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THE EFFECT OF COPPER ON NITROGEN FIXATION IN SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM*)

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SUMMARY

Copper deficiency decreased nitrogen fixation in subterranean clover. Three lines of evidence support this conclusion. Firstly, while both copper and nitrogen application increased the growth of the legume the interaction between copper and nitrogen on growth was negative. Secondly, the application of copper increased the concentrations of both total and protein nitrogen in the plant. Lastly, nitrogen fixation as measured by the acetylene reduction assay increased with copper application to the soil.

INTRODUCTION

Several authors have claimed that copper is specifically involved in nitrogen fixation by subterranean clover (Hallsworth, Wilson and Greenwood, 1960; Greenwood and Hallsworth, 1960) and by white clover (van der Elst, McNaught and Rolt, 1961). These claims are based on the observations that alleviating copper deficiency in these legumes increased nitrogen concentrations in tops as well as growth. However, alleviation of deficiency of nutrients not thought to be directly involved in nitrogen fixation may also increase nitrogen concentrations in tops (e.g. sulphur – Anderson and Spencer, 1950b; zinc – Lo and Reisenauer, 1968).

There are several other approaches for examining nutrient involvement in nitrogen fixation (Robson, 1978). In this paper we use evidence from three approaches to conclude that copper is required specifically for symbiotic nitrogen fixation in subterranean clover. In Experiment 1 the interaction between copper and combined nitrogen on the growth of subterranean clover is examined on three soils. In Experiment 2 the effect of copper on nitrogen fixation is assessed directly using the acetylene reduction technique. In both experiments the effect of copper supply on copper and nitrogen concentrations within the plant is determined.

EXPERIMENTAL METHODS

Experiment 1

A 7×2 completely factorial design of the following treatments was used, replicated 4 times:

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$CuSO_4.5H_2O$ at 0, 30, 100, 150, 300, 1000, 3000 μg Cu pot^{-1} (3000 μg Cu pot^{-1} is equivalent on a surface area basis to 5 kg $CuSO_4.5H_2O$ ha^{-1});

NH_4NO_3 at nil, or applied in solution at 257 mg pot^{-1} $week^{-1}$. First application was made 14 days after germination when plants were nodulated.

Three virgin soils from coastal areas north of Perth, Western Australia were used.

(i) Soil I, A virgin grey diatomaceous sandy soil from Dandaragan; originally carrying *Eucalyptus rudis*, pH 4·5 in 1/5 0·01M $CoCl_2$; total copper 1 to 2 p.p.m.; field capacity 28%.

(ii) Soil II, A virgin brown sandy soil from Lancelin carrying *Banksia* sp., *Xanthorrhoea preissii* and *Nuytsia floribunda*; pH 5·2, total copper 0·2 p.p.m., field capacity 15%.

(iii) Soil III, A virgin grey sand with loose gravel in the surface layer from Badgingarra, carrying a variety of heath type flora, including *Xanthorrhoea* sp. and *Eucalyptus macrocarpa*, pH 4·9, total copper < 0·02 p.p.m.; field capacity 21%.

All soils were sieved with a 2·5 mm stainless steel sieve, thoroughly mixed and potted into plastic pots with polythene liners to a capacity of 2 kg pot^{-1} in the case of soil I and 3 kg pot^{-1} for soils II and III. A pilot experiment was run to ascertain the optimum level of phosphorus to use on each soil and to test the soils for ability to produce copper deficiency in *Trifolium subterraneum* cv. Clare. All soils produced copper deficiency within 4 weeks from germination and maximum growth was obtained with 107 mg KH_2PO_4 pot^{-1} on soil I and 214 mg KH_2PO_4 pot^{-1} on soils II and III.

Basal dressings of nutrients applied, expressed as mg pot^{-1}, were as follows: KH_2PO_4, 107 (Soil I), 214 (Soils II and III); K_2SO_4, 428; $CaCl_2.2H_2O$, 428; $ZnSO_4.7H_2O$, 30; $MnSO_4.H_2O$, 30; $MgSO_4.7H_2O$, 30; H_3BO_3, 2; $CoSO_4.5H_2O$, 1; $Na_2MoO_4.2H_2O$, 0·5. On a surface area basis, 1 kg ha^{-1} is equivalent to 2·14 mg pot^{-1}.

All macronutrient salts were purified to remove any traces of copper by complexing with dithiazone in carbon tetrachloride (Hewitt, 1952). The ammonium nitrate treatment solution was analysed after purification and was found to contain no detectable copper.

All basal salts were applied in solution to the soil, allowed to dry and then thoroughly mixed throughout the soil. The soils were brought to 80% field capacity and allowed to equilibrate for 48 h before sowing each pot with 30 seeds of *Trifolium subterraneum* cv. Clare sieved for evenness of size. Each sown seed was inoculated with 1 ml of a heavy suspension (> 10^7 rhizobia per seed) of *Rhizobium trifolii* strain TAI in a 5% sucrose solution.

Plants were thinned to 25 per pot 10 days after germination on soil I, and 20 plants per pot 7 days after germination on soils II and III. Plants from soil I were harvested on 7 October, 38 days after germination and from soils II and III on 4 November, 32 days after germination.

Plant tops were weighed fresh and then placed in a forced draught oven at 68 °C prior to analysing for copper, total nitrogen and protein nitrogen. Roots were placed in a solution of 50% alcohol, 3% formalin and 1% acetic acid.

No analyses for copper or nitrogen were carried out on material from Soil I. Plant material from the other soils was analysed for copper after acid-digestion (Johnson and Ulrich, 1959), using atomic absorption spectrophotometry for samples over 0·1 g (Gladstones, Loneragan and Simmons, 1975), and using the heated graphite atomizer for samples with dry wt between 0·01 and 0·1 g (Simmons and Loneragan, 1975).

Total nitrogen was determined using the Kjeldahl digestion procedure, (McKenzie and Wallace, 1954). Protein nitrogen was estimated from total nitrogen determination after extraction of non-protein nitrogen (Loneragan, 1959).

Experiment 2

This experiment examined the effect of copper on growth and nitrogen fixation by subterranean clover on one soil at several harvests as plants became copper deficient. Nitrogen fixation was assessed at each harvest using the acetylene reduction technique. In this experiment three copper levels were used (0, 100, 1000 μg Cu pot^{-1}). These rates were selected on the basis of results of Experiment 1 to give deficient, marginal and adequate copper supply respectively. There were two replicates.

The experiment was conducted on the Lancelin soil collected from the same locality as soil II in Experiment 1. The soil was prepared and treated with basal nutrients as for Experiment 1.

Twenty-five seeds per pot of cv. Clare of subterranean clover were sown and thinned to 18 plants per pot, 7 days after emergence. At sowing the seeds were inoculated with 1 ml of a heavy suspension of *Rhizobium trifolii* strain TA1.

All plants in the experiment had to rely on the nitrogen fixed symbiotically for the major part of their nitrogen supply, as soil nitrogen has been shown to be negligible in virgin Lancelin soil, (Nualsri, 1978). Soil temperature was maintained at 15 °C ± 1 °C in a root cooling unit.

Four harvests were taken at the following times: 26, 33, 35 and 41 days after germination. The initial harvest time depended on the onset of copper deficiency symptoms when it was hoped that no appreciable differences in growth between treatments would have occurred. The time intervals between harvests were based on the acetylene reduction activity attained at the preceding harvest.

Determinations of acetylene reduction activity were made using 18 plants at the first two harvests and on 9 plants at the final two harvests. The method used was that of Trinick, Dilworth and Grounds (1976). At harvest after shaking the soil from the roots, intact plants were placed in vacola jars in a water bath at 15 °C for 30 min before introduction of acetylene. Duplicate gas samples were taken after a series of times for the estimation of acetylene and ethylene on a gas chromatograph. After assays of acetylene reduction were completed plants were separated into tops, roots and nodules for total and protein nitrogen analysis. At the final two harvests, nine plants from each pot were harvested and separated into the following plant sections: oldest pair of trifoliate leaves, youngest fully expanded leaf, new growth, rest of top, roots and nodules and then analysed for copper using the same procedure as for Experiment 1. Copper concentrations were also determined on the whole tops from the second harvest and on the nodules from the first two harvests.

Results

For simplification, tables and graphs may depict results from a single soil only. Data for other soils are similar unless otherwise stated.

Experiment 1

On all soils where nitrogen was applied but where copper was not applied, leaflets of young trifoliate leaves curled during the hottest part of the day. Eventually permanent wilting and necrosis of the leaflets occurred. Different amounts of copper

were required to eliminate these symptoms on each soil (300 μg Cu pot^{-1} Dandaragan soil; 100 μg Cu pot^{-1} Lancelin soil; and 30 μg Cu pot^{-1} Badgingarra soil. On Lancelin soil, in addition to these symptoms, new growth was stunted, malformed and necrotic.

When nitrogen was not applied, these symptoms were not observed except for Lancelin soil where no copper had been added. At low levels of copper application on all soils where nitrogen was not applied, plants showed a general paleness of colour extending to all leaves (Table 1).

Copper application increased yield of subterranean clover on all soils both with and without nitrogen application [Fig. 1]. However, on all soils growth responded less to applied copper when nitrogen was applied. Moreover, on two of the three soils the

Table 1. *The effect of ammonium nitrate and copper sulphate on the colour of nodulated subterranean clover grown on Dandaragan soil (Expt. 1)*

Ammonium nitrate (mg pot^{-1} week^{-1})	Copper sulphate (μg Cu pot^{-1})						
	0	30	100b	150	300	1000	3000
0	1·3a	2·3b	3·0bc	3·5c	3·6c	4·9d	5·5d
257	7·5efg	7·8efg	7·1efg	8·0ef	6·9g	7·9ef	8·0ef

Values are the means of 8 observations being the product of 4 replicates by 2 observers. Ranking is on the basis of colour: 1, pale green to 10, dark green. Main effects of copper and nitrogen and the interaction of copper and nitrogen significant $P < 0{\cdot}05$. Values with the same letter are not significantly different $P < 0{\cdot}05$.

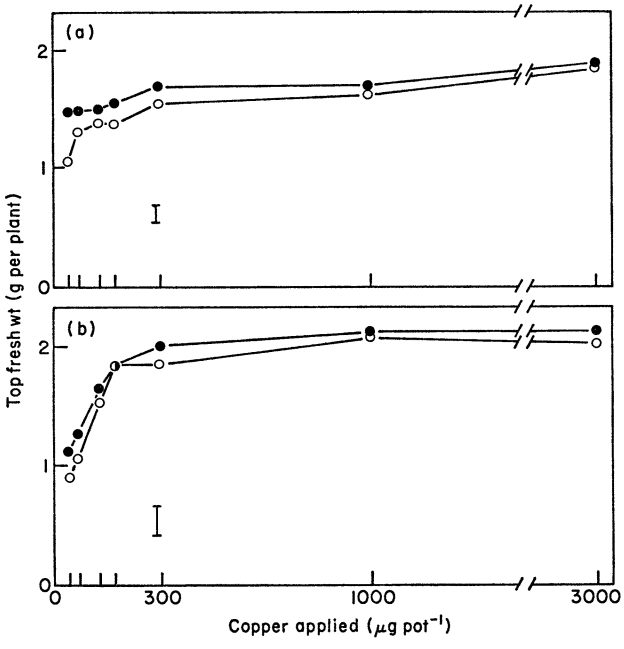

Fig. 1. The interaction of copper and nitrogen on the growth of subterranean clover grown on (a) Badgingarra and (b) Lancelin soils (Expt. 1). ○, No ammonium nitrate; ●, ammonium nitrate; bar represents L.S.D. at 5% level.

application of ammonium nitrate increased the growth of subterranean clover at low levels of copper application but not at high levels of copper application.

In general, all soils differed only in the degree of response to both copper and nitrogen applications.

Nitrogen application at low copper levels did not increase plant growth by increasing either copper concentrations or contents of plant tops on either Lancelin or Badgingarra soil (Table 2). Indeed on the Badgingarra soil nitrogen application decreased copper concentrations in tops without affecting copper contents. Hence the relationship between yield and copper concentrations in the whole tops depends markedly on whether nitrogen was applied.

Table 2. *The effects of ammonium nitrate and copper sulphate on the copper concentrations, total copper contents, percent total and protein nitrogen and the nitrogen contents of tops of subterranean clover grown on Lancelin soil (Expt. 1). Values are means of 2 replicates*

Copper sulphate (μg Cu pot^{-1})	Copper*				Nitrogen†					
	Concn (p.p.m.)		Content‡ (ng per plant top)		Total N (% dry wt)		Protein N (% dry wt)		Content (mg per plant top	
	−	+	−	+	−	+	−	+	−	+
0	0·7	0·7	60	70	3·0	4·5	2·7	3·5	2·1	4·5
30	0·8	0·7	90	70	3·1	4·0	2·7	3·6	3·3	4·3
100	1·0	0·8	140	140	3·3	3·1	2·9	2·9	4·9	5·2
150	1·1	1·1	200	230	3·2	3·2	3·1	2·9	6·1	6·6
300	1·9	1·7	370	410	3·4	3·1	3·1	2·8	6·7	7·5
1000	4·8	4·4	1150	1030	3·3	3·2	3·1	2·8	8·1	8·6
3000	10·1	9·4	1880	2620	3·6	3·3	3·1	2·9	6·9	8·9
L.S.D. (0·05)	1·24		—	—	0·37		0·31	0·49	0·86	

* Copper concn and content: Main effect of copper significant ($P < 0·05$). Main effect of nitrogen not significant ($P < 0·05$).

† Total N and N content: Main effect of copper and nitrogen significant ($P < 0·05$). Interaction of copper and nitrogen significant ($P < 0·05$). Protein N: Interaction of copper and nitrogen significant ($P < 0·05$). Effect of copper treated independantly, significant at $P < 0·05$.

‡ Data log transformed prior to statistical analysis.

− or + indicates absence or presence of ammonium nitrate.

On both soils copper application increased both copper concentrations and contents in tops at both nitrogen levels. Copper concentrations in whole tops were considerably lower on Lancelin soil than on Badgingarra soil when copper was not applied. This soil also produced the most marked symptoms of copper deficiency and the largest yield response to applied copper. On both soils copper concentrations in whole tops were 3 to 4 p.p.m. at near maximum yield.

Where copper was not applied, nitrogen application increased both total and protein nitrogen concentrations in tops for both soils (Table 2). Where nitrogen was not applied, copper application increased both total and protein nitrogen concentrations in tops on both soils. By contrast, where nitrogen was applied, copper application decreased total and protein concentrations in tops. Indeed on Lancelin soil, the interaction between copper and nitrogen application is significant ($P < 0·05$) and negative in relation to total and protein nitrogen concentrations in tops.

Copper applications to both soils irrespective of nitrogen application, increased the total nitrogen content of plants. However, nitrogen application generally increased nitrogen content of tops only at low levels of copper supply.

Experiment 2

Copper deficiency symptoms similar to those observed for Experiment 1 where plants were reliant on symbiotically fixed nitrogen, appeared on all nil copper pots 25 days after germination and remained for the duration of the experiment. The only symptom appearing on plants receiving 100 μg copper per pot was a paleness on comparison with the high copper level plants and this did not appear until 35 days after germination but remained until the end of the experiment.

Table 3. *Effect of copper sulphate on the copper concentration (p.p.m.) and on the nitrogen concentration (% dry wt.) both total and protein in tops, roots and nodules of subterranean clover grown on Lancelin soil (Expt. 2)*

Copper sulphate (μg Cu pot^{-1})		Copper concentration (p.p.m.)	Nitrogen concentration (% dry wt)	
			Total	Protein
Tops	0	0·8	2·6	2·2
	100	1·2	2·9	2·5
	1000	5·9	3·1	2·5
L.S.D. (0·05)		1·1	0·21	ns
Roots	0	3·0	2·6	1·9
	100	2·9	2·7	2·2
	1000	10·6	2·9	2·2
L.S.D. (0·05)		2·8	ns	ns
Nodules	0	2·9	*	4·8
	100	3·4	*	5·0
	1000	15·5	*	6·2
L.S.D. (0·05)		0·59		1·0

Values are means of 4 replicates.
* Insufficient material available for analyses.

Copper application increased the yield of subterranean clover tops at each harvest, the magnitude of this effect increased with time (Fig. 2). Copper application also increased the percentage total nitrogen in the tops and roots of subterranean clover at all but the first harvest (Table 3). Percentage protein nitrogen values tended to follow values for percentage total nitrogen.

Increasing copper supply from 100 to 1000 μg pot increased protein nitrogen concentrations of nodules markedly at both 35 and 41 days. However, protein nitrogen concentrations in nodules of plants receiving no copper were similar to those receiving 100 μg Cu pot^{-1}. In all copper levels protein nitrogen concentrations in nodules were considerably higher than those in either roots or tops.

At all harvests there was a marked effect of copper application on the amount of ethylene produced on a per plant basis. However, the effect of copper was to also increase the growth of the plants and the weight of nodules per plant at all harvests. When the rates of ethylene produced are expressed per gram of dry wt of nodule, the values obtained at harvests 2, 3 and 4 indicate a marked increase in nitrogen fixation by subterranean clover when copper is applied to the soil (Fig. 2).

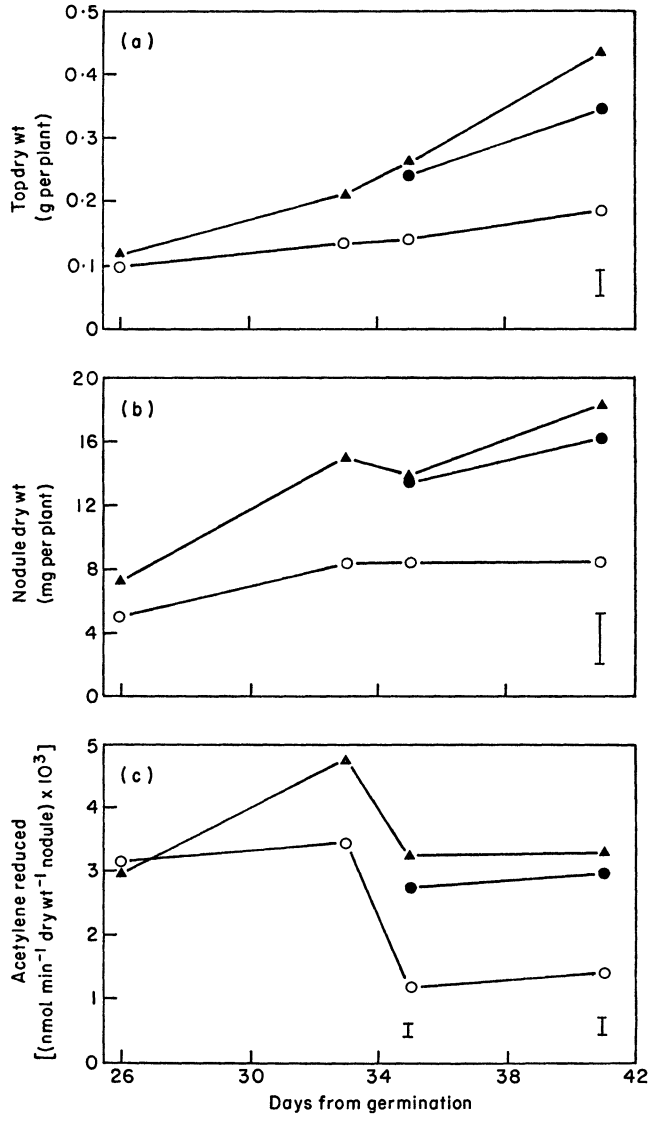

Fig. 2. The effect of copper on the growth of tops and nodules of subterranean clover grown on Lancelin soil over a period of time and the relationship of copper to nitrogen fixed as measured by the acetylene reduction assay (Expt. 2). ○, No copper; ●, copper 100 μg/pot^{-1}; ▲, copper 1000 μg/pot^{-1}; bar represents L.S.D. at 5 % level.

Discussion

The question of whether copper has a direct effect on nitrogen fixation is complex and can be resolved only by gathering evidence from a number of approaches. No one piece of scientific evidence reported here could substantiate the claim. Only in the case of an acetylene reduction assay, suitable replicated, showing a significant reduction in the production of ethylene due to a deficiency of copper, before there is any effect on the growth of the whole plant could one claim such a direct effect. This was not achieved here.

However, a number of approaches were used to demonstrate that copper has a role in nitrogen fixation. Anderson (1956) proposed that when two fertilizer treatments produce a positive response in plant growth and the interaction between them is negative then they correct the same deficiency; in fact he showed that nitrogen and molybdenum could replace one another in their effects on the yield of subterranean clover when these plants were using symbiotically fixed nitrogen. Other researchers working with different nutrients and legumes have shown similar negative interactions with nitrogen, e.g. Loneragan (1959) with calcium; Delwiche, Johnson and Reisenauer (1961) with cobalt. However, Greenwood and Hallsworth (1960) working with copper, showed a positive interaction between copper and nitrogen. One explanation for this could involve the timing of nitrogen application. They applied nitrogen at the beginning of the experiment before nodulation would be functional, giving an immediate advantage to those plants receiving fertilizer nitrogen.

In Experiment 1 of this paper, it was shown that either the addition of copper or nitrogen to plants of low copper status which were well nodulated produced greener plants and increased plant growth. The effect on yield of the addition of nitrogen was greater at low copper supply than at adequate copper supply. The interaction between copper and nitrogen was therefore negative. Adequate copper for plant growth did not however completely eliminate the response to added nitrogen.

Hence the first criterion for a direct effect of copper on nitrogen fixation was only partially met. Moreover, this negative interaction in terms of yield response could be explained readily if the addition of combined nitrogen resulted in an increase in the uptake of copper by the plant either through the direct addition of copper as a contaminant in the fertilizer nitrogen or through some release of copper in the soil. One could then expect an immediate response to added nitrogen where copper was limiting plant growth and this response need have no relationship to nitrogen fixation. However, the application of combined nitrogen to two of the soils in Experiment 1 did not increase either the concentration of copper or the total copper content in the plant tops at any level of copper supply.

The effect of a nutrient on the concentration of nitrogen in the tops of plants and the relationship of non-protein nitrogen to protein nitrogen is a further criterion for demonstrating a nutrient involvement in nitrogen fixation.

For other nutrients for which negative interactions with combined nitrogen on growth have been observed, alleviating deficiencies increases nitrogen concentrations in tops as well as increasing growth (e.g. Loneragan, 1958, for calcium; Chatel *et al.*, 1978, for cobalt; Anderson and Spencer, 1950a, and Johansen *et al.*, 1978, for molybdenum). In the current experiment, alleviating copper deficiency increased nitrogen concentrations in tops as well as increasing growth as has been previously reported (Greenwood and Hallsworth, 1960; Van der Elst *et al.*, 1962).

Copper deficiency did not effect the proportion of total nitrogen insoluble in alcohol (protein N) like molybdenum deficiency (Anderson and Spencer, 1950a) but unlike sulphur deficiency (Anderson and Spencer, 1950b). In the two experiments reported in this paper, although all nitrogen values were low, low levels of copper in the soil generally resulted in reduced concentrations of both total and protein nitrogen in the tops of plants when grown with symbiotically fixed nitrogen. Where combined nitrogen had been applied, low copper levels in the plant especially in the case of Lancelin soil resulted in higher concentrations of protein nitrogen in the tops which would certainly not implicate copper in the metabolism of non-protein nitrogen.

In our work reported here, we have used the acetylene reduction assay (Dilworth, 1966) as a further test to measure involvement of copper in nitrogen fixation. At all harvests nitrogen fixation per plant, as measured by acetylene reduction, decreased with decreasing copper supply along with yield and nitrogen content of the tops. However, at harvest 1 nitrogen fixation as measured by the rate of ethylene produced per gram of nodule tissue was not affected by copper treatment. Although yield had already been affected by harvest 1 copper taken up in the early stages of growth and present in the nodules in relatively high concentrations may not, like copper in old leaves, be mobile and probably remained sufficient for normal nodule functioning. From data on the respiratory rate for nodules it would seem that copper supply had little effect on the time period in which nodules remained physiologically active (Cartwright and Hallsworth, 1980). At subsequent harvests acetylene reduction per gram nodule was decreased by copper deficiency as well as acetylene reduction per plant.

The requirements of copper for symbiotic nitrogen fixation relative to its requirement for plant growth is not clear. For the elements molybdenum and cobalt, much greater amounts are required for nitrogen fixation than host plant growth. Where calcium is only moderately limiting plant growth, requirements for nodule function maybe greater than for plant metabolism. However in the case of copper we believe that the effects of copper on plant growth and nitrogen fixation are occurring simultaneously and at the same levels of copper supply.

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