A Cobalt Requirement for Symbiotic Growth of Azolla filiculoides in the Absence of Combined Nitrogen[1]

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Summary. Cobalt was shown to be essential for the symbiotic growth of *Azolla filiculoides* and *Anabaena azollae* in the absence of fixed nitrogen. Addition of 0.01 μg/liter cobalt resulted in large increases in yield, chlorophyll content and nitrogen fixation as compared to control cultures without cobalt. Cobalt was not required for the growth of *Azolla* when nitrate nitrogen was supplied. The number of *Anabaena azollae* cells in the fronds of *Azolla* appeared to be decreased by ommission of cobalt from the culture medium containing nitrate nitrogen. It is concluded that cobalt is essential for the symbiotic growth of *Azolla* in the absence of combined nitrogen and it is suggested that the cobalt requirement is associated with the growth of *Anabaena azollae*.

The aquatic fern *Azolla filiculoides* characteristically occurs with the blue-green alga, *Anabaena azollae*, enclosed within cavities in the dorsal lobes of the leaves of the fern (12). Atmospheric nitrogen is fixed symbiotically by *Azolla* and the associated *Anabaena* (4). *Azolla* cultured in the absence of the symbiotic alga requires a source of combined nitrogen (11). According to Bortels (4) *Anabaena azollae* isolated from *Azolla* plants, failed to grow alone. Venkataraman (13) however, has reported that the alga grew independently and fixed atmospheric nitrogen in pure culture. This report has not been confirmed.

Since cobalt is required for certain free-living blue-green algae (7) for symbiotic nitrogen fixation by legumes (1, 2) and for certain nonlegumes (3), it was of interest to investigate the influence of cobalt on the growth of *Azolla* in the presence and absence of fixed nitrogen. A preliminary report on this research has appeared (6).

Materials and Methods

The macronutrient salts and $MnSO_4 \cdot 4H_2O$ used in cobalt deficiency studies with *Azolla* were purified by extraction of the 1-nitroso-2-napthol complex of heavy metals with redistilled chloroform as described by Kliewer et al. (9). Calcium sulfate was crystallized from purified K_2SO_4 and $CaCl_2$. Micronutrient salts, other than $MnSO_4 \cdot 4H_2O$ were twice recrystallized. Purified Fe citrate was prepared from iron carbonyl as described by Ahmed and Evans (2). Water that had been twice distilled and then deionized was used in the purification of the chemicals and in all growth experiments. All glassware was washed with 3 N HCl followed by deionized water prior to use in cobalt deficiency experiments.

The nitrogen-free nutrient solution consisted of the following macronutrients in mmoles/liter: KH_2PO_4, 0.20; K_2PO_4, 0.05; K_2SO_4, 0.49; $CaCl_2$, 0.13; and $CaSO_4$, 1.00; and the micronutrients in μmoles/liter: B, 5.77 as H_3BO_3; Mn, 1.13 as $MnSO_4 \cdot 4H_2O$; Zn, 0.19 as $ZnSO_4 \cdot 7H_2O$; Cu, 0.08 as $CuSO_4 \cdot 5H_2O$; Mo, 0.05 as $NaMoO_4 \cdot 2H_2O$; and Fe, 4.33 as Fe citrate.

A solution with macronutrient composition similar to that used by Nickell (11) for the aseptic growth of *Azolla* was used in experiments with combined nitrogen. This solution consisted of the following macronutrients in mmoles/liter: $Ca(NO_3)_2$, 4.90; KH_2PO_4, 1.00; $MgSO_4$, 2.00; K_2SO_4, 2.00; and $CaCl_2$, 3.00. The purified micronutrients salts previously listed were added at the following concentrations in μmoles/liter: B, 23.10; Mn, 4.55; Zn, 0.76; Cu, 0.31; Mo, 0.20; and Fe, 18.22. Cobalt as $CoCl_2$ was added at the concentrations indicated in the tables.

Azolla filiculoides plants, 2 to 3 mm in diameter, and the associated *Anabaena azollae* were obtained from greenhouse cultures. *Azolla* plants used for cultures lacking combined nitrogen were surface sterilized in 1 % Chlorox (a commercial material containing 6 % sodium hypochlorite) for 2 minutes to prevent the growth of contaminating microorgan-

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isms. The plants were then washed with a large volume of purified KH_2PO_4-K_2HPO_4 buffer (2.5×10^{-4} M). The washed plants were subsequently placed in 200 ml of sterilized nitrogen-free nutrient solution in 400 ml beakers covered with petri dishes. The details of individual experiments are described in the tables.

Prior to culturing *Azolla* in a nutrient medium containing combined nitrogen, it was necessary to eliminate green algae which remained associated with *Azolla* after 2 minutes of surface sterilization with 1 % Chlorox. Green algae (largely *Chlamydamonas*) failed to grow appreciably in the nitrogen-free solution, but grew very rapidly in media containing combined nitrogen. In the initial experiment, *Azolla* was surface sterilized for 30 minutes in 1 % Chlorox. This treatment was followed by washing with purified buffer just prior to placing the plants in the experimental solutions previously sterilized in an autoclave. While this treatment was not detrimental to the fern, many of the replicate cultures were discarded as a result of the growth of green algae. In a subsequent experiment plants were surface sterilized in 1 % Chlorox for either 10 or 25 minutes and then cultured individually in test tubes in a sterile solution containing combined nitrogen. This solution had essentially the same inorganic composition as that used by Nickell (11). Cobalt was not added to this medium, however, unpurified reagent grade salts were used for the preparation of the medium.

After 3 weeks individual plants that appeared to be of the same size were treated with the following concentrations of antibiotics expressed in ppm: Na penicillin G, 25; streptomycin SO_4, 5; and terramycin, 1. During a 20-day period individual plants were transferred several times to sterile solutions containing antibiotics. At the end of this period fronds from individual plants that had remained free of green algae were cut into 2 mm sections, treated with 1 % Chlorox for 30 minutes and then washed with purified and sterile buffer. These plants were then transferred to the purified nutrient solution containing combined nitrogen. Experiments with combined nitrogen were conducted using 50 ml of sterile nutrient solution in 125 ml flasks covered with small beakers.

The cultures of *Azolla* were randomly arranged in a growth cabinet with a constant temperature of 23°. The cultures were illuminated 16 hours each day using fluorescent lamps. The intensity of the light at 1 m above the cultures was 400 ft.-c.

At the conclusion of each experiment the plants were removed from the beakers and blotted with paper towels. After the fresh weight of the plant material was obtained, the plants were cut into small pieces to provide representative samples for analysis. Chlorophyll was extracted with 80 % acetone and measured spectrophotometrically at 625 mμ (10). Vitamin B_{12} was determined by the Ochromonas malhamensis assay (7), nitrogen was determined by the micro Kjeldahl method, and dry weights were obtained after drying over night at 70°. Samples of fronds, with roots removed, were crushed in 10 volumes of a dilute buffer solution using a Ten Broeck tissue grinder. The number of *Anabaena* cells in this suspension were then counted in a hemocytometer.

Results and Discussion

The effect of cobalt on the growth and appearance of *Azolla* in the absence of fixed nitrogen is illustrated in figure 1. Without cobalt, growth was restricted and a severe chlorosis typical of nitrogen deficiency developed.

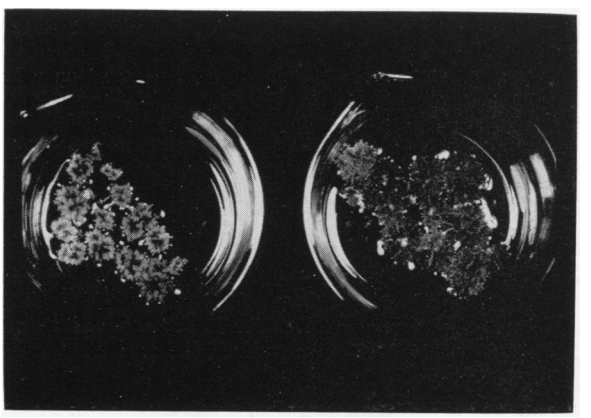

FIG. 1. The effect of cobalt on *Azolla* grown without fixed nitrogen. The plants on the left were from a culture lacking Co while those on the right were from a culture that received 10.0 μg/liter Co. The photograph was taken after 24 days of growth under the experimental conditions described under Materials and Methods.

The influence of the concentration of cobalt in the nutrient solution on the yield and on the contents of vitamin B_{12}, chlorophyll and nitrogen in *Azolla* is shown in table I. Addition of as little as 0.01 μg/liter of cobalt resulted in very large increases in yield, chlorophyll content and nitrogen fixation. The very limited amount of growth observed without the addition of cobalt is attributed to cobalt initially present in the plant tissue and traces of the metal present as an impurity in the culture medium.

Yield, vitamin B_{12} content and the amount of nitrogen fixed did not change significantly as the cobalt concentration in the nutrient solution was increased from 0.01 to 10.0 μg/liter. The chlorophyll content of *Azolla*, however, increased significantly as the cobalt concentration in the nutrient solution was increased from 0.01 to 1.0 μg/liter or higher. While the yields of the cobalt deficient cultures were much less than those receiving cobalt, the vitamin B_{12} concentration in the deficient plants was only slightly less than that in those receiving cobalt.

The addition of 1.0 μg per liter of each of 12 elements presumed to have been removed by the

purification procedure failed to replace cobalt in the growth of *Azolla*. Some symptoms of toxicity in plants receiving these elements were noted initially; however, they disappeared later in the experiment.

Data in table II show the effect of the addition of cobalt to *Azolla* grown in the absence of both cobalt and fixed nitrogen. In this experiment plants were cultured for 15 days in solution lacking added

Table I. *Influence of Cobalt on the Growth and Composition of* Azolla *Cultured Symbiotically on a Nitrogen-free Medium*

Treatment*	Fr wt g	Dry wt g	Vitamin B_{12}** mμg/g	Chlorophyll ** mg/g	Nitrogen mg/culture
—Co	0.27 ± 0.09†	0.019 ...	0.60 ...	0.09 ...	0.21 ...
0 01 μg/liter Co	1.28 ± 0.30	0.066 ± 0.018	0.66 ± 0.08	0.44 ± 0.07	2.52 ± 0.54
0.10 μg/liter Co	1.34 ± 0.27	0.065 ± 0.024	1.00 ± 0.31	0.53 ± 0.03	3.14 ± 1.20
1.00 μg/liter Co	1.08 ± 0.22	0 054 ± 0 017	1.17 ± 0.56	0.63 ± 0.05	2.98 ± 0.82
10.0 μg/liter Co	1.13 ± 0.07	0.059 ± 0 003	0.82 ± 0.24	0.58 ± 0.10	2.97 ± 0.30
—Co + 12 elements***	0.19 ± 0.07	0.012 ...	0.56 ...	0.17 ...	0.15 ...

* All cultures were supplied with the nitrogen-free medium (Materials and Methods) and the various treatments as indicated.
** Vitamin B_{12} and chlorophyll are expressed on a fresh weight basis.
*** Al, V, Cr, Ni, Ga, Ru, Pd, Ag, Sn, W, Bi, and U each at 1 μg/liter.
† Standard deviation. The reduced yield in the —Co and —Co + 12 elements treatments required that the replicate cultures lacking cobalt be combined to obtain sufficient material for determination of dry weight and for chemical analysis Hence, the standard deviations of the dry weight, vitamin B_{12}, chlorophyll and nitrogen data for the —Co and —Co + 12 elements treatments could not be calculated.

Table II. *Influence of Cobalt on Recovery of Cobalt Deficient* Azolla *Cultured Symbiotically on a Nitrogen-free Medium*

Azolla plants were cultured for 15 days on a nitrogen-free medium lacking Co. The fresh weight of *Azolla* plants at this time was 0.060 g per culture, the N content was 0.16 mg/culture and the vitamin B_{12} content was 0.55 mμg/g fresh weight. Cobalt was then supplied to 4 cultures at 1.0 μg/liter. After 15 days the cultures receiving Co showed a slight improvement in appearance. The level of cobalt was then increased from 1.0 to 10.0 μg/liter in these cultures. All cultures were harvested 26 days after the addition of Co at 10.0 μg/liter. All values are means of 4 replicate cultures of 5 plants each.

Treatment	Fr wt g	Dry wt g	Vitamin B_{12}* mμg/g	Chlorophyll* mg/g	Nitrogen mg/culture
—Co	0.61 ± 0.05**	0.026 ± 0.0009	0.61 ± 0.15	0.07 ± 0.00	0.30 ± 0.18
10 μg/liter Co	1.28 ± 0.15	0.065 ± 0.009	0.60 ± 0.09	0.27 ± 0.07	2.14 ± 0.54

* Vitamin B_{12} and chlorophyll are expressed on a fresh weight basis.
** Standard deviation.

Table III. *Influence of Cobalt on the Growth, Chlorophyll and Algal Content of* Azolla *Cultured Symbiotically on a Medium Containing Nitrate*

In experiment I, initial fresh weight of *Azolla* plants was 0.003 g/culture. Cultures were harvested after 5 weeks of growth. All values are means of 2 replicate cultures of 8 plants each.

In experiment II, initial fresh weight of *Azolla* plants was 0.003 g/culture. Cultures were harvested after 6 weeks of growth. All values are means of 4 replicate cultures of 8 plants each.

Treatment	Fr wt g	Dry wt mg	Chlorophyll* mg/g	Algal cells** No × 10^{-6}/g
Expt I**				
—Co	0 89 ± 0.13	48.2 ± 3.2	0.10 ± 0.03	142 ± 74
1.0 μg/liter Co	0.70 ± 0.11	43.4 ± 10.5	0.12 ± 0.01	722 ± 420
Expt II				
—Co	0.49 ± 0.07	28.0 ± 4.3	0.15 ± 0.05	...
1.0 μg/liter Co	0.55 ± 0.09	30.1 ± 4.2	0.13 ± 0.04	...

* Chlorophyll is expressed on a fresh weight basis.
** Algal content is based on the fresh weight of fronds alone. In experiment II *Anabaena azollae* was not found in the fronds and undoubtedly were destroyed by antibiotic treatment.

cobalt. When cobalt deficiency symptoms were apparent near the end of the period 1 μg of cobalt was added to each of the 4 replicate cultures. After 15 days the cultures receiving this level of cobalt showed only slight improvement in appearance. The level of cobalt was then increased to 10.0 μg/liter and rapid recovery ensued. Aerial portions of plants from cultures not receiving cobalt were almost completely necrotic at the conclusion of the experiment. It may be noted that while addition of cobalt to deficient cultures markedly increased yield, chlorophyll and nitrogen contents, the vitamin B_{12} concentration in the plant tissue was not higher than that in the deficient plants. The failure of *Azolla* to recover upon the addition of cobalt at 1.0 μg/liter may have been a consequence of nonspecific absorption of cobalt by the host tissue which composes the mass of the plant material. This would greatly limit the uptake of cobalt by the algal cells.

The influence of cobalt on the growth of *Azolla* when supplied with nitrate nitrogen was investigated in order to evaluate the essentiality of cobalt when nitrogen fixation was not obligatory. In the 2 experiments reported in table III it is seen that the addition of 1.0 μg per liter cobalt to the culture medium did not increase the yield or chlorophyll content of *Azolla*. In experiment I of (table III), a marked increase in the number of algal cells per g fresh weight of fronds occurred when cobalt was added to the culture medium. In experiment II the combination of surface sterilization and antibiotic treatments apparently eliminated *Anabaena azollae* from the cultures and the alga was not observed in the fronds.

Azolla cultured on the medium containing nitrate nitrogen had a much lower chlorophyll content than did *Azolla* grown symbiotically on the nitrogen-free medium. The reason for this difference is not known but it has been reported that the chlorophyll content of tobacco plants cultured with nitrate nitrogen was less than that of plants supplied with nitrogen in the ammonium form (5).

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