RELATIONSHIP OF NITRATE NITROGEN AND CHLOROPHYLL CONTENT OF WHEAT GROWN IN SAND CULTURES

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INTRODUCTION

In modern times work on so-called nutrient solution cultures as related to plant metabolism may be said to have started within the decade ending in 1920. Cultural experiments, in which certain mineral elements were deficient, have resulted, in addition to abnormal growth, in an alteration in the chlorophyll content of the leaves.

From the work of Willstätter and his co-workers it is known that nitrogen and magnesium are incorporated in the chlorophyll molecule. Thus, a variation in the quantity of either of these elements, or in their availability to the plant in the soil or the cultural medium, will cause a disturbance in the pigment content, providing that all other factors for pigment production remain ideal.

The purpose of this investigation was to study quantitatively the chlorophyll production in wheat as influenced by varying the nitrate nitrogen content of the nutrient solution when using sand cultures.

HISTORICAL

The quantitative study of chlorophyll as effected by environmental conditions was perhaps first made by VILLE (27) who worked with hemp. He noted that the increase in green coloring was due to increasing the amount of nitrogenous fertilizers, and that the intensity of the color varied as the nitrogen was increased or decreased.

In working with cultures of algae ARTARI (3) found that by adding nitrogen from organic sources to cultures growing in the dark he could keep them green. BORESCH (5) who also worked with algae found that when an inorganic source of nitrogen in the form of a nitrate solution was added, the brown colored cultures would regain their natural green color. He also found that as the cultures turned brown, the extracted chlorophyll was in reduced amounts.

McBETH (16), working with citrous plants, noted that extreme mottling is frequently associated with a high nitrate content, but that the correlation is by no means an invariable one. Other environmental factors may influence mottling. A summary of the various causes of mottling may be found in the publication of BRIGGS, et al (6).

In studying the mottling of Coleus leaves, SCHERTZ (22) reported that in the case of complete mottling, nitrate nitrogen had almost disappeared from the leaves.

SIR JOHN RUSSELL (21) discussed the results of fertilizers and their effects upon plant growth and concluded that nitrogenous fertilizers increased the leaf growth, thus producing larger leaves and stems and inducing a greater formation of green coloring matter. PINCKNEY (19) in studying the effects of nitrate applications upon the hydrocyanic acid content of sorghum noticed that the nitrogen differences would affect the greenness of these plants.

DEUBER (7) in working with soy bean seedlings noted a decrease of chlorophyll in plants grown in nutrient solution, when compared

with plants grown in distilled water. But he concluded that the size of the leaf, either fresh weight or area, was more reduced by the low plane of mineral nutrition. Results per leaf basis indicated that more chlorophyll was present in those seedlings grown in nutrient solution than in distilled water. GUTHRIE (10) reported that under greenhouse light conditions in the winter, minus nitrogen cultures tended to have more chlorophyll than the complete cultures, the opposite being true in the spring when the light intensity increases. This seems to check with the findings of DEUBER (7) in 1928 who also noticed an increase in chlorophyll during the winter months with cultures deficient in nitrogen.

SCHERTZ (23) found that results differed quite appreciably when working with various fertilizers in regard to chlorophyll production and nitrogen concentration. His high nitrogen values were associated with intermediate chlorophyll content in most of his results.

Of recent work, TAM and MAGISTAD (25), 1935, studying the effect of nitrogen fertilizers and chlorophyll content of pineapple plants, found a high correlation between the amount of nitrogen applied and the corresponding increase in chlorophyll concentration.

METHODS

The wheat used in this experiment was of the variety RED ROCK.* Approximately 400 seeds were germinated on moist paper toweling and after five days the seedlings that were to be used were carefully selected for uniformity. Six seedlings were then transplanted into each of 12 nine-inch glazed earthenware pots. These plants were grown in a pure, washed silica sand (99.82 per cent) with a Shive's R_3S_3 solution (4) which was modified in the concentration of nitrate nitrogen in each of the pots, thus resulting in twelve different nitrogen treatments. A modified method of continuous nutrient solution supply was used (2, 18) which was regulated to a 600 cc. drip per day. The solution flow was stopped once on every sixth day for a 24-hour period as a means of further aëration. A cotton plug prevented the loss of quartz sand through the drain of the pot.

The 19 liter nutrient solution bottles were first painted a flat black and then coated with an aluminum paint to prevent algal growth and to minimize temperature fluctuations of the nutrient solution in the bottles. Algal growths appeared only once in pots #1 and #2, and after once removing the top sand, no more growths were observed. The constant level reservoirs were frequently cleansed to minimize algal growths.

*Winter wheat of the 1935 crop.

The 12 pots were arranged in groups of four, each group being illuminated by a 1,000 watt electric bulb. The Benjamin reflectors were supplemented by cardboard reflectors to insure each group the same amount of illumination. At a distance of 54 1/2 inches from the bulbs to the surface of the sand, a Weston Photronic Foot-Candle Meter gave a reading of 350 foot-candles at the beginning of the experiment. At the end of the experiment the illumination had decreased to 200 foot-candles.

The amount of nitrate nitrogen supplied as calcium nitrate varied in each pot in increasing amounts of 40 p.p.m.* of nitrogen. The first pot received 40 p.p.m. and the twelfth, 480 p.p.m. of nitrate nitrogen. From preliminary studies this range of 40-480 p.p.m. of nitrogen gave apparently good growth, and it was thought that this range would also be sufficient to include any abnormalities which might occur.

During the course of the experiment, November 4 to December 12, 1936, the greenhouse temperature was maintained between 21°-25° C., and the sub-surface sand temperature remained rather constant, 22° C.± 1.0° C.

Since the sun, during this period, furnished only a small fraction of the illumination, as compared with spring or summer, additional illumination was furnished by the 1,000 watt electric light bulb to give a ten hour photo-period during the day.

*The abbreviation p.p.m. will be used to denote parts per million.

CHLOROPHYLL EXTRACTION

The method adopted for the extraction of the chlorophyll (α and β)* was that of ULVIN (26) which was a modification of the method used by SCHERTZ (22). From preliminary work it was found that 5.0 gram samples of the fresh tissue had to be used due to the limited quantity of the fresh material produced. Immediately after harvesting the plants, 5.0 gram duplicate samples were weighed and then placed in a refrigerator. The remaining tissue was weighed and dried at 45° C. (12).

For the details of the extraction, the original paper should be consulted. Briefly, the method is as follows. The fresh leaves were extracted by grinding the tissue with quartz sand and acetone, a twenty-one minute grinding was necessary for complete extraction of the pigments. The yellow mass of tissue was filtered and washed with petroleum ether. After washing out the yellow pigments, the ether-chlorophyll solution was saponified with a saturated methyl alcohol-potassium hydroxide solution. The ether-alkali-chlorophyll solution after remaining in the refrigerator overnight was washed, and then the chlorophyllin layer separated into a 100 cc. volumetric flask. The chlorophyll content was then compared with a prepared color standard (11) in a colorimeter.

*The term chlorophyll will be used to denote both the α and β forms.

EXPERIMENTATION

EXPERIMENT I.

EFFECT OF NITRATE NITROGEN ON CHLOROPHYLL CONTENT OF WHEAT

No differences in height could be detected in any of the 12 treatments as the experiment progressed. Visibly, all the plants appeared equally green to the eye. In the first two pots (the two lowest in nitrogen) small yellow spots appeared on the tips of a few of the plants, otherwise no mottling appeared.

After harvesting the plants of one treatment, the sand adhering to the stems was carefully removed and duplicate 5.0 gram samples were cut so as to include both stems and leaves. The duplicates were placed in small beakers, covered, and placed in the ice-cube compartment of a refrigerator. The remaining tissue was weighed and dried at 45° C. (12). Each treatment, or set of plants, was handled singly as above in order to minimize chlorophyll decomposition.

The pH of the nutrient solutions was determined after the experiment had begun. The range of pH was from 4.38 to 3.97, with an average increase of acidity of .03 pH per culture as the nitrogen was increased. Since this range was so small, no buffer was added. Unfortunately, no pH determinations were made on the solution after it had passed through the sand.

TABLE I

NITRATE NITROGEN, CHLOROPHYLL CONTENT
AND PER CENT MOISTURE OF WHEAT LEAVES

CHLOROPHYLL PERCENTAGES ON
FRESH AND DRY WEIGHT BASIS

p.p.m. Nitrate Nitrogen	Chlorophyll per 5 gm. Mg.	Chlorophyll Fresh Weight %	Chlorophyll Dry Weight %	Moisture %
40	5.87*	.117	.984	88.08
80	5.63	.112	.954	88.20
120	6.21	.124	1.11	88.91
160	6.64	.133	1.18	88.74
200	6.96	.139	1.29	89.22
240	7.07	.141	1.26	88.79
280	8.45	.169	1.41	87.99
320	9.49	.189	1.62	88.32
360	6.21	.124	1.04	88.08
400	6.91	.138	1.17	88.21
440	7.34	.146	1.28	88.58
480	7.72	.154	1.18	86.94

*Values are averages of duplicates

The results of the chlorophyll analyses are shown graphically in Fig. 1, the chlorophyll being on dry weight basis. It is interesting to note in Fig. 1 the gradual increase found in the chlorophyll content in the first eight treatments. This is indicated by the straight line. In culture 9, which had 360 p.p.m. of nitrogen, there was a sudden decrease in chlorophyll content, followed by another gradual increase with a falling off again in the last treatment.

The moisture content of the leaves remained rather constant, varying only 2.28 per cent between the highest and the lowest percentage. This lack of any great fluctuation may be due to the age of the plants, for, as yet, there had not been any outstanding accumulation of storage products.

With this dropping off of chlorophyll content in the high nitrogen cultures, it seemed advisable to make some nitrogen determinations as a means of attempting to find what adjustments might have taken place in the nitrogen metabolism of the plant.

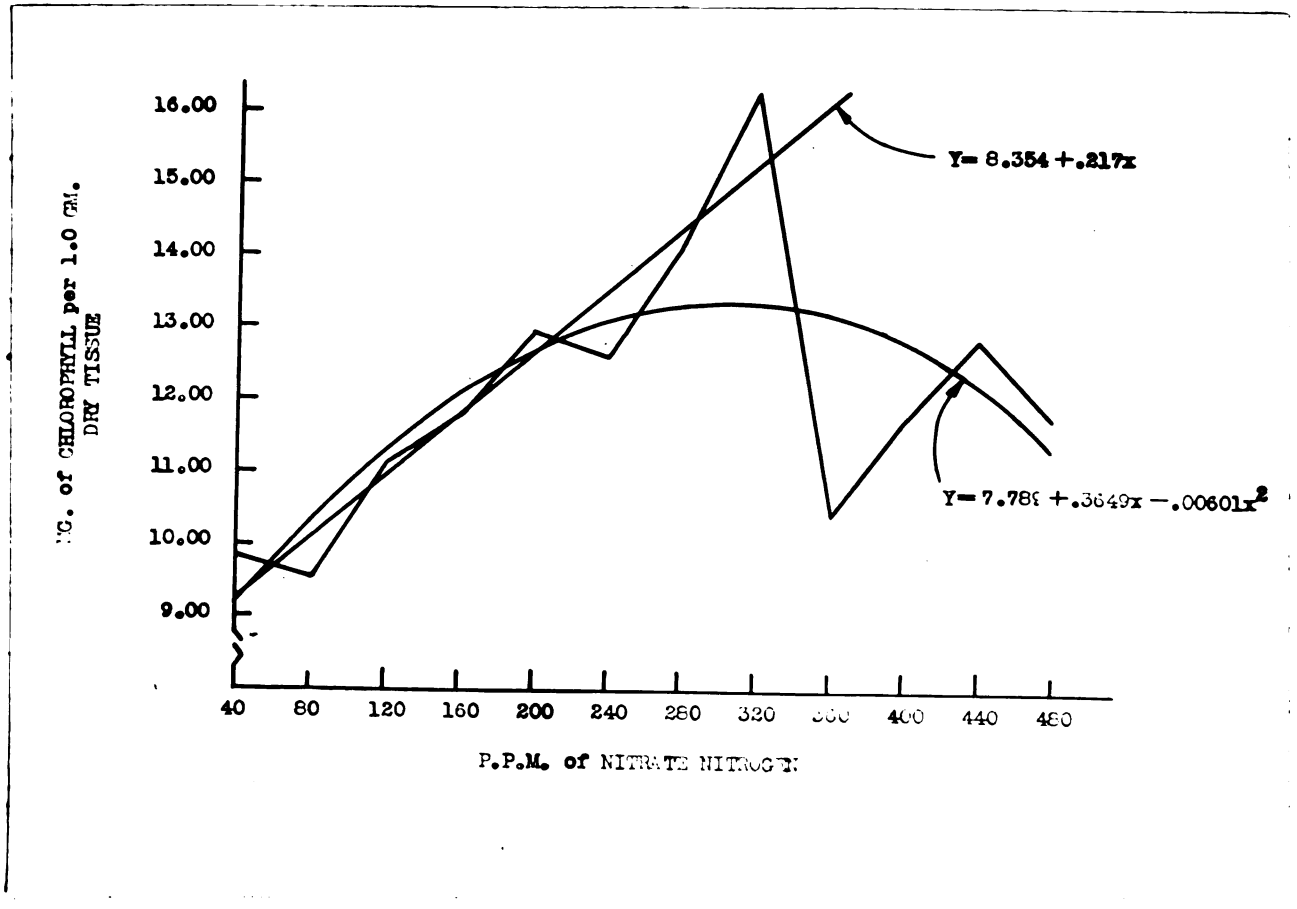

Fig. 1. Amounts of Chlorophyll produced on dry weight basis. Equations represent lines of best fit. The straight line includes only the first 8 treatments; the curve, all 12 treatments.

EXPERIMENT II.

EFFECT OF NITRATE NITROGEN ON PROTEIN, TOTAL NITROGEN, AND TOTAL ORGANIC CONTENT OF WHEAT

The oven-dried material (12, 14, 15) was ground and passed through a mesh screen. Triplicate samples weighing between 20-25 milligrams were weighed in small, glass vials (1 x .5 cm.). This amount was chosen as roughly containing one milligram of nitrogen. The samples were corrected for moisture by drying additional material at 95° C.

A modified method of PREGL (20) and DONEEN (8) was used to determine total nitrogen to include nitrate nitrogen. The samples were thoroughly mixed with 3 cc. of the acid mixture and allowed to stand for 20 minutes. A small amount of sodium thiosulfate was added and the mixture heated with a low flame until fumes appeared. After cooling, a few milligrams of a 3 to 1 copper sulfate-potassium sulfate mixture was added and the sample digested almost to clearness. A few drops of a 30 per cent hydrogen peroxide solution was used to complete the oxidation. The apparatus used for the distillation is one described by ALLEN (1).

Protein nitrogen was determined according to the technique described by VAN SLYKE (13) and used by McCALLA (17) on plant tissues. Preliminary precipitations with 2.5 per cent trichloracetic acid showed that when three extractions were used on a sample containing 50 milligrams of dry tissue, the filtrate of the last extraction

contained no nitrogen thus showing a complete precipitation of the proteins and a complete extraction of the soluble forms of nitrogenous compounds.

The extractions were carried out as follows: The acid (approximately 50 cc.) was added to a beaker containing the dried tissue, and the mixture allowed to stand for 30 minutes after which the acid was decanted off. A similar 30 minute period with fresh acid followed after which a 15 minute extraction completed the protein precipitation. The material was transferred to a filter paper and washed with more acid to remove traces of soluble nitrogens from the tissues and filter paper. The filter paper and sample were then digested with sulfuric acid and a 3 to 1 copper sulfate-potassium sulfate mixture. The final clearing was accelerated with a few drops of 30 per cent hydrogen peroxide. The distillate was run into a 4 per cent boric acid solution containing methyl red and titrated against .01 normal sulfuric acid.

A total organic content of the material was also desired. This was determined by an ashing technique (24). Since a round furnace was not available, a small (25 x 11 x 7.5 cm.) muffle furnace was used. Duplicate determinations were made using 100-125 milligram samples.

TABLE II.

NITRATE NITROGEN, TOTAL NITROGEN, PROTEIN AND ORGANIC CONTENT OF WHEAT LEAVES

p.p.m. Nitrate Nitrogen	Gm. Total Nitrogen per 100 Gm. Dry Tissue	Mg. Protein per 1 Gm. Dry Tissue	Mg. Organic Matter per .10 Gm. Dry Tissue
40	3.71*	81.06#	82.58#
80	4.42	93.25	81.77
120	4.01	81.69	79.87
160	4.29	90.06	82.17
200	4.59	99.94	80.63
240	4.52	83.63	81.89
280	4.26	93.19	82.13
320	4.34	87.00	81.51
360	4.46	81.88	80.92
400	4.55	80.94	80.76
440	4.73	100.56	80.25
480	4.59	90.00	80.58

*Average of triplicates
#Average of duplicates

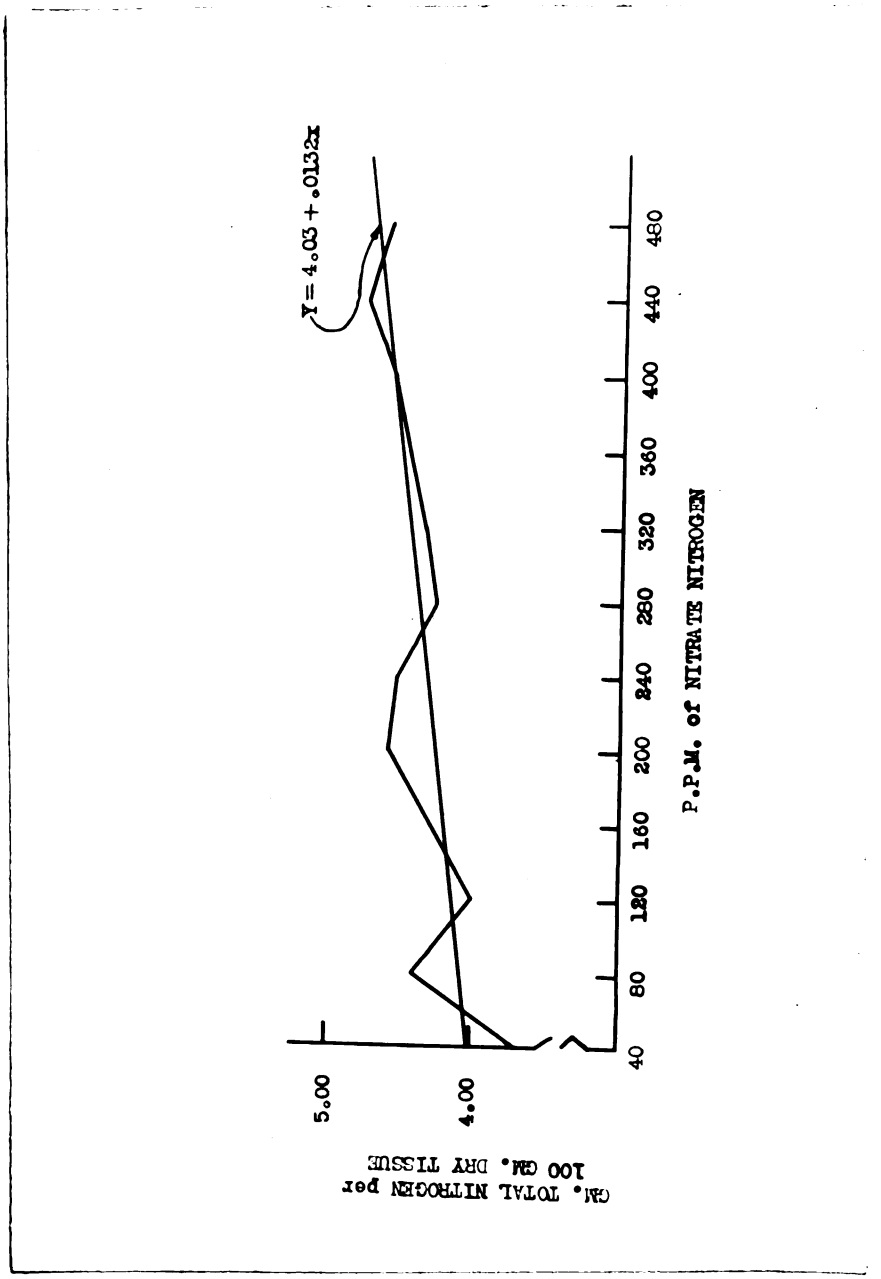

Fig. 2. Amounts of total nitrogen produced on dry weight basis. Equation represents best line of fit.

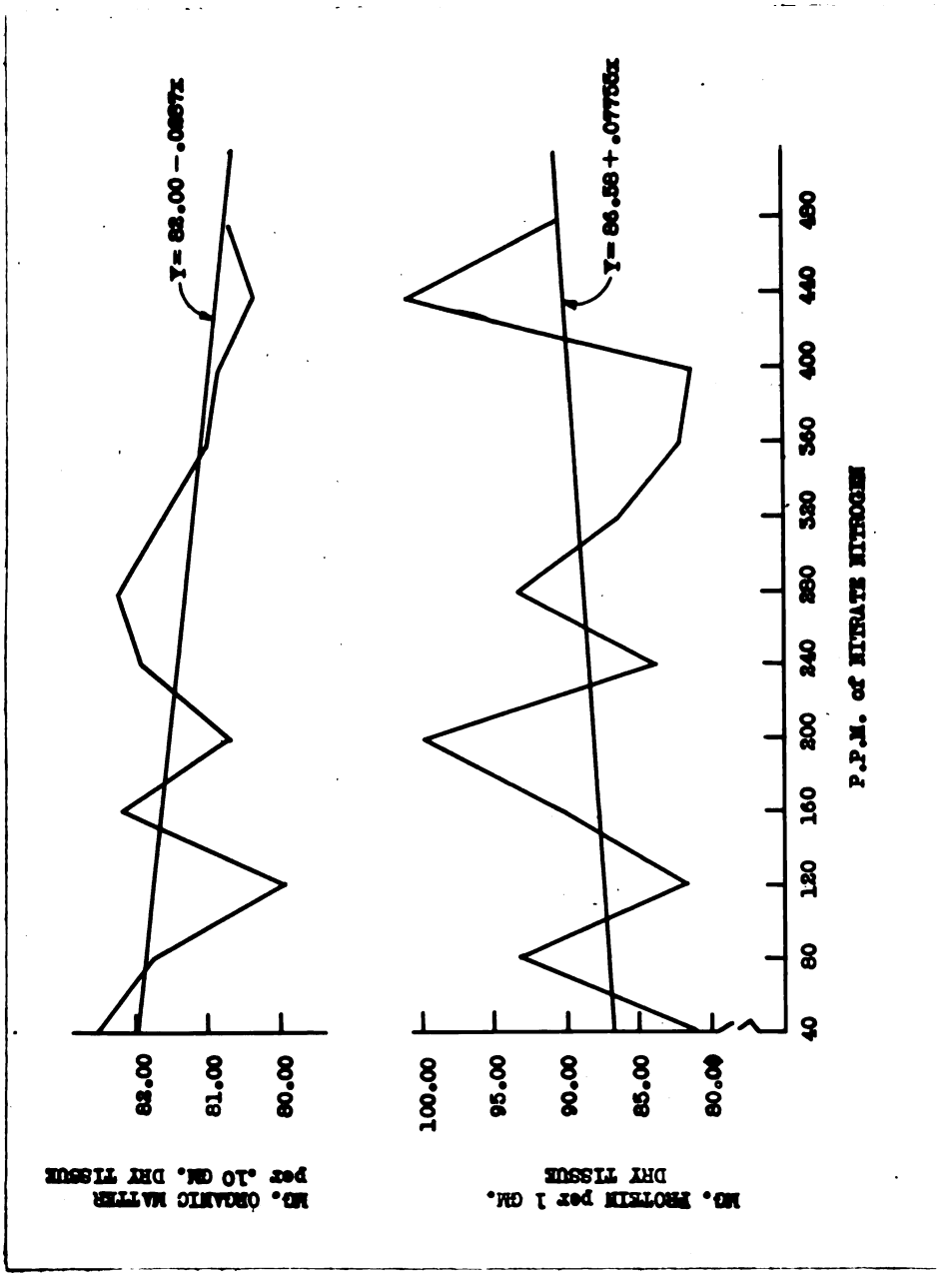

Fig. 3. Amounts of protein and total organic content based on dry weight basis. Equations represent best lines of fit.

STATISTICAL TREATMENT

Since the chlorophyll content of the leaves showed such a sharp decline in the ninth culture after a linear production, it seemed advisable to compute not only the 12 treatments, but also the first eight cultures. For the computation of the best line of fit for the whole series a second degree parabola was used; $Y = a + bx + cx^2$, while for the first 8 pairs of observations the formula, $Y = a + bx$[##], was used. From the data obtained, the coefficients of correlation were derived by the method of least squares. The same method of analysis was applied to the data obtained in the protein, total nitrogen, and total organic content of the leaves.

Tests of significance and the tables used were those of FISHER (9). Results are summarized in Table III.

TABLE III.

THE CORRELATION COEFFICIENTS
AND THEIR TESTS OF SIGNIFICANCE

Analysis	r	t_r	P_t (%)	z	P_z(%)
Chlorophyll	.695*	3.056	.01#	.8596	.03
Total Nitrogen	.768	3.793	.01	1.154	.01
Protein	.12	—	—	—	—
Total Organic Content	.752	3.614	.01	.9849	.02

*Coefficient of correlation using a second degree parabola
#Percentage figures represent probability values of results being equal to or surpassed in a 100 cases of random sampling.
##Coefficient of correlation = .975

DISCUSSION

From the statistical analyses, it seems evident that there is a significant correlation between the chlorophyll content of wheat and the concentration of nitrate nitrogen in the nutrient solution. The most sensitive test of the correlation coefficient gave an exceedingly low probability value.

Since the proteins showed no definite trend, there was no correlation between the nitrogen nutrition and the protein concentration.

The highly significant increase of total nitrogen and the significant decrease of total organic content (TABLE III.) suggests that the excess nitrogen, in the higher nitrogen concentrations, diverted the most condensed forms of carbohydrates to form organic nitrogenous compounds (28). Perhaps these transformation products of organic nitrogen, as they increased in the tissues, became the inhibitors of chlorophyll formation. This would explain the sharp decrease of chlorophyll in the ninth culture, and the decreasing tendency of chlorophyll content in the last four cultures.

As a further experiment in nitrogen, it would be interesting to study the effect of an excess of nitrate nitrogen nutrition on the various nitrogen fractions of the tissues, and to interpret their possible relationship as inhibitors of chlorophyll formation.

SUMMARY

Through the use of sand cultures an attempt was made to find the relationship between nitrate nitrogen nutrition and chlorophyll production in wheat. It was found that in a range of 40-480 p.p.m. of nitrogen, there was a significant correlation between the concentration of nitrogen and the amount of chlorophyll produced.

In excessively high nitrate nitrogen concentrations, a decrease in chlorophyll content was noted.

From the nitrogen analyses that were made, it seems possible that in the very high nitrogen cultures, the organic nitrogenous compounds produced by the carbohydrates became inhibitors to chlorophyll production.

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Fig. 4. Shows method of nutrient solution supply, arrangement of pots, and the Benjamin reflectors supplemented by cardboard.