Plant and Soil XIX, no. 3 December 1963

COBALT REQUIREMENT OF SYMBIOTICALLY GROWN ALFALFA*

by D. O. WILSON ** and H. M. REISENAUER ***

Department of Agronomy, Washington State University Pullman, Washington

INTRODUCTION

The essentiality of Co for legumes grown under symbiotic conditions has recently been established (Ahmed and Evans [1 2 3], Hallsworth *et al.*[7], Reisenauer [11], Delwiche *et al.*[6]); however little is known of the amount of the element required or of its function in symbiotic N-fixation. The Co-requirement of symbiotically grown legumes can be divided into that of the microorganism, that of the plant and that of the symbiotic association. Nicholas and Wilson [9] have reported that the *Rhizobium* spp. have a growth requirement, when utilizing nitrate-N, of at least 0.1 μg of Co per liter in the culture. The requirement of the plant *per se* is known to be considerably less than that for N-fixation (Ahmed and Evans [3], Delwiche *et al.*[6]). The role of Co in animal nutrition is centered around the Co-containing vitamin B_{12} which functions in the synthesis of hemoglobin and in protein metabolism (Smith [12]). The presence of both vitamin B_{12} and a form of hemoglobin in N-fixing nodule tissue (Levin *et al.*[8]) suggests a similar dependence.

This paper presents the results of an experiment conducted to determine the level of Co associated with maximum symbiotic

* Scientific Paper No. 2301, Washington Agricultural Experiment Station, Pullman. Project Number 1526. This investigation was supported in part by funds provided for biological and medical research by the state of Washington Initiative Measure No. 171.

** Present address: Biology Laboratory, General Electric Company, Richland, Washington.

*** Present address: Kearney Foundation of Soil Science, University of California, Davis, California.

N-fixation, and the relation of Co-level to the formation of leghemoglobin in the root nodules of a leguminous plant.

EXPERIMENTAL

Alfalfa (*Medicago sativa*) was grown in culture solutions prepared from purified chemicals and to which different amounts of radioactive Co were added. Ten days after the appearance of N starvation symptoms in the plants grown with no added Co, the crop was harvested. Treatment effects were evaluated by measuring the yield, N- and Co-content of the plant tops; and the weight, volume, and leghemoglobin content of the root nodules.

Culture methods

The chemical purification and plant growth procedures used were similar to those described by Delwiche *et al.*[6] Alfalfa (variety Vernal) was grown in 2-liter beakers fitted with paraffin-coated plaster of Paris lids in which three plants were supported.

The experiment was conducted during June and July in a greenhouse which was equipped with an air-circulation system that filtered the incoming air through activated carbon. The air used for aerating the culture solutions was filtered through glass wool and activated carbon.

After 46 days growth in the culture solution, the crop was harvested. The tops were dried at 60°C, separated into leaves and stems, and ground. The roots were immediately frozen and the nodules hand-picked from the frozen tissue.

Treatments

The composition of the culture solution is given in Table 1. In addition to the nutrients listed, all treatments received approximately 16 mg per liter of ammonia-N from the ammonium hydroxide added for pH adjustment during purification of the culture reagents. Calcium carbonate was added to maintain the pH of the culture solutions between 6.5 and 7.0 throughout the course of the experiment.

The introduced treatment variables are described in Table 2. Rhizobia were added as 1 ml of a suspension of *Rhizobium meliloti* containing approximately 10^6 organisms per ml shortly after the seedlings were transplanted. The no-rhizobia treatment (number 6) was included to measure the growth allowed by that N supplied in the culture solution and in the seed.

TABLE 1

Composition of nutrient solution			
Compound	Concentration in culture, *M*	Compound	Concentration in culture, *M*
KH_2PO_4	2×10^{-3}	H_3BO_3	2.5×10^{-4}
K_2SO_4	2×10^{-3}	$CuSO_4.5H_2O$	5×10^{-7}
$CaSO_4.2H_2O$	3×10^{-3}	$FeCl_3.6H_2O$	2×10^{-5}
$MgSO_4.7H_2O$	1×10^{-3}	$(NH_4)_2H_2EDTA$	2×10^{-5}
$MnCl_2.4H_2O$	5×10^{-6}	Na_2MoO_4	5×10^{-7}
$ZnSO_4.7H_2O$	2×10^{-6}	$CaCO_3$*	(1.0 g/liter)

* Added as dry salt for pH control.

TABLE 2

Treatment variables					
Treatment number	Rhizobia added	Concentration of added Co		Form of added Co	
				Radioactive*	Stable
		M	ppt**	ppt	ppt
1	yes	0	0	0	0
2	yes	1.66×10^{-12}	0.1	0.1	0
3	yes	1.66×10^{-11}	1	1	0
4	yes	1.66×10^{-10}	10	10	0
5	yes	1.66×10^{-9}	100	10	90
6	no	1.66×10^{-9}	100	10	90

* Supplied as Co^{60} of specific activity 100 $\pm$ 4c/g obtained from U. S. Nuclear Corp., Burbank, California.

** Parts per 10^{12}.

The rhizobia suspension used to inoculate the cultures was a mixture of two effective strains * of *Rhizobium meliloti*. The bacterial growth of separate agar slants of each strain was washed with redistilled water into a clean centrifuge tube. The suspension was centrifuged for 10 min at 10,000 $\times$ *g* to throw down the bacteria and the supernatant discarded. The bacteria were suspended with redistilled water and centrifuged as before for a total of three times. The washed bacterial suspension was then diluted with redistilled water to give a concentration of about 10^6 organisms per ml as determined by direct bacterial count.

* Strains 102F28 and 102F30 supplied by the Nitragin Co., Inc., Milwaukee, Wisconsin.

Statistical methods

The pots were arranged on the greenhouse bench in a randomized block design of three replications. Each replication contained two pots each of Treatments 1 and 2, and one pot each of Treatments number 3 through 6. Since the yield and N-content data from the individual pots contained two distinct variance populations, standard errors of the mean were calculated on the data from Treatments 1, 2, 3, and 6 as one population, and of Treatments 4 and 5 as another population.

Analytical procedures

Cobalt was determined from direct counts of 1 gram samples of plant material, or suitable evaporated aliquots from the culture solution, using a scintillation counter equipped with a 2-inch NaI (Tl) crystal. A single-channel scintillation spectrometer was used to reduce background and at least 10,000 disintegrations were counted on each sample. Nodule leghemoglobin was extracted with Drabkin's solution (Crosby *et al.*[4]) and measured colorimetrically as the cyanmetleghemoglobin complex on the centrifuged extract. Nodule volume was measured by liquid displacement using water or Drabkin's solution as appropriate. Nitrogen analyses were made by a Kjeldahl procedure.

RESULTS AND DISCUSSION

The fixed N supplied by the nutrient solutions was adequate for normal growth of the plants of all treatments for all but the last 10 days of the growth period. The growth and color differences illustrated in Fig. 1 developed during this period. The treatments divide into two groups: those in which the level of Co was insufficient for symbiotic N-fixation (1 ppt added Co and less), and those in which the supply was adequate (10 and 100 ppt of Co in the nutrient solution). The plants with insufficient Co showed the characteristic symptoms of N-starvation, whereas those that were nodulated and supplied adequate Co appeared normal.

The effect of the Co-concentration of the nutrient solution on N fixation, and on the yield and Co-content of the nodulated alfalfa plants is shown in Fig. 2, and of the plants of all treatments in Tables 3 and 4. No N was fixed by the plants receiving 0, 0.1, and 1.0 ppt of added Co, whereas 30 and 44 mg of N were fixed by the plants receiving 10 and 100 ppt respectively. The difference between the amounts of N fixed at the two higher levels of added Co is not statistically significant, and as illustrated is somewhat exaggerated

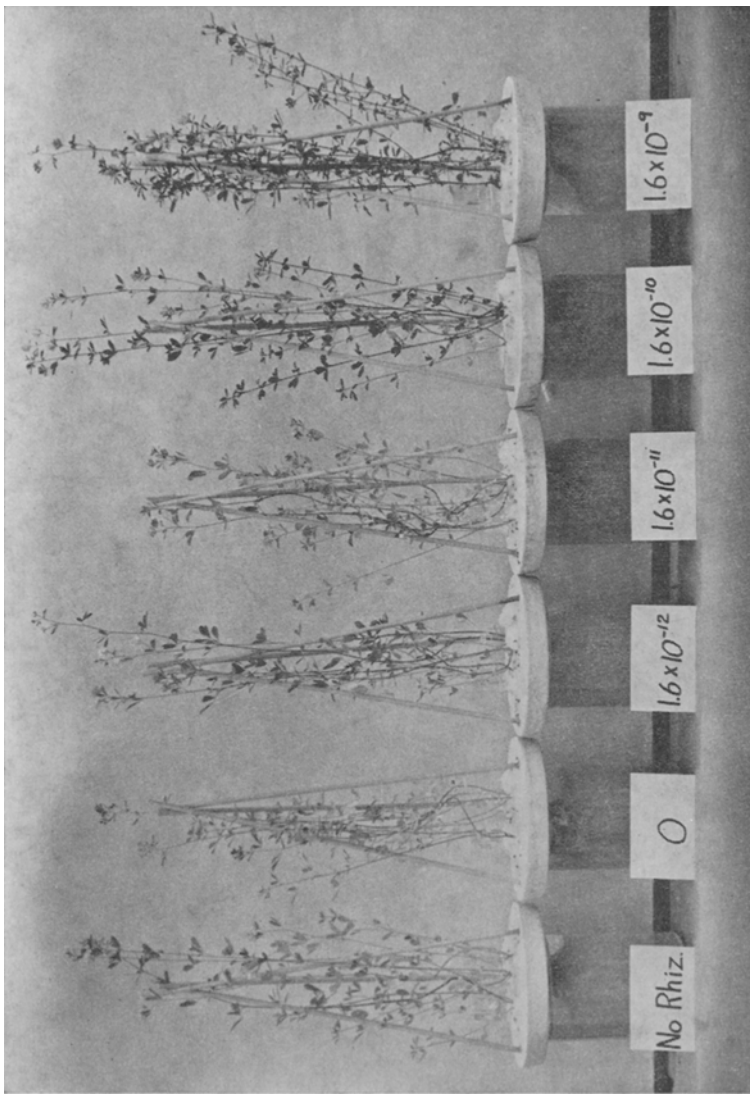

Fig. 1. The effect of Co-level and rhizobia inoculation on growth of alfalfa. (Treatments were: No rhizobia with 1.6×10^{-9} *M* Co, and rhizobia with the indicated molarities of added Co).

by the logarithmic scale of the *x*-axis. The plant-top yield curve is similar to the N fixed curve and again statistically significant differences exist only between the yields from those treatments of 1 ppt or less, and 10 ppt or more of added Co. Plant growth on the non-N-fixing treatments was very uniform whereas that on the

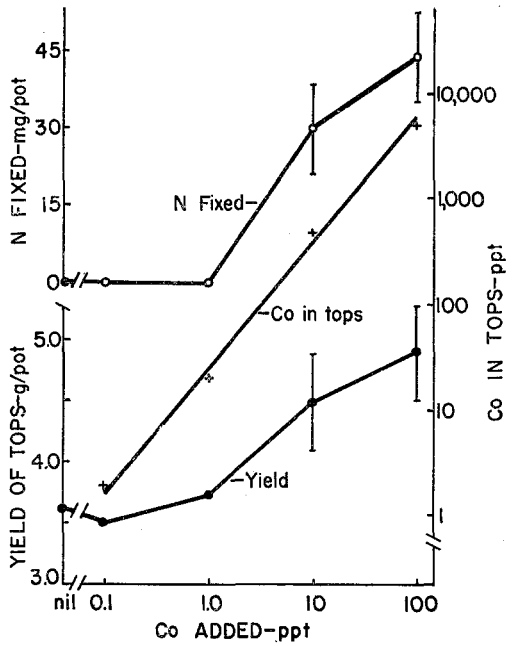

Fig. 2. The effect of Co-concentration of the nutrient solution on N fixation, and on the yield and Co-content of the tops of nodulated alfalfa plants.

TABLE 3

The Co-content of plant tops						
Treatment number	Co added μg/pot	Co in plant tops		Co-concentration in:		
		μg/pot	% of added	Stems ppt	Leaves ppt	Tops ppt
2	2×10^{-4}	6.4×10^{-6}	3	1	3.3	1.8
3	2×10^{-3}	7.5×10^{-5}	4	6	38	20
4	2×10^{-2}	2.2×10^{-3}	11	100	750	486
5	2×10^{-1}	2.4×10^{-2}	12	1700	9000	4900
6	2×10^{-1}	1.8×10^{-2}	9	2400	8200	4500

N-fixing treatments varied widely, resulting in two distinct plant yield variance populations (Table 4). Since N was the primary factor limiting growth, and since the plants were harvested soon after the initial meager supply of chemically fixed N was exhausted,

TABLE 4

Yield and N-content of plant tops								
Treatment		Yield of plant tops		N-content of plant tops				N fixed
Co added	Rhizobia added							
ppt		g/pot	s_x*	%	s_x	mg/pot	s_x	mg/pot
100	no	4.02	0.07	0.80	0.02	32.1	0.5	nil
0	yes	3.60	0.07	0.80	0.02	28.8	0.5	nil
0.1	yes	3.53	0.07	0.88	0.02	31.1	0.5	nil
1	yes	3.69	0.07	0.82	0.02	30.1	0.5	nil
10	yes	4.53	0.37	1.38	0.09	62.5	9.4	30.5
100	yes	4.90	0.37	1.56	0.09	76.2	9.4	44.2

* Standard error of the mean.

the time of initiation of symbiotic N-fixation and the rate of this process controlled growth on the N-fixing treatments. Considerable variation between plants existed in either the time of initiation or the rate of symbiotic N-fixation, or both.

The Co-concentration in the plant tops was an exponential function of the initial concentration of Co in the nutrient solution (Fig. 2). The approximate equation for this relation was found to be:

$$\text{Co in tops} = 25\ (\text{Co added})^{1.145}$$

Measures of the distribution of Co among the different phases of the culture systems at the conclusion of the experiment revealed that 3 to 12 per cent of the added Co was in the plant tops (Table 3), approximately 50 per cent in the solution phase, and about 25 per cent associated with the $CaCO_3$ at the bottom of the beaker.

In this experiment 10 ppt of Co in the rooting medium was adequate for maximum symbiotic N-fixation by alfalfa. There were no significant differences in any of the measurements made between the 10-ppt and 100-ppt levels. In an experiment with soybeans, Ahmed and Evans [2 3] obtained no significant differences between 100 ppt and 1000 ppt of Co added to the culture. Maximal functioning of the symbiotic N-fixing mechanism was obtained here with 10 ppt of Co in the rooting medium. This is only 1/10 of the reported growth requirement of the rhizobia (Nicholas and Wilson) and may explain the difficulties encountered in obtaining

adequate nodulation of plants growing in low-Co cultures (Delwiche *et al.*[6]). Cobalt accumulation by the plant roots would assure an adequate level within the root nodule. No N was fixed by the plants growing in the cultures receiving 0.1 and 1.0 ppt of Co, indicating that a minimum Co-level is necessary for the symbiotic N-fixing mechanism to become functional. This threshold Co-level is between 1 and 10 ppt in the rooting medium.

Maximum symbiotic-N fixation by alfalfa is associated with 0.0005 ppm of Co in the plant tops and 0.0008 ppm of the element in the leaves. Compared to the other plant nutrient elements, the Co-requirement of alfalfa is exceedingly low, being only 1/300 of that for molybdenum (Reisenauer [10]) on an atomic basis. The plant-top Co-content found here to be associated with maximum growth is approximately 1/100 of the concentration of the element in the forage considered adequate for the well-being of ruminants (Davis [5]). Since the Co-requirement of legumes is so much lower than that of animals, crop responses to fertilization with the element should be expected only on soils producing forage supplying insufficient Co for animals.

A measure of the parasitism of the rhizobia-legume association was obtained as a comparison of the growth of the non-nodulated and nodulated but non-N-fixing plants. All components of the symbiotic N-fixing mechanism except the Co-factor are present in the nodulated Co-deficient plants as evidenced by the rapid activation of N-fixation upon the addition of Co (Delwiche *et al.*[6]).

TABLE 5

Weight, volume, and leghemoglobin content of nodules

Treatment		Nodule volume	Nodule weight**	Leghemoglobin in nodules
Co added ppt	Rhizobia added	cc/pot	g/pot	mg/cc
0	yes	2.4	2.72	Trace
0.1	yes	1.7	1.96	Trace
1	yes	3.2	3.51	Trace
10	yes	1.7	1.92	0.73
100	yes	1.5	1.70	0.73
100	no	0	0	0

* Fresh weight.

Production and maintenance of the symbiotic N-fixing mechanism cost the plant 10 per cent of its growth capacity under the conditions of this experiment.

The data showing the effect of Co-level on the weight, volume, and leghemoglobin content of the nodules are in Table 5. The plants of the pots inoculated with rhizobia nodulated profusely throughout their entire root system. Only one nodule was found in the noninoculated series. The Co-level of the culture had no measurable influence on either the weight or volume of nodules formed. Leghemoglobin was produced in measurable quantities only at the two higher levels of added Co. The nodules from the plants receiving 0, 0.1 and 1.0 ppt of added Co were white and contained only traces of leghemoglobin. Plants receiving 10 and 100 ppt of added Co yielded nodules that were pink and contained 0.73 mg of leghemoglobin per cc of nodule tissue. Nitrogen was fixed only in those nodules containing leghemoglobin.

SUMMARY

Alfalfa was grown in purified culture solutions to which different amounts of radioactive Co were added. Treatment effects were evaluated by measuring the yield, N and Co content of the plant tops; and the weight, volume and leghemoglobin content of the root nodules. The Co requirement of symbiotic N fixation was met with 10 ppt of the element in the rooting medium; this amount gave plants containing 0.0005 ppm of Co in their whole tops and 0.0008 ppm of Co in their leaves. Leghemoglobin was found in measurable amounts only in nodules which were fixing N at the time of harvest. The level of added Co had no effect on the weight or volume of root nodules formed, nor were the Co levels supplied related to the leghemoglobin content of the nodules.

Received January 3, 1963

REFERENCES

1 Ahmed, S. and Evans, H. J., Effect of cobalt on the growth of soybeans in the absence of supplied nitrogen. Biochem. Biophys. Research Comm. **1**, 271–275 (1959).
2 Ahmed, S. and Evans, H. J., Cobalt: A micronutrient element for the growth of soybean plants under symbiotic conditions. Soil Sci. **90**, 205–210 (1960).
3 Ahmed, S. and Evans, H. J., The essentiality of cobalt for soybeans grown under symbiotic conditions. Natl. Acad. Sci. U.S. Proc. **47**, 24–36 (1961).
4 Crosby, W. H., Munn, J. I., and Furth, F. W., Standardizing a method for clinical hemoglobinometry. U.S. Armed Forces Med. J. **5**, 693–703 (1954).
5 Davis, G. K., Metabolic function and practical use of cobalt in nutrition. *In* Trace

Elements Proceedings Conference, Wooster, Ohio, 1957. Eds. C. A. Lamb, O. G. Bentley and J. M. Beattie, Academic Press, New York and London (1958).
6 Delwiche, C. C., Johnson, C. M., and Reisenauer, H. M., influence of cobalt on nitrogen fixation by *Medicago*. Plant Physiol. 36, 73–78 (1961).
7 Hallsworth, E. G., Wilson, S. B., and Greenwood, E. A. N., Copper and cobalt in nitrogen fixation. Nature **187**, 79–80 (1960).
8 Levin, A. P., Funk, H. B., and Tendler, M. D., Vitamin B_{12}, rhizobia and leguminous plants. Science **120**, 784 (1954).
9 Nicholas, D. J. D. and Wilson, P. W., Metabolism of inorganic nitrogen and its compounds in microorganisms. Science **136**, 328 (1962).
10 Reisenauer, H. M., Molybdenum content of alfalfa in relation to deficiency symptoms and response to molybdenum fertilization. Soil Sci. **81**, 237–242 (1956).
11 Reisenauer, H. M., Cobalt in nitrogen fixation by a legume. Nature (London) **186**, 375–376 (1960).
12 Smith, L. E., Vitamin B_{12}. John Wiley and Sons, London and New York (1960).