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ON THE USE OF MICRO-ORGANISMS IN MEASURING A DEFICIENCY OF COPPER, MAGNESIUM AND MOLYBDENUM IN SOILS[1])

by

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1. INTRODUCTION.

The fact that micro-organisms may be used in determining the amount of plant food in soils, results from their faculty to assimilate, generally speaking, the same foodstuffs as the higher plants. In adding a certain quantity of soil to a liquid medium, free of a certain nutrient, the rate of growth of a test organism, inoculated in this medium, is a measure of the amount of this particular nutrient present in the soil in an available form. In comparison with chemical analysis microbiological tests show the advantage that estimation of a certain element is possible without separating it from other compounds. This saves a great amount of time especially in the case of a determination of the so-called minor elements, such as copper, zinc, molybdenum etc. Another advantage is that the same organism and similar methods may be used in determining almost all of the elements essential for plant growth.

In the *Aspergillus niger* tests for potassium, phosphorus and magnesium, according to NIKLAS and collaborators (10), the fungus is cultivated in 75 ml Erlenmeyer flasks containing 30 ml of a nutrient liquid medium. The development of the fungus, estimated by weighing the mycelia, is a measure of the amount of plant-available potassium, phosphorus and magnesium, respectively.

In order to avoid the necessity of weighing the mycelia, 1000 ml Erlenmeyer flasks containing 40 ml of a nutrient solution were used

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in the determination of available copper and magnesium in soils. In these large flasks the *Aspergillus* mycelia have about 10 times the surface of those in the 75 ml vessels. This results in much more significant differences in the appearance of the mycelia and spores by increasing amounts of copper and magnesium, than in the case of the small flasks. Since these differences are reproducible, it is possible to estimate the concentration of these elements in the soil without weighing the mycelia only by comparing the cultures to which soil is given with those of a standard series, to which increasing amounts of copper and magnesium are added.

2. COPPER TESTS.

In the *Aspergillus* test for determining the copper which was recently worked out and described (6, 7, 8. Cf. also the earlier investigations of WOLFF and EMMERIE (12)), a nutrient solution of the following composition is used:

Water distilled from Jena glassware 1000 ml

Glucose	50 g	$FeCl_3 . 6H_2O$	0.050 g
KNO_3	5 g	$ZnSO_4 . 7H_2O$	0.020 g
K_2HPO_4	2.5 g	$MnSO_4 . 4H_2O$	0.003 g
$MgSO_4 . 7H_2O$	1 g	$Na_2MoO_4 . 2H_2O$	0.0015 g

In order to remove the small traces of copper which are found even in the purest commercial chemicals, 0.3 ml of a solution of NH_4SH and 5 g of charcoal (Norit) are added to the solution. After some 5 minutes' shaking the charcoal is separated by filtration. In order to avoid losses of the minor elements by adsorption on the charcoal, the iron, zinc and manganese salts and the molybdate are added after the filtration. Of this nutrient medium 40 ml portions are used in 1000 ml Erlenmeyer flasks of Jena glass. After sterilization of the solution for 5 minutes at 105°C., 1 gram of air-dry soil is added. The flasks are inoculated with a few drops of a suspension of *Aspergillus* spores in sterile, copper-free water and incubated at 31°C. After 4—5 days the amount of available copper can be estimated by comparing the colour of the mycelia with those of a standard series containing copper as sulphate in amounts of 0, 0.1, 0.2, 0.4, 0.6, 1, 1.3, 1.6, 2 and 2.5 γ Cu respectively [1]). Without copper the mycelia of *Aspergillus niger* are but insuffi-

[1]) It is advisable, in order to avoid the adsorption of the copper on the glass surface, to add the copper sulphate after the autoclaving of the solution.

ciently developed and quite sterile; increasing amounts of copper result in a more abundant spore formation, whereas the colour of the spores is changing from yellow to yellow-brown, gray-brown, black-brown and black (see the coloured plates in 6). Since the differences in colour of the mycelia of the standard series are clearly perceptible, the estimation of the available soil-copper may be made with a rather high degree of accuracy.

In these investigations a certain strain called "M" of *Aspergillus niger* was used throughout. Of 4 other strains two gave about the same results, the others, however, showed a different colour scale on account of increasing amounts of copper. So every strain has to be considered individually.

3. RESULTS OBTAINED WITH THE *ASPERGILLUS* TEST IN THE DETERMINATION OF COPPER.

Some 60 sandy and peaty soils from different parts of Holland were tested on their copper content. These estimations were carried out in connection with the investigation of the so-called "reclamation disease", occurring in many countries on peaty and sandy soils (6). This disease may be cured by the addition of copper sulphate in amounts of 50—100 pounds per acre. Cereals, especially oats, wheat and barley are very sensitive to the disease. The most typical symptoms are the rolled and shrunken youngest leaves and the white tips of the second highest leaves (fig. 1).

It appeared that soils on which the plants suffered from the disease had a very low content of available copper; soils producing healthy plants always showed much higher figures. Part of the results are recorded under Table I.

The data of Table I conform with the results of water culture experiments with cereals, according to which the symptoms of copper deficiency and those of the "reclamation disease" are identical (figures 1 and 2).

From these results and from copper analysis in healthy and diseased plant material it is concluded that the "reclamation disease" is caused by a deficiency of available copper.

Table I.

Available copper present in soils.

Soil	Plant growth	Available copper in γ per 1 g of soil
Sandy soil	wheat, healthy	> 2.5
„ „	white oats, healthy	> 2.5
„ „	„ „ , severely diseased	0.1
„ „	„ „ , slightly diseased	1.1
„ „	„ „ , healthy	1.5
„ „	„ „ , „	> 2.5
„ „	„ „ , diseased	0.3
peat soil	*a*) wheat, severely diseased	0.2
„ „	*b*) healthy spot in diseased field	2—2.5
„ „	wheat, severely diseased	0.2
sandy soil	„ „ , healthy	> 2.5
„ „	canary grass, healthy	> 2.5
peat soil	wheat, healthy	> 2.5
„ „	„ , „	> 2.5
sandy soil	„ , „	> 2.5
„ „	white oats, healthy	1.8
„ „	„ „ , diseased	0.4
peat soil	„ „ , severely diseased	0.1
sandy soil	„ „ , „ „	0.2
„ „	*a*) white oats, severely diseased	0.25
same field	*b*) „ „ , less diseased spot	0.8
„ „	*c*) „ „ , healthy spot	1.7
„ „	*d*) „ „ , cured by the addition of $CuSO_4$	> 2.5

4. MAGNESIUM TESTS.

For the determination of available magnesium in soils, a medium of the following composition is used:

Distilled water	1000 ml	$FeCl_3 . 6H_2O$	0.02 g
Glucose	50 g	$ZnSO_4 . 7H_2O$	0.01 g
KNO_3	5.0 g	$MnSO_4 . 4H_2O$	0.003 g
K_2HPO_4	2.5 g	$Na_2MoO_4 . 2H_2O$	0.0015 g
$Na_2SO_4 . 10H_2O$	1.0 g	$CuSO_4 . 5H_2O$	0.001 g

Three-gram portions of the air-dry soils are added to 1000 ml Erlenmeyer flasks containing 40 ml of the sterilized medium, and the 5-day-old cultures are compared with those of a standard series to

Fig. 1. "Reclamation disease" in barley. Left with the addition of 25 mg of copper sulphate per jar (100 pounds per acre).

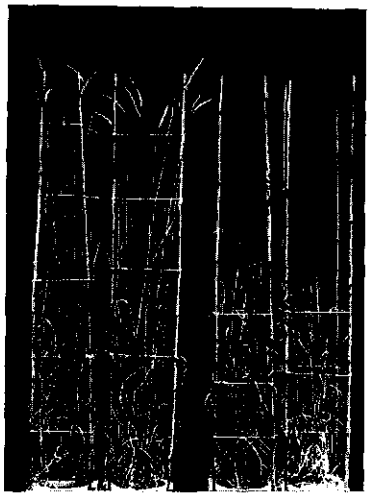


Fig. 2. Copper deficiency in barley. Right: without copper, left: with the addition of 0.2 mg of copper sulphate per 1000 ml of nutrient solution.

which magnesium sulphate is added in amounts of 0, 25, 50, 75, 100, 150, 200, 300, 400 and 500 γ of magnesium, respectively. Up to 100 γ no spores are formed in these standards, but there are significant differences in the thickness of the mycelia. With 150 γ a slight spore formation is shown, which gradually grows with increasing amounts of magnesium. Between the 400 γ and 500 γ cultures only very slight differences are observed. The appearance of these standard cultures is not changed by adding calcium salts to the medium or by changing the K:Na ratio.

For this determination other organisms than *Aspergillus niger* may be used. Good results were obtained with *Azotobacter chroococcum*. The bacterium was grown on a medium of the following composition:

Distilled water 1000 ml

Agar	20 g	$ZnSO_4 . 7H_2O$	0.001 g
Glucose	20 g	$MnSO_4 . 4H_2O$	0.0005 g
K_2HPO_4	1 g	$Na_2MoO_4 . 2H_2O$	0.0005 g
$Na_2SO_4 . 10H_2O$	0.2 g	$FeCl_3 . 6H_2O$	0.010 g
$CaCO_3$	1 g	$CuSO_4 . 5H_2O$	0.0005 g

For the purification of the agar from magnesia the following method

was used (3). The dry agar was cut into small pieces and covered with a solution of 10 per cent NaCl, which was refreshed after 10 hours. This treatment was repeated 10 times, after which the agar was washed with distilled water until no more Cl-ions could be detected.

Ten ml of this medium were mixed with 2 g of soil, boiled for some minutes and poured into 9 cm Petri dishes. After solidification the agar-soil-plates were covered with 5 ml of the same medium in order to get a smooth surface, and inoculated with a young culture of *Azotobacter chroococcum*. It appeared that without the addition of magnesium *Azotobacter* did not grow; with 10 γ of magnesium small colonies were formed, which much increased in size when 100 γ was given. The best growth, however, was obtained with 400 γ of magnesium per plate.

Another organism which may be used for the determination of small amounts of magnesium is *Bact. prodigiosum* which needs this element for its growth and for the development of its red pigment. In order to cultivate this bacterium an agar medium of the following composition was used:

Distilled water	1000 ml	$Na_2CO_3 . 10H_2O$	1 g
Agar	20 g	$ZnSO_4 . 7H_2O$	0.0005 g
Glucose	5 g	$FeCl_3 . 6H_2O$	0.001 g
K_2HPO_4	1 g	$MnSO_4 . 4H_2O$	0.0005 g
NH_4NO_3	1 g	$Na_2MoO_4 . 2H_2O$	0.0005 g
K_2SO_4	1 g	$CuSO_4 . 5H_2O$	0.0005 g

Plates, 9 cm in diameter, were prepared and inoculated with the bacterium. After 4 days at 20°C. very tiny, white colonies developed, due to the traces of magnesium present in the nutrient salts. With increasing amounts of magnesium a better growth appeared and the colour of the colonies gradually grew more reddish. Whether *Bact. prodigiosum* may be used for the estimation of the available magnesium in soils still remains to be investigated.

5. RESULTS DERIVED FROM THE MAGNESIUM TESTS.

Magnesium was determined in a number of sandy soils on which plants were suffering from the so-called "Hooghalen disease" or acidity disease, occurring especially on acid soils. The plants being very chlorotic are of poor growth. In cereals, dark green spots are shown on the yellow-green leaves: evidently the production of chlorophyl does not function properly.

Although a distinct relation is observed between the occurrence of the disease and the low pH of the soil, the latter is not the direct cause of the former. This is proved by cultivating plants in water or sand cultures provided with all of the necessary elements. A good development is obtained at pH-values which are lower than those of the soils on which the disease is found [1]).

In the publications of GEHRING (4) and VAN ITALLIE (5) good results of the application of magnesium salts to acid soils are shown. According to GEHRING the magnesium in these soils is very strongly adsorbed by the soil colloids and a neutralization of the soil is said to enhance the availability of the magnesium. VAN ITALLIE reports that plants growing on acid soils need more magnesium than those on neutral ones, whereas the adsorption of this nutrient by the roots does meet with more difficulties in the former case.

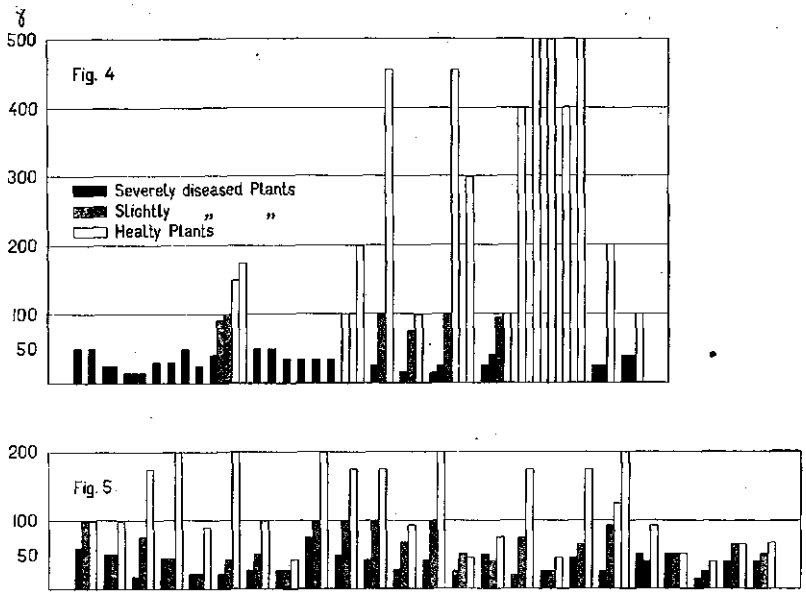
Fig. 3. Magnesium deficiency symptoms (white oats in a nutrient solution, a few days after the addition of a small amount of $MgSO_4$).

In studying the symptoms of magnesium deficiency in water cultures of cereals it appeared that these symptoms were very similar to those of the "Hooghalen disease". In both cases the plants grew poorly, with symptoms of chlorosis and showed dead lower leaves. The spotted leaves, which represent a milder form of the disease, could be obtained in the water cultures by adding small amounts of magnesium sulphate to the deficiency cultures (cf. fig. 3).

A great number of soil samples from fields with diseased crops were tested with *Aspergillus niger* on the available magnesium. Figs. 4 and

[1]) It must be emphasized that detrimental effects of low pH-values in media well provided with magnesium may be obtained. These injuries which will be discussed in a subsequent paper are not identical with those of the "Hooghalen disease".

5 contain the results of these investigations. Fig. 4 deals with samples from experimental plots and other fields of well-known properties; the soils of fig. 5 were received from farmers and agricultural advisers in different parts of Holland. The samples from one field are always placed together in the graphs.



Figs. 4 and 5. Available Magnesium in γ per 3 grams of soil.

It is revealed by these figures that soils bearing healthy plants have an available magnesium content being nearly always 100 γ or more in 3 grams of soil. The pH-value of these soils generally lies above 5. Soils with diseased plants, on the contrary, contain very little available magnesium. The pH-values of these soils are mostly between 4.2—5. Evidence that the bad growth of *Aspergillus niger* is really due to the low magnesium content, and not to the acid reaction of the soil added, is given by the fact that mixing the soil samples with 0.5 gram of magnesium-free calcium carbonate did not change the growth of the fungus; the addition of 500 γ of magnesium, however, brought about a normal growth.

The low content of available magnesium in acid soils is not due to the absence of magnesium in these soils. This could be proved by calcinating the samples during 2—3 hours at 500°C., and adding the ash to the *Aspergillus* flasks. It appeared that in many cases the amount

of available magnesium in the ash was more than 20 times higher than that in the soil.

The absence of available magnesium in acid soils is most probably due to the leaching of the Mg-ions under the influence of the low pH. This could be proved by the following experiment. To a slightly acid sandy soil with a high content of available magnesium, increasing amounts of dilute sulfuric acid were added, in order to bring the pH down to 4.1, 3.9, 3.6, 2.8, and 2.2 respectively. After 2 days the soils were dried and 6 days later 3-gram portions were added to *Aspergillus* flasks. A fixation of magnesium in the acid soils, as reported by GEHRING, could not be observed. The same acid soil, however, leached on a filter with distilled water till no more SO_4-ions could be detected in the filtrate, had lost practically all of the available magnesium.

6. EFFECT OF NEUTRALIZATION.

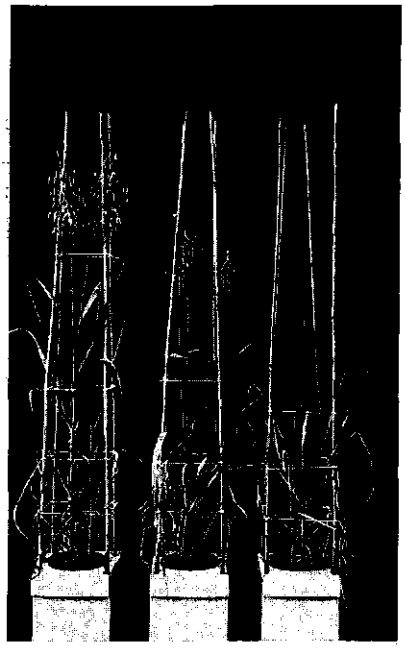


Fig. 6. White oats growing in acid soil. Right: untreated soil, center: addition of $CaCO_3$, and left: addition of $MgSO_4$.

Soils on which the plants are suffering from the "Hooghalen disease" generally may be improved by adding neutralizing substances such as $CaCO_3$, CaO or by fertilizing with $NaNO_3$. It is not yet known how this favourable effect of the neutralization is to be explained. Partly it is due to the magnesium content of the lime used (in two samples of $CaCO_3$ magnesium contents of 0.2 and 0.5 per cent were found). Very pure $CaCO_3$, however, although not increasing the amount of available magnesium, also gave a better growth of the plants (fig. 6, Table II).

Evidently the improved plant growth in the limed soils must be ascribed to an increased assimilation of magnesium which is due to the better developed root system or to an easier absorption of Mg-ions by the root cells in the less acid media.

Table II.

Effect of $CaCO_3$ and $MgSO_4$ on the available magnesium in soils and on the yield and magnesium content of oat plants.

Samples	Available magnesium per 3 g of soil in γ (after cropping)	seed		straw	
		dry matter in g [1])	Mg in mg [1])	dry matter in g [1])	Mg in mg [1])
acid sandy soil A	25—50	0	0	2.66	0.35
with $CaCO_3$	25	3.41	1.66	5.31	0.50
with $MgSO_4$	300	6.01	7.35	5.60	6.79
acid sandy soil B	25—50	1.08	0.62	4.66	0.44
with $CaCO_3$	25	4.75	3.77	7.31	0.52
with $MgSO_4$	300	7.81	9.81	7.17	7.45

[1]) average values from duplicate samples.

7. MICROBIOLOGICAL DETERMINATION OF SMALL AMOUNTS OF MOLYBDENUM.

Recent investigations from ARNON and STOUT (1) have shown the indispensability of molybdenum for the growth of tomato plants. From previous publications it appears that under certain conditions *Azotobacter chroococcum* (2) and *Aspergillus niger* (7, 8, 11) do not thrive well without the presence of small amounts of this element in the nutrient media. Although it is unknown whether molybdenum deficiency of higher green plants is found in natural soils, it may be of some importance to determine the available molybdenum in these media. For these investigations *Azotobacter chroococcum* (VAN NIEL (9)) as well as *Aspergillus niger* may be used.

Summary.

A description is given of some microbiological tests for the determination of plant-available copper, magnesium and molybdenum in soils. In these investigations *Aspergillus niger* and in a few cases *Azotobacter chroococcum* and *Bact. prodigiosum* were used. From the figures obtained it is revealed that soils on which the plants are suffering from the so-called "reclamation disease", have a much lower available copper content than those producing healthy crops. Soils on which the plants show the so-called "Hooghalen disease", have a very low

content of available magnesium. From these results and from experiments with cereals it is concluded that the "reclamation disease" is caused by a deficiency of plant-available copper and that a deficiency of available magnesium is the chief cause of the "Hooghalen disease".

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