GROWTH OF CUNNINGHAMELLA AS INFLUENCED BY FORMS OF PHOSPHORUS AND NITROGEN

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by

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GROWTH OF CUNNINGHAMELLA AS INFLUENCED BY FORMS OF PHOSPHORUS AND NITROGEN

The relative growth of the fungus *Cunninghamella* on properly controlled soil cultures has recently been proposed for measuring the level of available phosphorus in soils[15,16]. Because of the considerable promise which this method has, it seemed desirable to make a thorough study of the nutrition of this fungus, particularly as regard the suitability of different forms of nitrogen and phosphorus. An investigation dealing with this subject was, therefore, undertaken and the results are herein reported.

HISTORICAL

Very little specific information regarding the nutrition of *Cunninghamella* was found in the literature and studies were mainly restricted to morphological and genetical classification. The forms of nitrogen which various fungi preferably utilize have been widely investigated, and for a general review of the literature, the reader is referred to the texts of Löhnis (13), Waksman (31), Lafar (11), and to the papers of Klotz (10), and Janke (9). Whether the ammonia, nitrate, amid, or amino forms of nitrogen are used at all, or with preference, appears to depend upon the fungus, the reaction and carbon source of the culture medium, and isoelectric point (19). *Cunninghamella bertholletia* at a slightly acid reaction was able to use the nitrate form rather well (24). In the presence of one per cent citric acid and ten per cent sucrose, *Cunninghamella* sp. apparently was unable to develop with ammonium sulfate as the nitrogen source (23). *C. elegans* and *C. echinulata* used the ammonia and nitrate form equally well at neutral and slightly acid reactions (15).

The usual forms of phosphorus used by fungi are the salts of orthophosphoric acid, although the meta- and pyro-forms of phosphoric acid and organic forms have been found to be used (3). For a review of the literature on the forms of phosphorus and the extent of its utilization by fungi, the reader is referred to Löhnis (13), and Waksman (31). It will be found that the assimilation of water insoluble or slightly soluble forms of phosphorus by fungi is closely related to the formation of acid in the course of their metabolic processes and partly to the action of enzymes. In the assimilation of phosphorus reduction may also play a role (18). The acidity of the culture medium influences, considerably, the assimilation of soluble phosphates by *Aspergillus niger* (26). *Rhizopus* sp. utilized phosphorus from the soil approximately in proportion to the phosphorus soluble in one per cent citric acid (22). *C. elegans* and *C. echinulata* grown on soil cultures near the neutral point developed in relative proportion to the solubility of the applied phosphates (15).

METHODS AND MATERIALS

In this investigation the method closely followed was that of Mehlich, Fred, and Truog (15), in which a solid substratum is used and the fungus growth of the aerial development of the mycelium was measured.

Fungus Cultures

Of the *Cunninghamella* species it was found preferable to use *C. blakesleeana* minus strain (12), because it develops a dense aerial hypha and grows rapidly. Reserve stocks, carried on malt-extract-agar slants (2.5% malt extract, 2% agar), were transferred every four months and kept in a cool place. Cultures were also carried on the regular nutrient solution used for making the test for phosphorus, about to be described, but to which is added 2 grams of agar and 0.05 gram $CaH_4(PO_4)_2 \cdot H_2O$ per 100 cc. of H_2O.

Nutrient Solution

The nutrient solution used for studying the influence of forms of nitrogen on the growth of Cunninghamella was the same as previously described (15), except that 0.1 gram of $CaH_4(PO_4)_2 \cdot H_2O$ per 100 cc. of H_2O were added and the forms and amounts of nitrogen were varied as will be shown later. To study the influence of forms of phosphorus on growth, the nutrient solution as previously described was at first used, however, later, as a result of detailed studies with nitrogen, the following somewhat modified nutrient solution was adopted. A stock solution of organic salts which keeps indefinitely is first prepared, and then to avoid decomposition, the final diluted nutrient solution is made up fresh daily. These solutions are made up as follows:

Stock Solution Inorganic Salts	
K_2SO_4	. . . 10.0 grams
$MgSO_4 \cdot 7H_2O$	. . . 5.0 grams
$FeSO_4 \cdot 7H_2O$	. . . 0.05gram
$ZnSO_4 \cdot 7H_2O$	. . . 0.05gram
Distilled water.	.100.0 cc.

Final Nutrient Solution	
Sucrose or Glucose	. . 2.5 grams
Asparagin	. . . . 0.5 gram
l-Aspartic acid	. . . 0.1 gram
Stock solution of inorganic salts	. . . 0.5 cc.
Distilled water	. . 100.0 cc.

Culture Dish

In order to better control the moisture conditions, to lessen the amount of soil needed, and especially to make the readings more accurate, a special clay dish (16) was designed for use instead of the Petri dish (50 mm. diam., 100 mm. deep). This special dish consists of a clay slab (55 mm. diam. by 15 mm. thick) having a cavity (23 mm. diam. by 7 mm. deep). The surface of the cavity is glazed so as to be waterproof, but the rest of the slab is unglazed and has a porosity of 8 to 10 per cent, so that it will hold moisture and thus promote the growth of the fungus uniformly over its surface. The surface of the unglazed portion should be of a dark color, like dark red, against which the white mycelium may be easily seen. This dish may be purchased of the Coors Porcelain Company, Golden, Colorado.

Materials

Forms of nitrogen: The inorganic salts of nitrogen, asparagin, aspartic acid, glycine, cystine, and urea used were commercial, chemically pure products, and the glutamic acid and arginine were prepared in the Agricultural Chemistry Department of this institution. The fertilizers used were commercial products, and the mold tissues were obtained from nutrient liquid cultures. They were washed free of the inorganic salts, and ground to pass a 60 mesh sieve. Alfalfa hay was similarly treated.

Forms of phosphorus: With the exception of crude phytin, which was prepared in the Agricultural Chemistry Department of this institution, and meta phosphate, which was prepared at Muscle Shoals, all inorganic and organic phosphorus salts used were commercial, chemically pure products. Before using the iron and aluminum phosphates, they were washed to remove the water soluble portion. The basic iron phosphate, dufrenite, was an impure sample containing 19.2% P_2O_5, and before using was treated with CO_2 saturated water so as to remove any easily soluble phosphorus. The rock phosphate used was a 200 mesh sample of Ruhm's phosphate.

Sand Cultures

Optimum conditions for the growth of the fungus using sand cultures were provided by mixing 2 parts of quartz powder with 1 part of washed quartz sand. This mixture, from the standpoint of texture, serves as a very satisfactory substratum for growth. To buffer this sand at different reactions, bentonites were employed. The use of 6% natural bentonite from Wyoming containing some free $CaCO_3$ permitted the system to be buffered at pH 8.6; five per cent of a 96% K-saturated bentonite and 1% of the natural bentonite buffered the system at pH 7.54. With 6% of a 51% K-saturated bentonite the system was buffered at pH 6.72. By means of H-bentonite, systems at pH 5.26 and pH 2.9 were obtained. The latter was made possible by treating the sand mixture with dilute citric acid and washing with distilled water to remove the excess of acid.

Systems of intermediate points of reaction were obtained by making mixtures of these systems. The K- and H-bentonites were prepared in this laboratory by Tyner (28), and Cook (4), respectively. For the determination of pH the glass electrode and Truog's triplex indicator methods were used.

Soil Cultures

Fresh soil samples were thoroughly air dried (employing at times a fan) and screened through a 20 mesh sieve. Samples may be dried in an oven, but the temperature should not exceed 40° C. If samples are to be stored for use later, it was found important that the well dried soil be placed in air-tight containers and stored in a cool, dry place. Soil stored improperly should never be used for a test of this kind, and fresh soil is always preferable.

Method of Addition of Different Forms of Nitrogen and Phosphorus

Unless otherwise indicated, the soluble forms of nitrogen and phosphorus were added in the desired concentration to the nutrient solution and a definite volume, usually 2 cc. added to 10 grams of sand or soil. The preparation of the plaque is brought to its optimum moisture condition by adding some of the nutrient solution free of nitrogen or phosphorus. To obtain a satisfactory distribution between the non-water soluble forms of nitrogen and phosphorus and the substratum (sand or soil), the required amount of material was first mixed in a mortar with a small amount of fine quartz sand, and vigorously stirred with a pestle. This larger sample can then be more thoroughly distributed with the soil or sand.

Details of Cunninghamella Culture Tests

Ten grams of soil or sand are thoroughly mixed with sufficient nutrient solution for optimum growing conditions. The mixture is then packed into the cavities of the special dishes with a spatula, and the surface smoothed and pressed just slightly below the rim of the cavity. These dishes are then placed in a pan, supplied with water to a depth of 6 to 8 mm. The pan is then covered with a glass plate, which is removed only for making inoculation and final measurements. A spore suspension is prepared by adding 1 cc. of the nutrient solution to a test tube culture of the fungus and stirring with a wire. This suspension is poured into a Petri dish. By means of a wire loop (3 mm. diam.) a very small drop of the spore suspension is placed in the center of each soil plaque. In order to make these drops uniformly small, the loaded wire loop is touched to the clean glass surface of the Petri dish, thus removing any excess. A piece of paper or wood is then placed between the glass cover and dish to provide for aeration. The cultures are incubated at 29° C. for 64 hours. At the end of the incubation period, the diameter of growth is determined by making two or three measurements with a pair of calipers and measuring rule. Measurements should include only the main or actual body of the growth, ignoring a few individual hyphae which project beyond the main mass. Accuracy in reading is obtained with the aid of a large magnifying glass. Daylight readings are usually more satisfactory.

INFLUENCE OF FORMS OF NITROGEN ON GROWTH

It was found previously (15), that within a moderate range of reaction, both the ammonia and nitrate forms of nitrogen were used by Cunninghamella. In this study organic forms of nitrogen were included and the range of reaction extended.

In some of the early tests for available phosphorus, with ammonium nitrate, as a source of nitrogen, the natural fungus flora of the soil greatly suppressed the growth of Cunninghamella. This was avoided when the sample of soil was heated at 40° C. for several days. In extending the test to include a greater variety of soils from regions where the natural fungous flora was more prolific, particularly if the soil samples were taken in the fall after green manures were incorporated, or undecomposed organic matter from the previous crop was present, the natural flora, interferred considerably, with the proper development of Cunninghamella. It was for this reason that the present investigation was extended to include the influence of several forms of nitrogen on the growth of the natural fungous flora of soil.

After the concentration of soluble nitrogen necessary to obtain maximum growth of the fungus was determined, different forms of nitrogen were added at a rate to provide 5 mgm. of nitrogen per 100 grams of sand-bentonite mixtures. The results in table 1, indicate that different forms of nitrogen are utilized differently and that the reaction plays an important role. Cunninghamella blakeleeana is able to use the nitrate form equally well throughout a wide range of reaction, while the ammonium form is used equally well only above pH 5 and to a considerably less extent below pH 4. That

Table 1

Growth of *C. blakesleeana* as Influenced by Forms of Nitrogen at Different Reactions. Rate of Application in All Cases Gave 5 mgm. of Nitrogen per 100 g. Sand

Reaction	Diameter of Lateral Growth with Different Forms of Nitrogen										
	Ammonium nitrate	Sodium nitrate	Ammonium sulfate	Urea	Calurea	Glutamic Acid	Glycine	Arginin	Casein	Asparagin	1-Aspartic Acid
pH	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
3.00	33	50	21	32	38	30	45	34	46	46	51
3.45	34	53	36	42	47	38	54	36	47	48	54
4.30	53	54	47	52	45	43	55	36	48	50	54
5.25	52	53	54	54	49	44	55	28	49	50	53
6.20	54	52	56	51	50	45	53	28	48	48	52
6.90	56	50	54	50	54	45	54	22	42	48	54
7.54	56	52	56	51	54	44	54	17	39	47	52
8.78	53	51	52	48	53	40	50	14	27	47	52

ammonium nitrate is not fully used at a reaction more acid than pH 5 is due to the lower concentration of the nitrate nitrogen form. It is interesting to note that urea and, to a lesser extent, calurea, which contains about 1/5 of the total nitrogen in the nitrate form, both of which yield ammonia as their first product of decomposition, influenced growth much like ammonium sulfate. That reaction plays an **important role** in the assimilation of different forms of nitrogen by fungi has been clearly demonstrated by Rippel (19), Sakamura (20), and others (13, 31).

Of the monoamino-monocarboxylic acids used, the glycine, $CH_2NH_2{\cdot}COOH$, was utilized equally well at all reactions. The cystine, $HOOC.CHNH_2.CH_2SSCH_2.\ CHNH_2{\cdot}COOH$, (table 2) was <u>considerably less</u> assimilated, notably toward the alkaline side of the reaction. The reason is obvious, glycine is quite soluble in water whereas cystine is insoluble. Of the monoamino-dicarboxylic acids used, aspartic acid, $HOOC.CH_2.CHNH_2.COOH$, permitted maximum growth throughout the range of pH 3.0 to 8.7, whereas the glutamic acid, $HOOC.CH_2.CH_2.CHNH_2.COOH$, while not permitting maximum growth was equally well utilized at reactions above pH 4. The diamino-monocarboxilic acid, arginine, $HN\begin{smallmatrix}NH_2\\NH.CH_2.CH_2.CH_2.CHNH_2.COOH\end{smallmatrix}$, did not permit maximum growth, and was best utilized on the acid side and least on the alkaline side of the pH scale. The acid amide, asparagine, $HOOC.CH_2.CHNH_2.CONH_2$, was assimilated equally well at all reactions. The casein containing a variety of amino acids was utilized best on the slightly acid side.

To determine to what an extent <u>C. blakesleeana</u> can develop in the presence of more complex nitrogenous materials,

Table 2

Relative Growth of Cunninghamella on Cyanamide & Organic Forms of Nitrogen Added on the Basis of 20 mgm. Nitrogen per 100 grams Sand

Reaction	Diameter of Lateral Growth with Different Nitrogen Materials									
	Cystine	Calcium Cyanamide	Cotton Seed Meal	Tankage	Dried Blood Meal	Milorganite	Nettolin	Mold Tissue (A. niger)	Mold Tissue (A. sydowy)	Alfalfa Hay
pH	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
5.2	26	12	45	31	29	40	54	44	53	31
6.0	27	18	50	26	27	37	54	46	54	34
6.8	25	17	47	30	31	40	53	40	50	33
7.5	22	12	48	19	30	40	50	39	50	33

a number of organic fertilizers, calcium cyanamide, mold and plant tissue were added to sand-bentonite mixtures. The results obtained are given in table 2. The nitrogen most accessable for fungus growth in the case of the organic fertilizers is found in Nettolin, which contains some nitrate nitrogen, followed by cotten seed meal. With $CaCN_2$ no growth was at first obtained, however, when a second sample was taken and wetted during a period of 14 days and then inoculated with spores of Cunninghamella, growth took place as shown in table 2. That the fungus can utilize nitrogen from mold tissue and alfalfa hay is also indicated.

The results obtained in general would lend support to the idea that C. blakesleeana could be used for measuring the nitrogen deficiencies of soil. The fungus not only responded to the plant available forms of nitrogen, namely, nitrate and ammonia, but also to the forms of nitrogen which are considered potential sources for plants, notably the monoamino acids. The writer made tests with soils of different fertility, and considerable differences in the growth of the fungus were obtained. The results, however, were not fully satisfactory, because too frequently the native micro-flora of the soil influenced the growth of the fungus.

Influence of forms of nitrogen on the natural fungous flora of soils: For this purpose soils were used with which the native flora had greatly suppressed the growth of Cunninghamella when ammonium nitrate was added as the source of nitrogen. The use of glycine and glutamic acid favored the growth of fungi less than did ammonium nitrate, however, they favored the growth of actinomycetes. Ammonium sulfate and urea also strongly favored the development of the fungous flora. With asparagin as the nitrogen source, the soil plaques remained uncontaminated after 72 hours, but the growth of Cunninghamella was also comparatively slow. Aspartic acid, on the other hand, greatly increased the rate of growth of Cunninghamella, although it also promoted the growth of other fungi slightly. Finally different ratios of asparagin to aspartic acid were tried, with the aim of finding a combination which would permit Cunninghamella to grow rapidly, but not favor the growth of the native soil fungous flora. The mixture which met these requirements best was found to consist of 0.5 gram asparagin and 0.1 gram of aspartic acid per 100 cc. of H_2O. Results with this nutrient solution, which was previously fully described, will be given later in this paper.

INFLUENCE OF FORMS OF PHOSPHORUS ON GROWTH

To study the influence of different forms of phosphorus on the growth of Cunninghamella, sand-bentonite mixtures, and soils were used as the substratum. The influence of iron, aluminum, and calcium, on the availability of phosphorus to growth was also investigated. Finally, the availability of phosphorus applied to soils, and slightly decomposed rock materials, was compared to their capacity of these soils and rock materials to fix phosphorus in difficultly available forms.

The influence of forms of phosphorus on growth in relation to reaction is shown in table 3. Of the inorganic phosphates the mono- and di- calcium phosphates are available throughout a large range of reaction. Magnesium phosphate

Table 3

Growth of Cunninghamella as Influenced by Forms of Phosphorus and Reaction

Reaction	Diameter of Lateral Growth with Different Forms of Phosphorus											
	Rate: 10 mgm. P_2O_5 per 100 g. sand						Rate: 5 mgm. P_2O_5 per 100 g. sand				Rate: 20 mgm. P_2O_5 per 100 g. sand	
	$Ca_2H_2PO_4 \cdot 4H_2O$	$Ca_3(PO_4)_2 \cdot H_2O$	$Mg_3(PO_4)_2 \cdot H_2O$	$AlPO_4$	$FePO_4 \cdot 4H_2O$	Meta-phosphate	Na-Glycero-phosphate	Ca-Hexose-phosphate acid ester	Lecithin	$CaH_4(PO_4)_2 \cdot H_2O$	Crude phytin	Dufrenite
pH	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
2.9	58	54	54	50	46	36	52	59	50	58	52	28
3.4	60	56	57	49	48	31	50	60	52	60	50	30
4.4	57	56	58	46	45	33	51	60	52	60	50	24
5.2	58	46	56	36	32	33	56	54	50	56	32	14
6.1	60	36	57	44	29	40	60	56	52	56	18	12
6.8	60	30	56	46	30	43	60	55	51	58	16	10
7.5	56	26	55	46	28	46	62	56	49	55	18	10
8.6	52	18	50	47	30	44	58	58	48	54	12	8

becomes slightly less available above pH 8.6. The tricalcium phosphate is readily available on the acid side and becomes gradually less available toward the neutral and alkaline side. _Cunninghamella_ can use to an appreciable extent the Al- and Fe-phosphates and the metaphosphate. Particularly $AlPO_4$ is absorbed on the alkaline side better than $Ca_3(PO_4)_2$. In the case of plants some investigators obtained similar results (1, 14, 25). That plants can use the metaphosphate has also been shown (7, 32). With the exception of phytin, which is appreciably utilized only on the acid side, the organic phosphates are readily available throughout the range of reaction.

In table 4, the influence of forms of phosphorus on growth of Cunninghamella on soil cultures are given. The presence of free calcium carbonate in soil appreciably renders the tri- calcium phosphate less available. To quite an extent also the di- calcium phosphate and iron phosphate are made less available. Other phosphates, notably the organic forms of phosphorus, are considerably less affected by the calcium carbonate. Soil 4 is a red soil, having a phosphorus fixing capacity of 80 per cent, as determined by the method of Heck (8). As a result of this high fixing capacity, the availability of the soluble phosphates was considerably reduced. Particularly the Fe- and Al-phosphates, the rock phosphate, and phytin are unavailable. It will be noted that appreciable growth was obtained after 48 hours in the case of the organic phosphates and particularly on soils 2 and 3, both of which are comparatively high in organic matter.

Table 4

Influence of Different Forms of Phosphorus on the Growth of Cunninghamella Applied to Different Soils at the Rate of 100 ppm. (P)

Kind of Soil	Re-action	Period Phosphorus was in contact with the Soil	No phosphorus	$CaHy(PO_4)_2 \cdot H_2O$	$Ca_2H_2(PO_4)_2 \cdot 4H_2O$	$Ca_3(PO_4)_2 \cdot H_2O$	$Mg_3(PO_4)_2 \cdot H_2O$	$AlPO_4$	$FePO_4 \cdot 4H_2O$	Rock phosphate	Crude Phytin	Alfalfa Hay	Mycelium (A.niger)	Casein	Na-glycerophosphate	Ca-hexose-phosphate acid ester	Lecthin
			Diameter of Lateral Growth with Different Phosphorus Materials														
	pH	Days	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Calcareous Soil with 1.5% $CaCO_3$	7.9	2	14	45	19	16	45	31	19	15	16	32	49	26	49	48	33
		3	14	45	21	16	45	31	20	15	17	40	50	26	50	48	43
Dodgeville Silt Loam	6.7	2	11	54	47	21	51	36	24	20	20	23	42	24	54	48	32
		3	14	54	49	24	52	39	28	23	23	39	44	29	54	48	44
Carrington Silt Loam	5.7	2	11	35	27	22	46	21	18	16	16	40	38	22	37	35	29
		3	16	45	46	26	52	34	27	24	23	48	47	39	50	50	36
Saugatuck Fine Sand	5.0	2	6	28	31	23	29	7	7	7	8	26	32				
		3	6	30	34	25	33	8	7	8	8	29	35				
Vesper Silt Loam	4.6	2	10											18	54	48	24
		3	13											21	54	48	34

Influence of Iron and Aluminum on the Availability of Phosphorus

The extent to which the fungus can use the different forms of phosphorus as has been shown appears to depend mainly on the solubility of the different forms and upon the factors which influence the solubility during the growth of Cunninghamella. To observe how iron and aluminum influence the availability of a soluble phosphorus, hematite, goethite, limonite, Al_2O_3, $Al(OH)_3$, and diorite were added to sand bentonite mixtures, containing 5 mgm. P_2O_5 per 100 grams, as $CaH_4(PO_4)_2$. The results in table 5 indicate that hematite has no effect on the availability of the phosphate, the goethite is more reactive and particularly the limonite reduces the availability of phosphorus, especially in the case when the soluble phosphorus is in contact with the limonite for 12 hours at 100° C. The Al_2O_3 was particularly effective in rendering the phosphorus difficultly available at a pH 5.2 to pH 6.0. When $Al(OH)_3$ was added in concentrations of ohly 0.02 per cent it markedly reduced the availability at pH 5.2 if the phosphorus was added to fresh colloidal aluminum hydroxide. Adding the $Al(OH)_3$ to the sand cultures, then drying, and finally applying the phosphorus, its efficiency in rendering the applied phosphorus less available was greatly reduced. In the presence of limonite and Al_2O_3, the availability of sodium-glycerophosphate was also appreciably reduced. The addition of peat apparently did not affect the availability of phosphorus. On adding colloidal SiO_2 or K_3SiO_3 to the system containing limonite or Al_2O_3, the

Table 5

Availability of $CaH_4(PO_4)_2$ as Measured by the Growth of Cunninghamella in the Presence of Fe, and Al- Oxides and Peat

Treatment: 5 mgm. P_2O_5 plus the following:	Diameter of Lateral Growth							
	Reaction of Substratum (pH)							
	2.9	3.4	4.4	5.2	6.1	6.8	7.5	8.6
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
No further addition	58	59	60	56	56	58	55	54
4% Hematite	56	50	56	54	56	55	55	52
4% Browm Goethite	47	52	49	48	46	43	40	42
4% Limonite	44	45	45	37	42	40	40	42
4% Al_2O_3	45	45	46	18	24	30	36	40
2% Diorite	56	58	58	52	54	53	52	50
2% Peat	53	50	52	50	56	56	57	54
0.02% $Al(OH)_3$	52	54	36	28	42	47	50	48
4% Limonite heated at 100° C.	24	28	32	28	29	32	34	34
4% Limonite air dried	38	40	42	40	38	39	39	42
4% Limonite Na-glycero-phosphate	50	52	48	40	41	46	50	52
4% Al_2O_3 Na-glycero phosphate	48	47	38	25	36	38	38	41

phosphorus became slightly more available if the concentration of limonite or Al_2O_3 was reduced to one per cent. At the higher concentration the effect was negligible. (Data not presented)

Influence of $CaCO_3$ & CaO on the Availability of Phosphorus

When to a sand culture, containing no bentonite, increasing amounts of $CaCO_3$, and varying amounts of $CaH_4(PO_4) \cdot H_2O$ are added, fungus growth as given in table 6, was reduced. The decrease in growth was greatest with the lower concentrations of phosphorus; the increased concentrations of $CaCO_3$ played a minor role. The presence of one-half per cent of $CaCO_3$ reduced growth nearly as much as 10 per cent. On adding 6 per cent of a 96 per cent K-saturated bentonite the efficiency of $CaCO_3$ to reduce the growth of Cunninghamella became greatly less at all concentrations. The addition of a soluble salt had an influence similar to bentonite when the $CaCO_3$ concentration was small. At the higher concentrations of $CaCO_3$ the K_2SO_4 tended to decrease growth.

The addition of CaO to sand cultures containing no bentonite prevented the germination and growth of *Cunninghamella.* If, on the other hand, small amounts of CaO were added to the sand bentonite mixtures, growth was obtained as shown in table 7.

Relation of the Phosphorus Fixing Capacity of Soils to Increase in Growth of Cunninghamella on Additions of Phosphorus

Method: The phosphorus fixing capacity of partially decomposed rocks from the state of Maine, and soils from the

Table 6

Growth of Cunninghamella as Influenced by Different Concentrations of Phosphorus and $CaCO_3$ and K-Bentonite

Treatment	Diameter of Lateral Growth — Amount $CaCO_3$ added (per cent) 0	0.5	1.0	2.5	5.0	10.0
	mm.	mm.	mm.	mm.	mm.	mm.
1.25 mgm. P_2O_5	13	8	8	7	7	5
1.25 mgm. P_2O_5 6% K-Bentonite	14	13	12	13	11	10
2.50 mgm. P_2O_5	25	11	10	11	10	8
2.50 mgm. P_2O_5 6% K-Bentonite	28	27	28	26	26	24
3.75 mgm. P_2O_5	36	18	18	17	16	14
3.75 mgm. P_2O_5 6% K-Bentonite	39	38	37	35	36	33
5.0 mgm. P_2O_5	48	30	31	28	28	25
5.0 mgm. P_2O_5 6% K-Bentonite	52	46	45	43	43	40
5.0 mgm. P_2O_5 2% K_2SO_4	48	42	40	32	24	18

Table 7

Growth of Cunninghamella as Influenced by CaO and 5 mgm. in form of $CaH_4(PO_4)_2 \cdot H_2O$

Reaction		CaO added per 100 g. sand	Diameter of growth
Initial	Final		
pH	pH	mgm.	mm.
2.9	2.9	0	56
2.9	3.6	50	57
2.9	4.5	100	51
2.9	5.6	200	36
6.8	6.8	0	55
6.8	7.3	50	38
6.8	7.6	100	37
6.8	8.0	200	40
7.5	7.5	0	55
7.5	7.9	50	46
7.5	8.2	100	42
7.5	8.6	200	44

Hawaiian Islands and Wisconsin were determined with a method proposed by Dean (5). Half gram samples of the 30 meshed materials were shaken with 50 cc. water containing 500 ppm. phosphorus in the form of $KH_2(PO_4)_2$, allowed to go to dryness on the hot plate, and then the phosphorus determined according to the method of Truog (27), excepting that the ratio of soils to extracting solution was 1 to 400. Using the same ratio and the method of Truog, the available phosphorus in the untreated sample was also determined. The amount of phosphorus fixed was then obtained from the relationship as follows:

$$\frac{\text{Amount of phosphorus extracted from treated sample}}{\text{Amount of P added, plus amount P in untreated sample}} \text{ times } 100$$

equals, amount phosphorus recovered from treated sample. This amount subtracted from 100 represents the phosphorus fixed in per cent at pH 3.

The test for phosphorus with Cunninghamella was carried out as already described, using the modified nutrient solution was employed. A parallel culture was introduced to which 5 mgm. of P_2O_5 per 100 cc. of soil were added. To do this, 44.4 mgm. of $CaH_4(PO_4).H_2O$ were added to 100 cc. of the regular nutrient solution in the test for phosphorus. Two cc. of this solution were then added to exactly 10 cc. of soil and the plaque prepared in the usual way. This plaque was brought to its optimum moisture condition by adding some of the regular nutrient solution. The phosphated and non-phosphated cultures were then placed into the same pan and incubated at 29° C. for at least 65 hours and then the diameter of the lateral growth measured. To express the growth increase in terms of availability of applied phosphorus, the following scheme was found helpful:

Growth increase due to the addition of 5 mgm. P_2O_5 per 100 cc. soil	Availability of applied phosphorus
less than 3 mm.	very low
4 mm.-6 mm.	low
7 mm.-9 mm.	moderate
10 mm.-14mm.	high
greater than 15 mm.	very high

Results: With the exception of the red sandstone, all other slightly decomposed rocks fixed more than 80 per cent of the applied phosphorus (table 8). The availability of the applied phosphorus is also low. Fixation by the soils from the Hawaiian Islands varied from about 50 per cent to about 100 per cent, and the availability of the applied phosphorus varied from very low to very high. For these soils and rocks, no relationship existed between fixing capacity, or phosphorus availability, and pH. The color of the soil, indicating the presence of hydrated iron oxides, helps better to explain differences in the phosphorus fixing capacities of these soils. In the case of the yellow soil, No.114, the availability of phosphorus at pH 7 is low, while at the same reaction, the availability is high in the case of the red-brown soil, No.113. Soils No.208 to 216 fix in general less phosphorus, and vary in availability of applied phosphorus from moderate to very high. In the case of these soils, the phosphorus is usually more available when the reaction is slightly acid or neutral.

Table 8

Growth of Cunninghamella on Slightly Decomposed Rocks, and Soils, Phosphated and Non-phosphated, and Their Phosphorus Fixing Capacities of the Materials

No.	Kind of Rock or Soil	Reaction	Fixing Capacity for Phosphorus	Diameter of Lateral Growth of Cunninghamella: No Phosphorus added	Diameter of Lateral Growth of Cunninghamella: 5 mgm. P_2O_5 per 100 cc. soil added	Availability of Applied Phosphorus
		pH	per cent	mm.	mm.	
200	Shale	4.1	85.0	12	17	low
201	Quartz trachite	4.4	82.3	4	8	low
202	Diorite	5.5	81.1	8	16	moderate
204	Rhyolite	5.5	93.3	7	10	very low
205	Andesite	5.8	91.2	9	12	very low
206	Basalt	6.2	83.0	16	20	low
207	Red sand stone	6.2	39.7	27	38	high
631	Yellow soil (Hawaii)	4.5	94.5	8	13	low
121	Yellow red soil (Hawaii)	4.8	96.0	20	20	very low
102	Yellow brown soil (Hawaii)	5.2	94.0	16	20	low
116	Yellow brown soil (Hawaii)	5.4	96.8	9	13	low
112	Yellow brown soil (Hawaii)	5.6	92.2	13	18	low
103	Light brown soil (Kauai)	5.9	93.7	13	30	very high
105	Yellow soil (Hawaii)	6.1	99.2	4	4	very low
108	Yellow soil (Kauai)	6.4	96.0	5	7	very low
111	Red brown soil (Oahu)	6.8	79.4	21	31	high
113	Red brown soil (Maui)	7.0	61.2	30	40	high
114	Yellow soil (Kauai)	7.0	95.2	8	13	low
117	Red brown soil (Maui)	7.2	59.4	26	36	high

Table 8 (Cont'd)

Growth of Cunninghamella on Slightly Decomposed Rocks, and Soils, Phosphated and Non-phosphated, and Their Phosphorus Fixing Capacities of the Materials

No.	Kind of Rock or Soil	Reaction	Fixing Capacity for Phosphorus	Diameter of Lateral Growth of Cunninghamella: No Phosphorus added	Diameter of Lateral Growth of Cunninghamella: 5 mgm. P_2O_5 per 100 cc. soil added	Availability of Applied Phosphorus
		pH	per cent	mm.	mm.	
120	Gray brown soil (Oahu)	7.7	83.7	6	13	moderate
123	Dark soil with free $CaCO_3$ (Oahu)	8.0	57.5	12	13	very low
203	Caribou loam	6.2	77.0	10	19	moderate
208	Marshall siltloam	5.0	49.0	12	19	moderate
209	" " "	6.2	35.9	20	34	high
210	" " "	6.9	37.1	20	30	high
211	Miami silt loam	5.8	44.7	30	41	high
212	" " "	7.0	43.1	29	39	high
213	Carrington silt loam	5.5	46.3	22	28	moderate
214	Carrington silt loam	7.1	43.6	24	37	very high
215	Dodgeville silt loam	5.3	51.8	9	16	moderate
216	Dodgeville silt loam	6.2	50.0	21	36	very high
217	Calcareous soil 2.3% $CaCO_3$	7.8	14.0	14	26	high
218	Calcareous soil 6.1% $CaCO_3$	8.0	11.5	10	17	moderate
219	Calcareous soil 20.0% $CaCO_3$	8.2	16.0	7	12	low
220	Calcareous soil 25.4% $CaCO_3$	8.2	9.4	6	17	high

In the case of calcareous soils the availability of the applied phosphorus varies, without a definite relationship to the $CaCO_3$ content. A fairly satisfactory relationship existed in general between fixing capacity of soils at pH 3 and availability of phosphorus as measured by the increased growth of Cunninghamella at the natural pH in the case of non-calcareous soils. Of the results given in table 8, only those from soil 103 show poor agreement. Although at pH 3, its fixing capacity is high, at pH 5.9 the applied phosphorus remains quite available.

DISCUSSION

Cunninghamella will grow over a wide range of reaction, pH 2.9 to 8.8, and is not easily influenced directly by acid or alkali, but is very sensitive to form and concentration of phosphorus. Five mgm. P_2O_5 of a soluble form added to 100 grams of sand will permit maximum growth. With decreasing solubility, the concentration of phosphorus necessary for maximum growth has to be increased in proportion to its solubility. However, the fungus can grow quite well on water insoluble forms of phosphorus. This it does probably because of the production of acid during its metabolic process. The substratum is usually more acid below the center of growth than the area surrounding it. While thus the rate of growth in the presence of soluble phosphorus is progressively uniform, in the presence of H_2O insoluble phosphorus, it, at first will be slow, but becomes rapid, usually after thirty hours.

Factors which influence the solubility of phosphorus will indirectly influence the growth of the fungus. Thus in an acid medium, water insoluble phosphates will be readily utilized. The presence of the hydrogen ion greatly aids solubility, and since the fungus is not affected by moderate acidity, good growth is obtained. The presence of hydrated iron oxides or aluminum oxides will greatly reduce the solubility of phosphorus and hence reduce growth. This is particularly marked at reactions where insoluble aluminum or iron phosphates are formed. Large~~r~~ amounts of iron or aluminum oxides may influence the availability of phosphorus from a very acid to a neutral range of reaction. Very small amounts of fresh, colloidal $Al(OH)_3$, particularly affect the solubility of phosphorus at pH 5.2 and somewhat less at pH 6.0.

On the alkaline side it is largely $CaCO_3$ that influences the solubility of phosphorus and hence the growth of Cunninghamella. The influence of $CaCO_3$ in depressing growth is most marked at the lower concentrations of phosphorus and becomes less with higher levels. The effect of $CaCO_3$ on growth is modified in the presence of a soluble salt or ~~the presence of an~~ exchange material containing a mono-valent cation. Relatively large amounts of a soluble salt, like K_2SO_4, at the lower concentrations of $CaCO_3$ will promote growth; at higher concentrations of $CaCO_3$ growth is not promoted. In the presence of an exchange complex, the $CaCO_3$ is mobilized, Ca ions are removed from the immediate sphere of growth and even at the higher concentrations of $CaCO_3$ growth is not reduced. That in the presence of an exchange complex, certain insoluble salts become quite soluble has been shown by Ungerer (29, 30).

Although Pierre found that higher plants cannot use organic phosphorus directly (17), good yields ~~by others~~ were obtained by others with organic forms of phosphorus which were readily mineralized by microorganisms (2, 21). Phytin can be used by plants largely in proportion to biological activity in which the catalitic action of phytose apparently plays an important part (6). It is significant to note that Cunninghamella can use to an appreciable extent only those forms of organic phosphorus which are readily mineralized.

While the growth of Cunninghamella on properly controlled soil cultures is ~~largely~~ approximately proportional to the concentration of readily available phosphorus, the rate of increase in growth due to the addition of a definite amount of soluble phosphorus is ~~largely in proportion~~ related to ~~the~~ factors in the soil which render the applied phosphorus difficultly available (Table 8 and Fig. 1). In carrying out the test for phosphorus, it is therefore desirable to introduce a parallel culture to which phosphorus is added. If the addition of 5 mgm. of P_2O_5 (preferably $CaH_4(PO_4)_2 \cdot H_2O$) per 100 cc. of a soil does not increase the diameter of the colony by more than 4 mm., a high fixing power for phosphate is indicated, and this is then taken into consideration in determining the rate and method of fertilization of this soil.

SUMMARY

An investigation dealing with the nutrition of Cunninghamella blakesleeana minus strain, particularly as regards the suitability of different forms of nitrogen and phosphorus was undertaken. Both sand and soil cultures were used and the growth of the aerial hyphae measured.

At neutral or slightly acid reaction, the fungus used equally well the nitrate and ammonia forms, urea, calurea, and aspartic acid. Somewhat less advantageously used were asparagin, glutamic acid, casein, arginine, and considerably less cystine. A relatively good growth was obtained with different organic nitrogen fertilizers, mold tissue, and alfalfa hay.

At a distinctly acid reaction, growth was considerably less with ammonium sulfate, urea, calurea, and glutamic acid.

At a more alkaline reaction, growth was appreciably less with arginine and casein.

Soluble phosphates were utilized equally well over a wide range of reaction, pH 2.9 to 8.6. The utilization of slightly soluble or insoluble phosphates varied with reaction. The production of acid during its metabolic processes apparently enabled Cunninghamella to make appreciable growth on water insoluble phosphates. Organic forms of phosphorus were utilized largely in proportion to the ease with which they can be mineralized.

Factors which influence the solubility of phosphates influenced growth. High concentrations of iron and aluminum oxides reduced the solubility of phosphates over a wide range of reaction. Freshly precipitated aluminum hydroxide reduced

growth particularly at a neutral and alkaline reaction.

A fairly close relationship was found to exist between the phosphorus fixing capacity of soils and increased growth of Cunninghamella on adding a soluble form of phosphorus.

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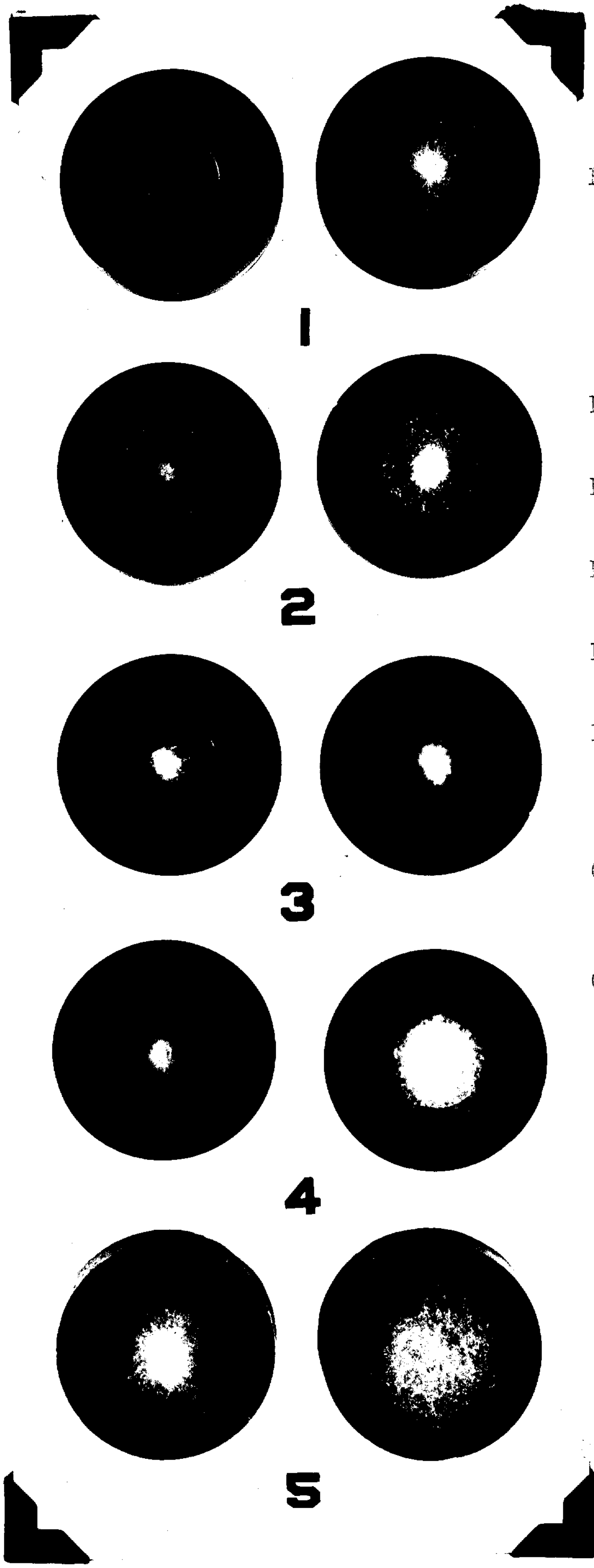


Figure 1 - Growth of *Cunninghamella* on soils untreated and treated with 5 mgm. P_2O_5 per 100 cc.

No.1. Knox silt loam, pH 5.3

No. 2. Knox silt loam, pH 6.2

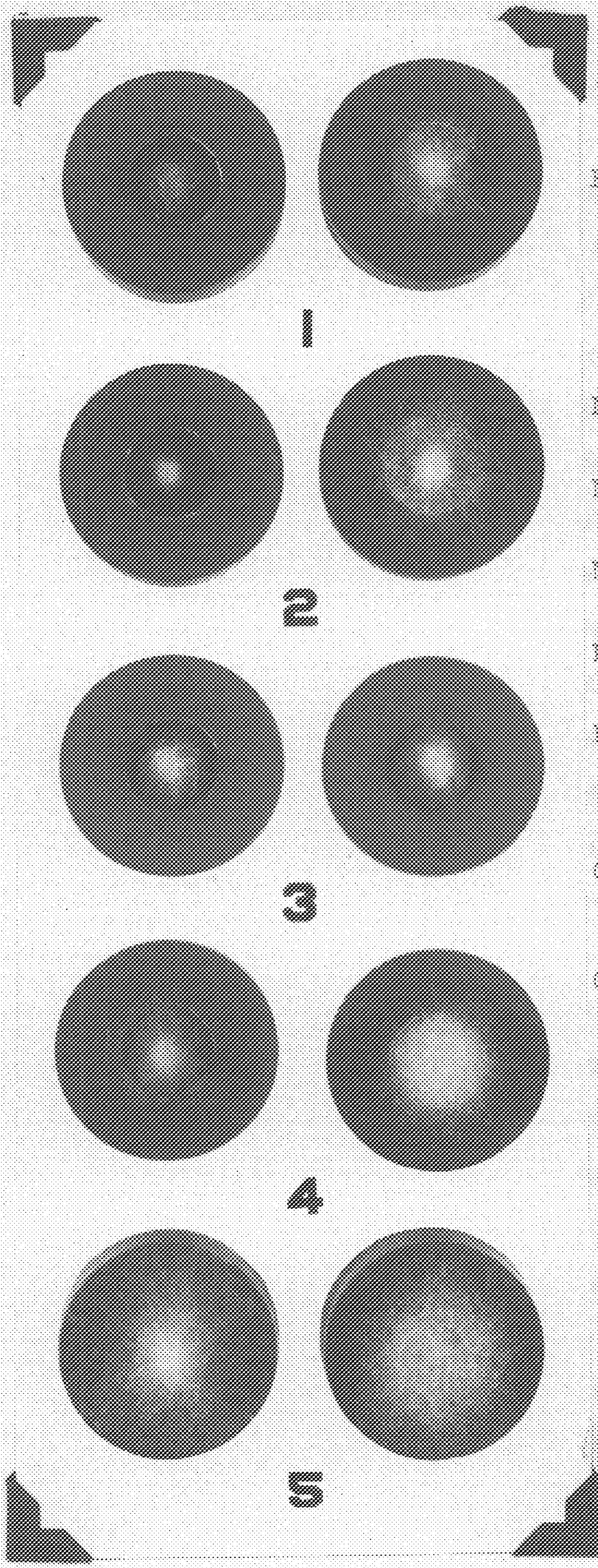
No. 3. Yellow brown soil, (Hawaii) pH 5.2

No. 4. Dodgeville silt loam, pH 6.2

No. 5. Soil from Germany, pH 6.8

Cultures on the left received no phosphorus.

Cultures on the right received 5 mgm. P_2O_5 per 100 cc. soil in the form of $CaH_4(PO_4)_2 \cdot H_2O$



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GROWTH OF CUNNINGHAMELLA AS INFLUENCED BY FORMS OF PHOSPHORUS AND NITROGEN

Adolf Mehlich

The relative growth of the fungus Cunninghamella on properly controlled soil cultures has recently been proposed for measuring the level of available phosphorus in soils (Mehlich, A., Fred, E. B., and Truog, E.). Because of the considerable promise which this method has, it seemed desirable to make a thorough study of the nutrition of this fungus, particularly as regards the suitability of different forms of nitrogen and phosphorus. An investigation dealing with this subject was, therefore, undertaken.

Spores of Cunninghamella blakesleeana minus strain were seeded on soil or sand plaques to which were added all the nutrients necessary for growth, excepting phosphorus and nitrogen, which were varied in form and concentration as desired. The plaques were made in specially designed clay culture dishes which permitted good control of moisture conditions, and made the measurements of the fungus colony more accurate. The cultures were incubated at 29°C. for at least 64 hours after which the diameter of the lateral fungus growth was measured.

At neutral or slightly acid reaction, the fungus used equally well the nitrate and ammonia forms, urea, calurea, and aspartic acid. Somewhat less advantageously used were asparagin, glutamic acid, casein, arginine, and considerably less cystine. A relatively good growth was obtained with different organic nitrogen fertilizers, mold tissue, and alfalfa hay.

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Factors which influence the solubility of phosphates influenced growth. High concentrations of iron and aluminum oxides reduced the solubility of phosphates over a wide range of reaction. Freshly precipitated aluminum hydroxide reduced growth mainly between pH 5.0 and 6.0. Calcium carbonate reduced growth particularly at a neutral and alkaline reaction.

A fairly close relationship was found to exist between the phosphorus fixing capacity of soils and increased growth of Cunninghamella on adding a soluble form of phosphorus.

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This abstract is approved as to form and content. I recommend its publication by the University of Wisconsin.

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Professor in charge of the thesis.