NUTRIENTS AND PIGMENTATION

Some Factors Affecting Pigmentation of Azotobacter Chroococcum

G. R. ANDERSON and J. V. JORDAN
University of Idaho, Moscow, Idaho

Boron is utilized by the azotobacter cell and exerts a positive effect upon pigment production and pigment coloration of *Azotobacter chroococcum*. Optimum pigmentation in nitrogen-free media occurred at concentrations of 70 p.p.m. of boron and 1.0 p.p.m. of copper. *Azotobacter chroococcum* appears to contain a Dopa oxidase system which produces the pigment, melanin. High concentration of boron appears to inhibit pigment production when an optimum concentration of copper is present. When used as the source of nitrogen, tyrosine and glutamic acid increased pigmentation by *Azotobacter chroococcum*.

THE ROLE OF MINOR ELEMENTS in the nutrition of plants and animals is an important one. The essentiality of these elements is unquestioned but their exact role in life processes is yet to be determined. The minor element, boron, and its influence on higher plant life have been the subject of intensive study.

Leggatt (*10*) reported that boron applications corrected abnormal sprouting of peas grown in a boron-deficient soil. Boron was suggested by Woodbridge (*18*) to be of importance in the formation of the middle lamella of plant cells. Brenchley (*3*) indicated that boron is important in the growing tips of plants, as boron deficiencies appear first in this region. Bobko *et al.* (*2*) concluded that in presence of boron, nitrogen and ash elements were utilized more efficiently and were assimilated more rapidly. Furthermore, Scripture and McHargue (*17*) and Briggs (*4*) noted that in boron-deficient plants soluble organic nitrogen compounds accumulated. This indicated that the amination of carbohydrate derivatives is inhibited. In the microbiological field, Mulder (*13*) and Brenchley (*3*) have shown that nodules are not formed on *Leguminosae* in boron-deficient soil. Jordan and Anderson (*9*) found that the addition of boron increased the amount of nitrogen fixed by *Azotobacter chroococcum*.

Copper is another micronutrient of importance in the metabolism of plants and microorganisms (*6*, *14*), and its role in the formation of melanin pigments has been established (*5*, *15*). Copper is concerned in the enzyme activity that brings about the conversion of tyrosine and other precursors to melanin (*8*). Copper is also known to be important in the conversion of 3,4-dihydroxyphenylalanine (Dopa) to melanin (*7*). Increased pigment production by *Azotobacter chroococcum* in the presence of added boron was noted in earlier experiments and emphasis has been placed on a study of pigmentation because of the wide importance of melanoid pigments, which are implicated in reactions occurring in such areas as soil maturation, food spoilage, and malignant melanomas.

The object of this experiment was to investigate further the effect of boron on *Azotobacter chroococcum*. Studies were made on the absorption of boron by the azotobacter cell. The effect of boron, copper, and amino acids upon pigmentation was investigated and corollary studies were made of the influence of boron and copper in the 3,4-dihydroxyphenylalanine oxidase system.

Experimental Methods

Bacterial cultures used in this study were two strains of *Azotobacter chroococcum*. Strain 1 was a freshly isolated culture selected for its high pigment production. Strain 2 was a laboratory culture carried on laboratory media for many years, selected for its low pigment production. Unless indicated all results reported were obtained with strain 1.

The basal medium used was a nitrogen-free agar described by Martin (*11*). This served as the basic culture medium for studying microbial responses to metallic ions and added nitrogenous substances. The mineral nutrients added to the basal medium were sterilized separately and added to the Petri plates in solution form. Boric acid was added at rates of 14, 70, and 112 p.p.m. of boron. Hydrated cuprous sulfate was added at rates of 0.1 and 1.0 p.p.m. of copper. These ranges of concentration were considered sufficient to cover the beneficial effects of these ions on pigment production without serious inhibition of microbial growth. For the study of pigment production in the presence of available nitrogen, 0.1% of the nitrogenous substances was added to the basal medium described above. It was necessary to add certain amino acids such as cysteine at a concentration of 0.05% because of lower solubility. The basal medium and the added mineral nutrients were then mixed in the Petri plates and allowed to solidify, and the inoculum was added.

The inoculums used were prepared by culturing the organisms on nitrogen-free

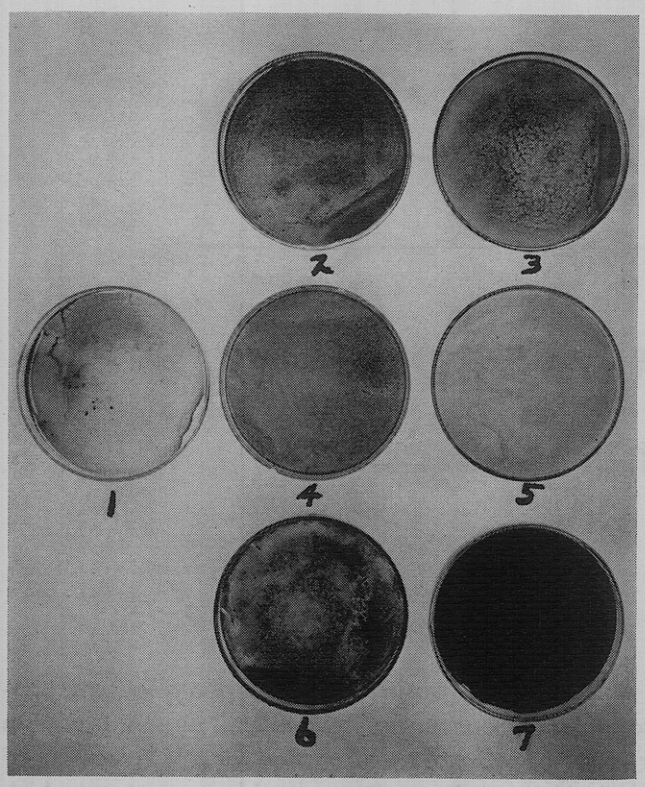


Figure 1. Pigment production by *Azotobacter chroococcum* grown on media varying in boron, copper, and nitrogen source

Basal (N-free) media

1. No boron (control)
2. 28 p.p.m. boron
3. 112 p.p.m. boron
4. 28 p.p.m. boron plus 1.0 p.p.m. copper
5. 112 p.p.m. boron plus 1.0 p.p.m. copper
6. 0.1 p.p.m. copper
7. 1.0 p.p.m. copper

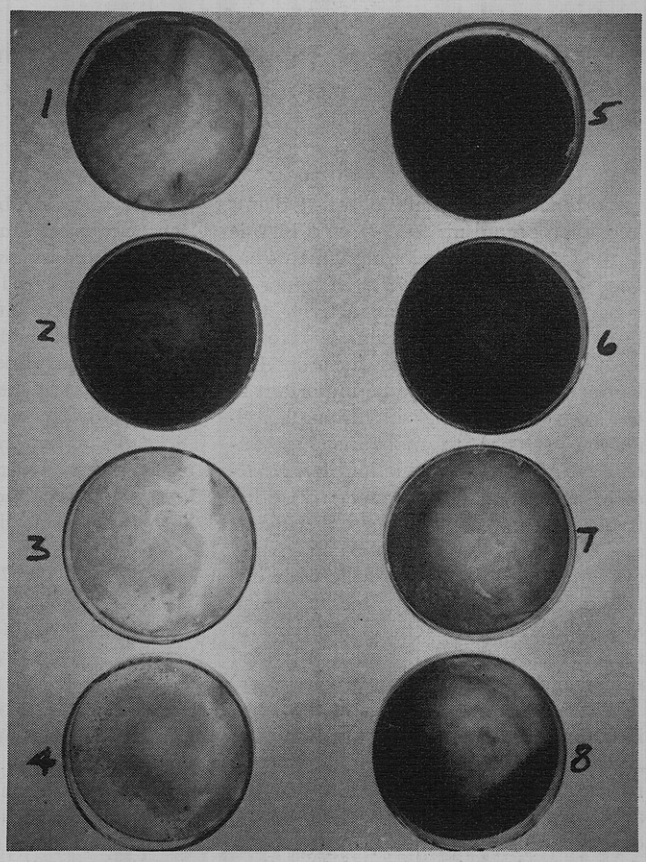


Figure 2. Pigment production by *Azotobacter chroococcum* grown in media varying in boron, copper, and nitrogen source

Basal media

1. No boron (control)
2. 1.0 p.p.m. copper
3. 112 p.p.m. boron
4. 112 p.p.m. boron plus 1.0 p.p.m. copper

Phenylalanine media

5. No boron (control)
6. 1.0 p.p.m. copper
7. 112 p.p.m. boron
8. 112 p.p.m. boron plus 1.0 p.p.m. copper

agar. After 3 day's incubation at 25° C., the cells were harvested by washing them from the plates with copper- and boron-free distilled water. The harvested cells from two Petri plates were made up to a volume of 30 ml., and 0.25 ml. of this suspension was used to inoculate the test plates. Each test was replicated three times.

The inoculated plates were incubated at 28° C. until pigmentation was definite and differences were observed. This period was generally 7 to 10 days. A tintometer (Model 610, Photovolt Corp., Chicago, Ill.) was used to measure the differences between the amounts of pigment produced by *Azotobacter chroococcum* under the various conditions mentioned. A red filter was used to reduce interference by tints other than that under study. Values obtained with this instrument were numerical only but enabled one to make comparisons between plates.

In the experiment to determine whether boron was actually held by the cell, the organisms were cultured on media with and without added boron, then harvested as described. The suspensions of organisms were then centrifuged and washed with 0.1 *N* hydrochloric acid. The washings and centrifugations were repeated several times to ensure that no free boron remained in the centrifugate. The cells were dried and ashed in platinum dishes and boron was determined by the method of Berger and Truog (*1*).

The 3,4-dihydroxyphenylalanine oxidase activity of *Azotobacter chroococcum* was determined by the method of Glick (*7*). In this procedure, a 3-day-old culture grown on the basal medium was added to the buffered 3,4-dihydroxyphenylalanine solution at a pH of 7.4. The effect of added mineral ions was determined by adding these to the 3,4-dihydroxyphenylalanine solution and then introducing the azotobacter culture. The mixtures were incubated at room temperature, and pigment production was noted at regular intervals.

Results and Discussion

In previous work (*9*) it was reported that boron increased nitrogen fixation by *A. chroococcum*. As boron-free media could not be prepared to test the ability of the organism to grow without boron, large numbers of cells were grown on media, with and without added boron. Boron is a common impurity in most chemicals, so that the media contained about 0.5 p.p.m. of boron as impurity. However, only a part of this is likely to be available for cell use. Data in Table I indicate that the cells retained boron added to the culture medium. This retention amounted to a tenfold increase of boron in the ash of cells from strain 1, and a twofold increase of boron in the ash of cells from strain 2. In both cultures, whether or not boron was added, *A. chroococcum* absorbed the boron present in the medium.

The influence of boron on pigmentation was studied because of repeated observations in earlier experiments that

Table I. Boron Retention by *Azotobacter chroococcum* Grown on Nitrogen-Free Media

		Harvested Cells	
A. chroococcum Culture	Boron Added, P.P.M.	Ash, mg.	Boron, mg./g. dry wt.
Strain 1	0	0.0557	0.005
	28	0.0432	0.050
Strain 2	0	0.0185	0.013
	28	0.0100	0.023

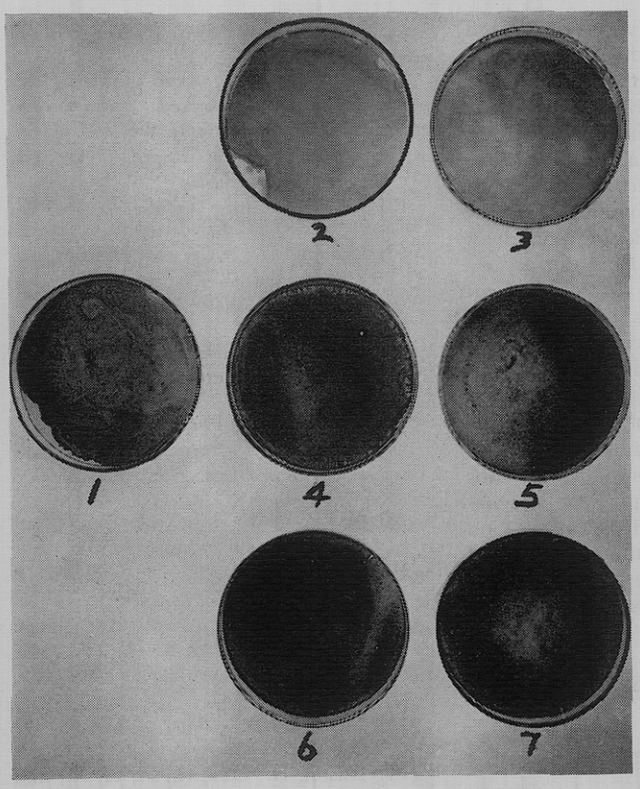


Figure 3. Pigment production by *Azotobacter chroococcum* grown on media varying in boron, copper, and nitrogen source

Basal media

1. No added nitrogen source (control)
2. Cysteine
3. Cysteine plus 1.0 p.p.m. copper
4. Tyrosine
5. Tyrosine plus 1.0 p.p.m. copper
6. Serine
7. Serine plus 1.0 p.p.m. copper

added boron increased pigmentation. Copper was included in this study because of its known influence on pigment production (*5*). The basal medium contained about 0.01 p.p.m. of copper as impurity. In Figure 1 are shown the effects of varying combinations of added boron and copper on pigment production by the organisms. The control plates carried only the minimum amounts of boron and copper as impurities. Figure 1 shows that the copper and boron treatments exert a definite influence on pigment production. However, the combination of 112 p.p.m. of boron and 1.0 p.p.m. of copper in the medium caused a marked inhibition of the mechanism of pigment production. Increased pigmentation induced by the copper ion is illustrated in Figure 2. The decreased pigmentation sometimes brought about by 112 p.p.m. of boron is evident. This inhibition is evident even in the medium carrying the optimum concentration of 1.0 p.p.m. of copper. As shown in Figure 3, there was darker pigmentation on the media containing tyrosine or serine than on the control media. However, the cysteine medium shows decreased pigmentation even in the presence of an optimum concentration of copper. It is evident that the copper caused little or no increase in the production of pigment when these amino acids were present.

Tintometer measurements of pigment production by azotobacter growing on the various media are shown in Table II. The data indicate that there was a significant increase in pigment production by the organisms growing on the media containing 14 p.p.m. of boron and tyrosine, and 14 p.p.m. of boron and glutamic acid. Evidently 14 p.p.m. of boron enhanced pigment production on the tyrosine and glutamic acid media, but not significantly on the control and phenylalanine media. Pigment production on the tyrosine and glutamic acid media was somewhat inhibited at 70 p.p.m. of boron, while in the nitrogen-free media (control) there was a very significant increase in pigment production between 0 and 70 p.p.m. of boron, and between 0 and 70 p.p.m. of boron plus 1.0 p.p.m. of copper. These results suggest that boron may stimulate the production of pigment by azotobacter. However, in Figure 1, where higher levels of boron and copper induced pigmentation separately, the combined action of the two ions caused a decrease in pigment production. This suggests that boron does not influence the reaction in the same manner as does the copper ion.

Other data from Table II indicate that the addition of the amino acids, tyrosine, phenylalanine, and glutamic acid to the media as nitrogen sources increased pigmentation. This may be due to breakdown into forms which are readily acted upon by the monophenol oxidases. Although not included in Table II, cysteine, cystine, and glycine were found to inhibit pigmentation by azotobacter. This can be explained in the case of the thioamino acids as being due to the phenolase inhibition properties of the —SH or sulfhydryl groups. The inhibitive action of glycine, however, is different and not understood. Other amino acids included in this study, such as alanine, isoleucine, valine, *l*-aspartic acid, and tryptophan, caused little if any change in pigment production.

Data in Table III present further evidence on the role of boron in pigment production by *Azotobacter chroococcum*. When 3,4-dihydroxyphenylalanine was added to a 3-day-old culture of the organism, tyrosinase was shown to be present, as the 3,4-dihydroxyphenylalanine solution changed to pink and later to a blue-black color. This is generally accepted as being indicative of the production of melanin pigment (*7*). When copper, which is known to influence the conversion of 3,4-dihydroxyphenylalanine to melanin, was added, the change followed the pattern of the 3,4-dihydroxyphenylalanine and *A. chroococcum* mixture, but the blue-black pig-

Table II. Effect of Boron and Copper in Different Media on Pigment Production by Azotobacter

Boron and Copper in Media, P.P.M.	N Source Added to Media				Averages of Means	
	Control	Tyrosine	Phenylalanine	Glutamic acid	B and Cu rates	N sources
0 B	42[a]	40	38	33	38 (no B)	36 (control)
14 B	37	25	34	20	29	34 (tyrosine)
70 B	32	36	31	27	32	35 (phenylalanine)
70 B, 1 Cu	32	36	36	33	34	28 (glutamic acid)
	L.S.D. for interaction between B and Cu rates, and N source: (0.01) = 9; (0.05) = 7				L.S.D. (0.01) = 3 (0.05) = 2	L.S.D. (0.01) = 3 (0.05) = 2

[a] Tintometer readings (pigmentation varies inversely with the reading):

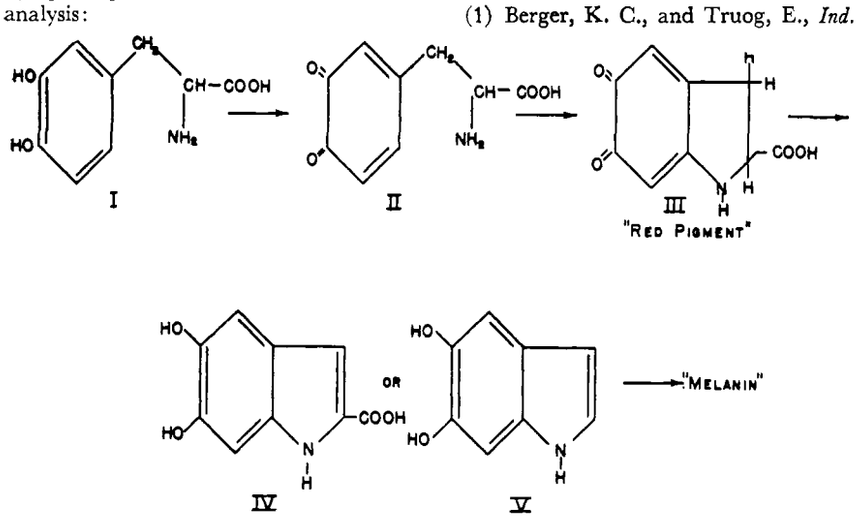
Table III. Enzymatic Action of Azotobacter on 3,4-Dihydroxyphenylalanine as Influenced by Boron and Copper

Time for Pigmentation, Hours	Dopa Solution	*A. chroococcum* Suspension and Dopa (1.0%)[a]			
		Control	Plus boron (0.1%)	Plus boron (1.0%)	Plus copper (0.001%)
1	Colorless	Pink	Pink	Red	Pink
2	Colorless	Pink	Red	Red	Blue-gray
4	Colorless	Blue-black	Red-brown	Red-brown	Blue-black

[a] 3-day-old culture.

ment formed in one half the time. After boron was added to the 3,4-dihydroxyphenylalanine–azotobacter mixture, a change was noted in the conversion of 3,4-dihydroxyphenylalanine. The final color produced was a red-brown pigment which did not change upon further incubation. It is evident from these tests that boron has a definite influence in the conversion of melanin precursors to melanin.

The various data have shown that the presence of boron, copper, and specific amino acids causes increasing pigmentation of azotobacter, with boron causing the production of a definitely redder pigment in the basal medium. This indicates that boron inhibits the complete oxidation of pigment precursors to melanin. The inhibitive action evidently occurs after hallochrome formation (red pigment, III), according to the following scheme of Raper (*16*), which has been substantiated by Mason (*12*) by spectrophotometric and manometric analysis:



The effect of copper both in the basal medium and in the 3,4-dihydroxyphenylalanine solution was to influence the rapid formation of a dark brown to black melanoid pigment. This indicates that the above scheme was not inhibited but enhanced by the presence of copper. In the experiments where boron and copper were combined, a moderate amount of boron (14 to 70 p.p.m.) did not greatly inhibit the oxidation of the pigment precursors to melanin. However, at higher ranges of boron concentration the pigment-producing ability of the accompanying copper was restricted and frequently no appreciable pigmentation was noted. These results suggest that the presence of boron in higher amounts blocks the activity of copper by influencing the enzymatic activity in a direction which produces a substance that is not readily and completely oxidized to melanin even in the presence of copper.

Literature Cited

(1) Berger, K. C., and Truog, E., *Ind. Eng. Chem., Anal. Ed.*, **11**, 540–50 (1939).
(2) Bobko, E. V., Syvorotkin, G. S., and Filippov, A. I., *Bodenkunde u. Pflanzenernähr.*, **4**, 334–9 (1937).
(3) Brenchley, W. E., *Agr. Progr.*, **3**, 104–5 (1926).
(4) Briggs, G. B., *Plant Physiol.*, **18**, 415–32 (1943).
(5) Cohen, G. N., *Trav. membres soc. chim. biol.*, **23**, 1504–7 (1941).
(6) Evans, W. C., *Biochem. J.*, **41**, 373–82 (1947).
(7) Glick, D., "Techniques of Histo- and Cytochemistry," Vol. I, pp. 90–1, New York, Interscience Publishers, 1949.
(8) Gregg, D. C., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 2500–5 (1940).
(9) Jordan, J. V., and Anderson, G. R., *Soil Sci.*, **69**, 311–19 (1950).
(10) Leggatt, C. W., *Sci. Agr.*, **28**, 131–9 (1948).
(11) Martin, W. P., Ariz. Agr. Expt. Sta., *Tech. Bull.* **83** (1940).
(12) Mason, H. S., *J. Biol. Chem.*, **172**, 83–99 (1948).
(13) Mulder, E. G., *Plant & Soil*, **1**, 179–212 (1948).
(14) Nason, A., Oldewurtel, H. A., and Propst, L. M., *Arch. Biochem. Phys.*, **38**, 1–13 (1952).
(15) Parkinson, G. G., Jr., and Nelson, J. M., *J. Am. Chem. Soc.*, **62**, 1693–7 (1940).
(16) Raper, H. S., *Physiol. Revs.*, **8**, 245–82 (1928).
(17) Scripture, P. N., and McHargue, J. S., *J. Am. Soc. Agron.*, **35**, 988–92 (1943).
(18) Woodbridge, C. G., *Sci. Monthly*, **70**, 97–104 (1950).

Received for review March 17, 1953. Accepted June 10, 1953. Published with the approval of the director of the Idaho Agricultural Experiment Station as Research Paper 364.

AIR POLLUTION EFFECTS

Lime Papers and Indicator Plants in Fluorine Air Pollution Investigations

V. L. MILLER, D. F. ALLMENDINGER, FOLKE JOHNSON, and DOROTHY POLLEY
Western Washington Experiment Station, Puyallup, Wash.

Industrialization has increased the possibility of air contamination from effluent fluoride in a number of areas of the state of Washington. This paper reports results of a search for inexpensive methods that may be used to detect atmospheric fluoride and to delineate areas where it may be an economic factor with respect to farming. The use of gladiolus and lime-treated filter paper is described as a method of estimating areas where sufficient fluorides are in the air to increase the fluoride content of forage used for cattle pasture.

THE INCREASING INDUSTRIALIZATION of the state of Washington as well as other parts of the nation has resulted in the problem of air pollution damage to agricultural crops. As there are at least five locations in the state of Washington where there are possible atmospheric fluoric effluents, an inexpensive and reliable method of estimating damage areas is needed. The agricultural interests involved in these areas in this state include dairy and beef cattle, gladiolus plantings, and Italian prune and other orchards.

Adams *et al.* (*1*) described an air-