population, and *Penicillium* was in the minority. When the moisture content went below 50 %, the situation was almost the opposite. It looks as if the increase of the moisture content is favorable for the *Mucor* fungi (OPURT and CURTIS 1957, MEYER 1959); on the contrary, the *Penicillium* fungi, as soil fungi in general, dislike a high moisture content (MENON and WILLIAMS 1957).

In the temperature requirements of the microfungi, unlike that for moisture, similar specific characteristics were not seen. It is generally known that soil microfungi, at least the ones mentioned above, can adapt themselves to very low temperatures. They are quite active at + 5° C, and still at high temperatures, although their competing ability against other microfungi and other organisms decreases (MISHUTIN 1956). Thus, the differences in abundance of the *Mucor* and *Penicillium* fungi in relation to season must be considered to reflect different requirements for the environmental moisture regime.

Table 3. Composition (%) of the microfungal flora in the samples of forest floor cultured in rose bengal agar.

Genetic groups in samples	Sample plots 1, 2, 3, 4, and 5													
	Date of sampling													
	1958								1959					
	18. VI.	8. VII.	2. VIII.	20. VIII.	9. IX.	21. X.	7. XI.	10. XII.	19. I.	16. III.	28. V.	25. VI.	20. VII.	14. IX.
Mucor	67 ± 5	35 ± 7	18 ± 2	24 ± 6	45 ± 8	46 ± 7	49 ± 6	52 ± 8	52 ± 3	45 ± 5	57 ± 8	37 ± 6	16 ± 4	29 ± 5
Mortierella	7 ± 1	9 ± 4	5 ± 2	9 ± 3	14 ± 4	7 ± 1	7 ± 3	8 ± 3	7 ± 2	8 ± 2	9 ± 2	6 ± 2	3 ± 1	10 ± 3
Other *Phycomycetes*	3 ± 2	—	—	—	—	—	—	—	—	—	4 ± 1	3 ± 1	2 ± 0	2 ± 1
Trichoderma	—	—	2 ± 0	—	—	4 ± 1	6 ± 2	6 ± 3	9 ± 3	5 ± 3	3 ± 1	2 ± 1	5 ± 2	4 ± 2
Penicillium	13 ± 3	43 ± 9	70 ± 4	53 ± 6	38 ± 6	33 ± 9	27 ± 8	22 ± 7	23 ± 9	24 ± 9	23 ± 5	45 ± 8	64 ± 9	50 ± 12
Scopulariopsis	—	—	2 ± 1	1 ± 0	—	3 ± 1	—	—	—	—	—	—	—	—
Mycelia sterilia ..	10 ± 3	13 ± 3	13 ± 2	13 ± 2	3 ± 1	7 ± 3	11 ± 8	12 ± 2	9 ± 3	18 ± 2	4 ± 1	7 ± 2	10 ± 3	5 ± 2

The behavior of *Mortierella* genus, the other members of the *Phycomycetes* order, and the non-spore-forming microfungi under changes moisture content and temperature can not be determined from the data. However, it is apparent that they have a greater amplitude than the *Mucor* and the *Penicillium* microfungi for the variations in this factor combination.

Although the results given clearly show the composition of the microfungal populations in the material studied, they can be generalized only with certain precaution. It is good to remember that the isolation of microfungi depends not only on the sample and its handling, but also to a considerable extent on the culture medium. Compared to the natural environment of the microfungi of the organic layer, any synthetic medium is to an extent selective, favorable to some species, unfavorable to others. A complete medium suitable to all the thaxonomic groups of the natural microfungal population has, so far, not been devised. Thus the microfungi isolated in this investigation do not represent the total microfungal population of the pine forests studied, but possibly the qualitatively and quantitatively most important subpopulations in the total population.

The fungi on the cellulose medium

The study of the microfungi decomposing cellulose was carried out during only a part of the period devoted to the whole study. The composition of this group of fungi and the quantity of the species-populations in the samples taken at different times are shown in table 4; the numbers given indicate the average number of colonies in the cultures:

1 = 1— 2 colonies
2 = 3— 5 »
3 = 6—10 »
4 = 11—20 »
5 = over 20 »

In addition to the qualitative analysis, the changes caused by the microfungi in almost pure cellulose, the only carbon source in the medium, were examined.

The most abundant and regularly occuring microfungus was *Trichoderma* sp. The cellulose started to change brown under, and around, the colonies immediately after 10—12 days of incubation when the first very small white fungal colonies appeared on the cellulose surface. As soon as the fungus began to form spores, cellulose completely disappeared from under and around the colonies of *Trichoderma* sp. to the distance of up to 4 mm.

On cellulose agar, *Trichoderma* sp. colonies started to develop after incubation period of about two weeks, but on rose bengal agar they developed in only five days, despite competition from faster-growing fungi like *Mucor spp.* The experimental results suggest, that this was not due to the different incubation temperatures. The slow initial development of *Trichoderma* sp. on the cellulose agar resulted apparently from its need first to hydrolyse cellulose into a more easily soluble form, glucose, before being able to use it as nutritive material (Mandels and Reese 1957). The same is true of all organisms utilizing cellulose as a secondary, alternative carbon source (Reese and Levison 1952, Reese 1956).

Table 4. The cellulose decomposing microfungi and their quantity in the forest floor (cellulose medium).

	Sample plots 1, 2, 3, 4, and 5								
	Date of sampling 1958								1959
	18. VI.	8. VII.	2. VIII.	20. VIII.	9. IX.	21. X.	7. XI.	10. XII.	19 I.
Phycomycetes ..	1	—	—	1	—	—	—	—	—
Trichoderma sp.	5	5	5	5	5	2	4	3	3
Penicillium spp.	—	1	2	1	—	—	1	—	—
Scopulariopsis sp.	1	1	1	1	1	—	1	—	—
Verticillium eff. + terr.	2	2	1	2	1	—	1	—	—
Pullularia sp. ..	1	—	1	1	2	—	1	1	—

In cellulose-decomposing capacity of the microfungi ocular estimates place *Trichoderma* sp. clearly in first place. Below the colony, only *Pullularia* sp. decomposed cellulose completely, although *Verticillium terrestre* accomplished this almost completely; the activity of the others was noticeably smaller. Also *Trichoderma* sp. brought about a more rapid decomposion than the other fungi, which were not active before about 15—18 days of incubation.

Phycomycetes-fungi have generally been considered incapable of using cellulose for their nutrition. Apparently some of them, however, are able to do this to some extent, as is also evident in some earlier observations (Smith 1948).

The *Trichoderma* populations were of about the same abundance in all sample plots, and their seasonal fluctuations were relatively small. During the growing season the other fungi populations remained essentially equal, but at the end they differed clearly from each other. The first ones to disappear in the autumn were *Penicillium* spp. and *Verticillium effusum*; somewhat after these and about the same time *Verticillium terrestre* and *Scopulariopsis* sp., the last one to disappear was *Pullularia* sp. Apperently *Penicillium* spp. were the least tolerant of unfavorable moisture-temperature combinations and *Pullularia* sp., the most.

Relatively few microfungi grew on the cellulose agar, and their composition of the population was different from that on the rose bengal agar; this outcome was not unexpected, since the population was limited to those able to hydrolyse

cellulose. The results indicate that certain cellulose decomposing microfungi are found in the organic layer of the types of pine forests sampled in this study, although their significance, excluding the *Trichoderma* and *Pullularia fungi*, is apparently negligible.

Summary

The dilution plate method was used in studying the density and composition of the microfungal populations of the organic layer of pine forests, and the soil-plate method in studying the part of these populations decomposing cellulose. The media used were rose bengal agar (Martin's medium for fungi) and cellulose medium.

The microfungal density depended to a considerable extent on the moisture content and temperature of the organic layer. It was interesting to observe that only the combination of a relatively high moisture content and temperature, but neither of these factors alone, influenced considerably the microfungal population density. The correlation of the populations to the changes in this combined factor was stronger than the correlation to the seasonal variations of spring, summer, and autumn.

The microfungal population consisted of only a few species. *Mucor*, *Mortierella*, and *Penicillium* were the most common genera isolated from the rose bengal agar. The first and the last of these comprised almost 90 per cent of the total population. For the *Mucor* fungi, increases in the moisture content up to the maximum falues found (75 %) were favorable; the *Penicillium* fungi, on the contrary, were intolerant of high moisture contents.

Among the cellulose decomposing microfungi grown on cellulose medium, *Trichoderma* sp. was the most common; also it formed the most colonies, tolerated the lowest temperature, and was the most efficient. The others were of the genera *Pullularia*, *Verticillium*, *Scopulariopsis*, and *Penicillium*; in addition, there were some unidentified *Phycomycetes* fungi. Only the two first-mentioned caused observable changes in cellulose.

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