

CALCIUM IN THE NITROGEN METABOLISM OF SUBTERRANEAN CLOVER

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Summary

It has been shown that calcium has a specific effect upon nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.).

Under conditions of moderate calcium deficiency the yield of plants was low, percentage protein and percentage non-protein nitrogen were reduced, and the plants were pale green. Addition of either combined nitrogen or calcium caused the plants to become dark green and increased the yield, the percentage protein, and the percentage non-protein nitrogen. Molybdenum deficiency produced similar effects on yield, protein, and non-protein nitrogen as moderate calcium deficiency.

The dry weight of nodules was decreased by calcium deficiency. The nodules of pale green calcium-deficient plants also showed signs of degeneration while those of molybdenum-deficient plants were not affected. The percentage calcium content of the nodules of the pale green calcium-deficient plants was much less than that of the tops and, unlike the tops, did not increase with calcium treatment. The effects of calcium deficiency on nitrogen fixation were therefore not necessarily brought about by a shortage of calcium in the nodules, but may have been due to a restricted supply of metabolite, possibly carbohydrate, resulting from calcium deficiency in the host plant.

Under conditions of severe calcium deficiency, growth of the host plant was stunted by calcium deficiency rather than by nitrogen deficiency. The host plant contained a higher than normal percentage of nitrogen and did not respond to nitrogen fertilizer.

I. INTRODUCTION

The level of calcium in the plant is known to affect nitrogen fixation in legumes indirectly through its effect on plant growth (Horner 1936; Albrecht 1937). Calcium level is also known to affect nodulation (Albrecht and Davis 1929; Loneragan and Dowling 1958). There is, however, no evidence in the literature that calcium has a specific effect upon nitrogen fixation by legumes. This paper presents evidence that calcium has a specific effect upon nitrogen fixation in subterranean clover.

II. METHODS

(a) General

Plants were grown on calcium-deficient soils in pots in a glass-house. The soils used were:

Soil A: a light brown sandy clay from Black Mountain, Canberra, of pH 5.6, exchange capacity 5.4 m-equiv/100 g, calcium <0.05 m-equiv/100 g.

Soil B: a light brownish grey loamy sand derived from Hawkesbury Sandstone, near Robertson, N.S.W., designated "Birrilee sand" by Walker (1959), of pH 5.0, exchange capacity 5.4 m-equiv/100 g, calcium <0.05 m-equiv/100 g.

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"Bacchus Marsh" strain of subterranean clover (*Trifolium subterraneum* L.) was used in experiments 1, 2, and 4. Wimmera rye grass (*Lolium rigidum* Gaud.) was used in experiment 3. Ten plants were grown per pot in all experiments.

The clover seed was inoculated with a dense suspension of *Rhizobium trifolii* strain NA 30 immediately before sowing. Four seeds were planted in each of 10 holes. Two milligrams of a mixture of calcium carbonate (1 part by weight) and the soil used in the experiment (9 parts) were placed on the seed before covering with soil. In this way nodules were formed on every plant of each treatment despite the acidity and the low calcium status of the soils.

In experiments 1, 2, and 3, 1900 g of soil A was used in glazed earthenware pots of 4½ in. diameter. In experiment 4, 1120 g of soil B was used in enamel pots of the same diameter.

Basal dressings (expressed in weight per acre on a surface area basis) common to all experiments were:

K_2HPO_4	Equivalent in phosphorus to 8 cwt of superphosphate per acre ($\equiv 563$ mg per pot).
K_2SO_4	3 cwt per acre ($\equiv 408$ mg per pot).
H_3BO_3	2 lb per acre ($\equiv 2.4$ mg per pot).
$ZnSO_4 \cdot 7H_2O$	7 lb per acre ($\equiv 8.5$ mg per pot).

The basal dressings were applied in solution prior to sowing.

All plants were harvested before flowering. The tops were dried rapidly under forced draught at 50°C. Roots of subterranean clover plants were washed from the soil and kept at 5°C until the nodules had been examined. Nodules which formed on the first 5 cm of tap root were designated "crown nodules". The remaining nodules were designated "distal nodules". Crown nodules were designated as "degenerating" when they showed definite brown discoloration or appeared burst and flaccid.

(b) Details of Experiments

Experiment 1.—To test the effect of calcium on subterranean clover grown at different levels of sodium molybdate, in the presence and absence of ammonium nitrate. Soil A. Sown January 29, 1958, and harvested April 15, 1958. A $3 \times 3 \times 2$ factorial design of the following fertilizers was used:

$CaSO_4$	Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre ($\equiv 0, 206, \text{ or } 824$ mg per pot mixed through dry soil prior to sowing).
Na_2MoO_4	Nil, 4, or 16 oz per acre ($\equiv 0, 0.3, \text{ or } 1.2$ mg per pot added in solution).
NH_4NO_3	Nil or 5 cwt per acre ($\equiv 0 \text{ or } 515$ mg per pot). Applied in solution at the rate of 1 cwt per acre per week from the beginning of the seventh week after sowing.

A further two treatments, one with and one without ammonium nitrate were included: in each case calcium was supplied as calcium sulphate plus calcium chloride, equivalent respectively to 2 plus 6 cwt of superphosphate per acre ($\equiv 206$ mg $CaSO_4$ plus 507 mg $CaCl_2$ per pot). Replicates 4. Seven weeks after sowing, a dressing of H_3BO_3 at the rate of 2 lb per acre ($\equiv 2.4$ mg per pot) was applied to two replicates of each treatment.

Experiment 2.—To test the effect of calcium chloride on subterranean clover grown in the presence and absence of ammonium nitrate. Soil A. Sown and harvested with experiment 1. Sodium molybdate at 4 oz per acre was applied to the pots of all treatments. A 2×2 factorial design, with four replicates, of the following fertilizers was used:

- CaCl₂ Equivalent in calcium to 2 or 8 cwt of superphosphate per acre
 (≡169 or 676 mg per pot added in solution before sowing).
 NH₄NO₃ Same as experiment 1.

Experiment 3.—To test the effect of calcium on Wimmera rye grass grown in the presence and absence of ammonium nitrate. Soil A. Sown June 3, 1958, and harvested July 22, 1958. Sodium molybdate at 4 oz per acre was applied to the pots of all treatments. A 3×2 factorial design, with four replicates, of the following fertilizers was used:

- CaSO₄ Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre
 (≡0, 206, or 824 mg per pot mixed through the dry soil prior to sowing).
 NH₄NO₃ Nil or 3.75 cwt per acre (≡0 or 386 mg per pot). Applied in solution
 at weekly intervals in increasing rates from 2 weeks after sowing.

Experiment 4.—To test the effect of calcium on subterranean clover grown in the presence and absence of ammonium nitrate. Soil B. Sown on September 12, 1957, and harvested on November 13, 1957. The following additional basal fertilizers were applied to the pots of all treatments:

- CuSO₄.5H₂O 7 lb per acre (≡ 8.5 mg per pot).
 Na₂MoO₄ 4 oz per acre (≡ 0.3 mg per pot).
 MgSO₄.7H₂O 14 lb per acre (≡ 17 mg per pot).

A 3×2 factorial design, with four replicates, of the following fertilizers was used:

- CaSO₄ Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre
 (≡ 0, 206, or 824 mg per pot mixed through the dry soil before sowing).
 NH₄NO₃ Nil or 4.5 cwt per acre (≡ 464 mg per pot). Applied in solution at
 weekly intervals in increasing rates from 2 weeks after sowing.

(c) Analytical Techniques

Plant tops which were dried rapidly at 50°C immediately after harvest were finely ground, dried overnight at 105°C, and cooled in a desiccator prior to chemical analysis.

Total nitrogen was determined on 0.2-g samples by the Kjeldahl digestion and steam-distillation procedures of McKenzie and Wallace (1954).

Protein nitrogen was determined on 0.2-g samples after extraction of the non-protein nitrogen with 100 per cent. ethanol and 70 per cent. ethanol (three times) on a water-bath at 60°C. Each extraction was carried out for 15 min. For the extraction the plant material was wrapped in filter paper: nitrogen was determined on the plant residue plus filter paper as described for total nitrogen.

Non-protein nitrogen was calculated by difference between the total nitrogen and protein nitrogen fractions.

Calcium was determined on 0.2-g samples in an "E.E.L." flame photometer after ashing at 550°C and removal of interfering anions by exchange on chloride-saturated "Amberlite IR4B" resin.

III. RESULTS

(a) Yield and Nitrogen Content of Legumes

(i) *Effects of Calcium*.—At lowest calcium levels plants exhibited acute calcium-deficiency symptoms, identical in both the young leaves and the old leaves with those described by Millikan (1953) for Dwalganup variety of subterranean clover. The growth of these plants was restricted by calcium deficiency and not by nitrogen deficiency. The plants did not respond to combined nitrogen treatment (Fig. 1). Also the percentage protein and percentage non-protein nitrogen were higher in these calcium-deficient than in the calcium-sufficient plants (Fig. 2).

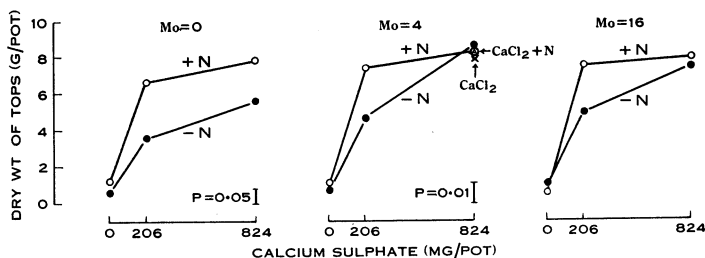


Fig. 1.—Effects and interactions of ammonium nitrate and calcium sulphate on yield of the tops of subterranean clover supplied with sodium molybdate at 0, 4, and 16 oz per acre. The values obtained where calcium chloride was added in addition to calcium sulphate are also given (expt. 1).

In plants which were moderately calcium deficient, calcium had a specific effect upon nitrogen fixation (Figs. 1, 2, and 3). Subterranean clover plants not provided with ammonium nitrate showed typical symptoms of nitrogen deficiency (pale green to yellow leaves with red petioles) with no symptoms of calcium deficiency. Percentage protein and percentage non-protein nitrogen were lowered and yield was reduced. Addition of ammonium nitrate restored to normal the percentage protein, percentage non-protein nitrogen, colour, and the yield: symptoms of calcium deficiency appeared in some of the older leaves (red leaves with withered petioles). The interaction between ammonium nitrate and calcium sulphate was strongly negative. Each fertilizer replaced the other in its effects on yield, colour, percentage protein, and percentage non-protein nitrogen (negative interaction significant for all effects at 5 per cent. level at least). This effect of calcium sulphate was shown to be due to the calcium ion. Calcium chloride was as effective as calcium sulphate in replacing ammonium nitrate in its effects on yield, colour, percentage protein, and percentage non-protein nitrogen (Figs. 1 and 2; Table 1).

The increase in percentage protein and percentage non-protein nitrogen of the severely calcium-deficient plants to which no combined nitrogen was added indicates that calcium became limiting to growth before soil nitrogen was exhausted.

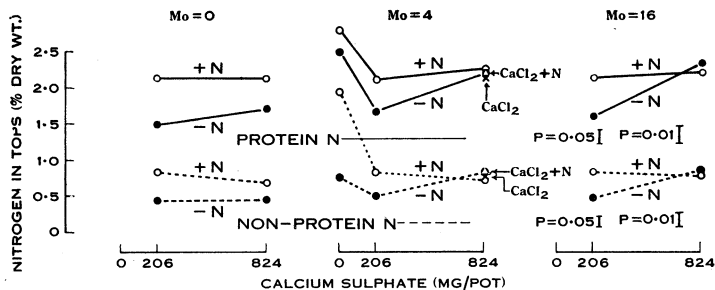


Fig. 2.—Effects and interactions of ammonium nitrate and calcium sulphate on percentage protein and percentage non-protein nitrogen of the tops of subterranean clover supplied with sodium molybdate at 0, 4, and 16 oz per acre. The values obtained where calcium chloride was added in addition to calcium sulphate are also given (expt. 1).

(ii) *Effects of Molybdenum.*—Molybdenum, like calcium, gave a negative interaction with combined nitrogen (significant at the 5 per cent. level at least) on yield, colour, percentage protein, and percentage non-protein nitrogen (Figs. 1 and 2).

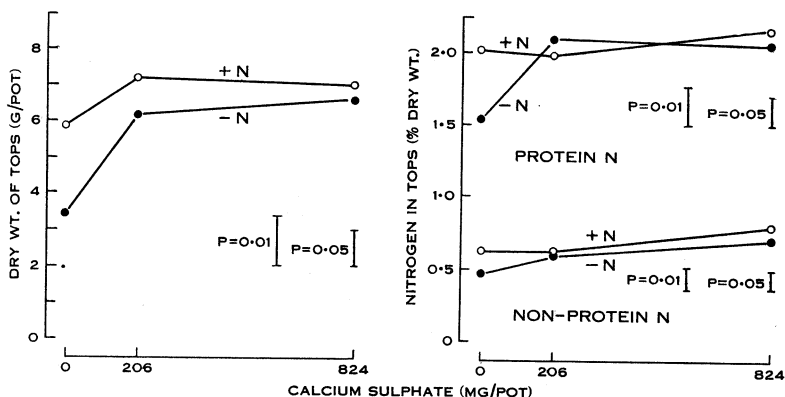


Fig. 3.—Effects and interactions of ammonium nitrate and calcium sulphate on yield, percentage protein, and percentage non-protein nitrogen of the tops of subterranean clover (expt. 4).

In the absence of ammonium nitrate, plants given no molybdenum and inadequate calcium were similar in colour, yield, and nitrogen content to plants deficient in only one of these elements. Dressings of ammonium nitrate alone produced dark green plants, and increased the yield, percentage protein, and percentage non-protein nitrogen to normal levels. However, where no combined nitrogen was applied, dressings of both molybdenum and calcium were required to give maximum increase in colour, yield, or percentage nitrogen. Thus molybdenum and calcium were both separately required for nitrogen fixation by subterranean clover.

(iii) *Effects of Boron.*—The direct effect of calcium on nitrogen fixation cannot be explained through any effect of calcium on the uptake or utilization of boron.

TABLE 1

EFFECTS OF AMMONIUM NITRATE AND CALCIUM CHLORIDE ON THE YIELD, PERCENTAGE PROTEIN NITROGEN, AND PERCENTAGE TOTAL NITROGEN OF THE TOPS OF SUBTERRANEAN CLOVER (EXPERIMENT 2)

	Without Ammonium Nitrate		With Ammonium Nitrate	
Calcium chloride (mg/pot):	169	676	169	676
Dry matter (g/pot)*	6.4	8.1	8.1	7.7
Total nitrogen (per cent. dry wt.)†	2.72	3.10	3.02	3.05
Protein nitrogen (per cent. dry wt.)‡	2.05	2.28	2.21	2.21

*Significant difference for $P = 0.05, 0.8$; for $P = 0.01, 1.1$. Interaction of calcium and nitrogen significant at $P < 0.01$.

†Significant difference for $P = 0.05, 0.23$; for $P = 0.01, 0.32$. Interaction of calcium and nitrogen significant at $P < 0.05$.

‡Significant difference for $P = 0.05, 0.20$; for $P = 0.01, 0.26$. Interaction of calcium and nitrogen not significant.

An application of boron (experiment 1) had no effect on yield, colour, percentage protein, percentage non-protein nitrogen, or even total nitrogen.

TABLE 2

EFFECTS OF AMMONIUM NITRATE AND CALCIUM SULPHATE UPON THE YIELD OF TOPS OF WIMMERA RYE GRASS (EXPERIMENT 3)

Results given as grams dry matter per pot. Mean log transformations given in parenthesis. Significant differences at $P = 0.05$ and $P = 0.01$ are 0.10 and 0.14 respectively (log transformations). Interaction of calcium and nitrogen significant at $P < 0.001$

Calcium sulphate (mg/pot):	0	206	824
Without ammonium nitrate	0.27 (0.42)	0.36 (0.56)	0.45 (0.65)
With ammonium nitrate	0.56 (0.74)	2.99 (1.47)	3.12 (1.49)

(b) *Yield of Non-legumes*

The stimulating effects of calcium on the nitrogen content and growth of subterranean clover cannot be explained by an effect of calcium upon soil nitrogen. Wimmera rye grass responded markedly to combined nitrogen in the presence of adequate calcium (Table 2).

Where neither calcium nor ammonium nitrate was applied, plants showed a marked withering of the tips of the leaves. These symptoms were very similar to those of nitrogen deficiency except that a few of the withered tips showed a characteristic sickle-shaped curling. In the presence of ammonium nitrate the withering of the tip and the sickle-shaped curling were also present. In addition, at the junction of the withered tip and the green blade, the green leaf often exhibited a peculiar and marked puckering for a short distance.

(c) *Calcium Content of the Tops of Subterranean Clover*

Calcium content of the tops of subterranean clover plants increased markedly with increasing calcium supply (Table 3). Tops containing 0.08–0.09 per cent. of calcium showed severe symptoms of calcium deficiency. In the absence of combined nitrogen, plants containing 0.25–0.34 per cent. calcium showed symptoms of nitrogen

TABLE 3

EFFECTS OF AMMONIUM NITRATE, CALCIUM SULPHATE, AND CALCIUM CHLORIDE UPON THE CALCIUM CONTENT OF THE TOPS OF SUBTERRANEAN CLOVER (EXPERIMENTS 1, 2, AND 4)

Calcium content expressed as per cent. dry weight. Mean log transformations given in parenthesis

	Expt. No.	Calcium Salt	Without Ammonium Nitrate			With Ammonium Nitrate		
			Calcium (mg/pot)			Calcium (mg/pot)		
			0	60	240	0	60	240
Soil A*	1	Sulphate	0.09	0.25	0.64	0.08	0.32	0.73
	2	Chloride		0.28	0.71		0.34	0.82
Soil B†	4	Sulphate	0.34 (53)	0.57 (76)	0.85 (93)	0.33 (52)	0.62 (79)	0.86 (93)

*Significant difference for $P = 0.05$, 0.11; for $P = 0.01$, 0.15. Effect of nitrogen treatment at calcium levels 60 and 240 mg/pot significant at $P < 0.05$.

†Significant difference (log transformation) for $P = 0.05$, 9; for $P = 0.01$, 12.

deficiency and not of calcium deficiency. In some cases the application of combined nitrogen to these plants caused small increases in the concentration of calcium in the tops: the tops became green and showed slight but definite symptoms of calcium deficiency. Since this application of combined nitrogen was associated with an increase in yield there was an appreciable increase in the total calcium content of the tops. Only those plants containing 0.57–0.86 per cent. calcium in the tops were free of either nitrogen- or calcium-deficiency symptoms.

The increase in the total amount of calcium in the tops may have accounted in part for the stimulating effect of combined nitrogen on yield but could not account for the stimulation in protein content of moderately calcium-deficient plants. Where

combined nitrogen was applied calcium deficiency did not reduce percentage protein despite the presence of definite calcium-deficiency symptoms and, in some cases, depression in yield. In fact, severe calcium deficiency even increased the percentage protein while at the same time it lowered the percentage calcium of the tops to

TABLE 4

EFFECTS OF AMMONIUM NITRATE, CALCIUM SULPHATE, AND SODIUM MOLYBDATE UPON THE NODULES OF SUBTERRANEAN CLOVER (EXPERIMENT 1)

Mean log transformations shown in parenthesis; — = not determined

Nodules Measured	Sodium Molybdate (oz/ac)	Without Ammonium Nitrate			With Ammonium Nitrate		
		Calcium Sulphate (mg/pot)			Calcium Sulphate (mg/pot)		
		0	206	824	0	206	824
Distal (No./pot)*	0	0	81 (1.87)	105 (1.99)	2	16 (1.18)	17 (1.23)
	4	1	98 (1.97)	28 (1.43)	2	9 (0.91)	16 (1.18)
	16	2	76 (1.85)	22 (1.33)	1	12 (1.05)	10 (0.96)
Crown (No./pot)†	0	10 (0.95)	76 (1.88)	63 (1.80)	15 (1.17)	44 (1.64)	44 (1.63)
	4	17 (1.20)	70 (1.84)	55 (1.73)	12 (1.08)	45 (1.64)	40 (1.60)
	16	23 (1.30)	77 (1.88)	44 (1.62)	8 (0.81)	48 (1.67)	39 (1.58)
Crown (mg/pot)‡	0	—	122	155	—	—	—
	4	—	106	176	—	50	59
	16	—	96	134	—	—	—
Crown (mg/nodule)§	0	—	1.59	2.43	—	—	—
	4	—	1.51	3.20	—	1.01	1.50
	16	—	1.29	3.27	—	—	—
Crown (No. degenerating/pot)	0	3 (0.44)	27 (1.32)	0	8 (0.93)	6 (0.62)	0
	4	10 (0.99)	46 (1.67)	3	5 (0.65)	11 (0.81)	1
	16	14 (1.01)	52 (1.70)	2	6 (0.81)	9 (0.74)	6

*Significant difference (log transformation) for $P = 0.05, 0.28$; for $P = 0.01, 0.37$.

†Significant difference (log transformation) for $P = 0.05, 0.19$; for $P = 0.01, 0.26$.

‡Significant difference for $P = 0.05, 44$; for $P = 0.01, 60$.

§Significant difference for $P = 0.05, 0.81$; for $P = 0.01, 1.10$.

||Significant difference (log transformation) for $P = 0.05, 0.55$; for $P = 0.01, 0.73$.

one-third its level in the moderately calcium-deficient plants. The stimulating effect of ammonium nitrate on the percentage protein of moderately calcium-deficient plants must therefore have been due to the increased supply of nitrogen available for metabolism. This indicates that calcium functions specifically in the fixation of nitrogen.

(d) Nodulation

(i) *Nodule Numbers*.—Those treatments which reduced the percentage nitrogen content of the tops increased the number of nodules on the roots (Table 4). Moderate calcium deficiency and molybdenum deficiency, separately or together, decreased nitrogen content and increased nodule numbers. This suggests that the effect of moderate calcium deficiency in promoting nodule formation was a secondary effect of induced nitrogen deficiency. The marked reduction in nodule numbers by the addition of ammonium nitrate in all treatments supports this view.

(ii) *Nodule Weight*.—The dry weight of the crown nodules was reduced by moderate calcium deficiency (Table 4). Molybdenum deficiency had no effect on the total dry weight of the crown nodules, but, through its effect on nodule numbers, decreased the dry weight per nodule.

(iii) *Nodule Degeneration*.—Calcium deficiency had a specific and marked effect upon nodule degeneration (Table 4). This degeneration varied from brown discoloration to a burst, deflated appearance of the nodules. This was not a secondary

TABLE 5
EFFECTS OF AMMONIUM NITRATE AND CALCIUM
SULPHATE UPON THE PERCENTAGE CALCIUM CONTENT
OF THE NODULES OF SUBTERRANEAN CLOVER
(EXPERIMENT 1)

Calcium content given as per cent. dry weight.
Differences not significant

Calcium sulphate (mg/pot):	206	824
Without ammonium nitrate	0.16	0.17
With ammonium nitrate	0.16	0.20

effect of nitrogen deficiency since the proportion of degenerating crown nodules on severely calcium-deficient plants containing a high percentage nitrogen was as high as on moderately calcium-deficient plants of low percentage nitrogen. Moreover, plants suffering from nitrogen deficiency induced by lack of molybdenum showed no nodule degeneration.

(iv) *Calcium Content of Nodules*.—Moderate calcium deficiency had no significant effect on the percentage calcium content of the crown nodules (Table 5). This lack of response to calcium treatment is in striking contrast to the effects of these treatments on the calcium content of the tops. It suggests that calcium might not function in nitrogen fixation in the nodule itself but rather through the supply of metabolites from the plant tops.

IV. DISCUSSION

Under conditions of extreme calcium deficiency, growth of subterranean clover plants was stunted by calcium deficiency rather than by nitrogen deficiency. The host plant contained higher than normal percentage nitrogen and did not respond

to nitrogen fertilizer. These findings are in keeping with the results of Horner (1936) and Albrecht (1937) who found that the total amount of nitrogen fixed by soybean plants decreased with decreasing concentrations of calcium in the plant: as calcium decreased, percentage nitrogen in the plants increased, so that the effect of calcium on the amount of nitrogen fixed was through its effect on growth of the host plant.

Under conditions of moderate calcium deficiency, nitrogen fixation was specifically inhibited. The yield of plants was low, percentage protein and percentage non-protein nitrogen were reduced, and the plants were pale green. Addition of ammonium nitrate caused the plants to become dark green and increased both the percentage nitrogen content and the yield of the plants. In these respects ammonium nitrate replaced additional dressings of calcium.

Molybdenum deficiency affected nitrogen metabolism of the tops of subterranean clover in a similar manner to moderate calcium deficiency. The effects of molybdenum on the nitrogen metabolism of subterranean clover have been described previously (Anderson and Thomas 1946; Anderson and Spencer 1950*a*). Molybdenum deficiency decreased both the percentage and the total amount of nitrogen in subterranean clover plants. The percentage protein and the percentage non-protein nitrogen were each decreased by deficiency of molybdenum. Addition of combined nitrogen to molybdenum-deficient plants gave dark green plants, yield increases, and increases in both percentage protein and percentage non-protein nitrogen. Under some conditions, addition of nitrate nitrogen produced these effects concurrently with a decrease in both percentage and total molybdenum contents of the tops of the plants (Anderson and Spencer 1950*a*). Thus it was established conclusively that, in the soils used, molybdenum was required only for the fixation of nitrogen.

Sulphur deficiency of subterranean clover produced pale green plants indistinguishable in appearance and in total nitrogen content from molybdenum-deficient plants (Anderson and Spencer 1950*b*). However, sulphur deficiency differed from molybdenum deficiency in that although it decreased percentage total and percentage protein nitrogen, it increased percentage non-protein nitrogen. Addition of sulphur increased the percentage protein nitrogen but decreased the percentage non-protein nitrogen. Furthermore, although addition of combined nitrogen increased the percentage total nitrogen it did not affect the colour or the percentage protein nitrogen. Thus sulphur was required for the conversion of non-protein into protein nitrogen so that the inhibition of nitrogen fixation by sulphur deficiency was caused by the non-utilization of the nitrogen fixed.

The effects of moderate calcium deficiency and its interactions with combined nitrogen on percentage protein and percentage non-protein nitrogen of subterranean clover were similar to those of molybdenum and quite distinct from those of sulphur deficiency. Calcium then did not affect conversion of non-protein to protein nitrogen. An effect of calcium upon the metabolism of the first-formed products of nitrogen fixation could conceivably lead to the observed low protein and non-protein nitrogen contents in moderately calcium-deficient plants. Increasing the supply of combined nitrogen did, however, replace dressings of calcium in restoring the percentage protein and non-protein nitrogen to normal. Nevertheless, this evidence does not establish direct participation of calcium in nitrogen fixation since the dressings of

combined nitrogen increased the amount (although not always the percentage) of calcium in the tops of the plants. However, if calcium is directly concerned in the metabolism of non-protein nitrogen, calcium deficiency would lead to low percentage protein in plants supplied with combined nitrogen. There was no evidence of any inhibition of protein synthesis where combined nitrogen was supplied, even in plants showing definite symptoms of calcium deficiency. There was, in fact, a stimulation in percentage protein in those plants showing the most severe symptoms of calcium deficiency and with the lowest percentage of calcium in the tops. Furthermore, the failure of calcium deficiency to inhibit protein formation has been reported for many plants grown in the presence of combined nitrogen (Ginsberg and Shive 1926; Nightingale *et al.* 1931; Hibbard and Grigsby 1934). The low percentage protein of 3-day-old calcium-deficient wheat seedlings observed by Burström (1954) was due almost entirely to inhibition of nitrate uptake. The present results cannot be explained by any effect of calcium deficiency on nitrogen uptake. The evidence therefore suggests that calcium functions specifically in nitrogen fixation in subterranean clover.

Effects of boron deficiency in legumes (Brenchley and Thornton 1925; Mulder 1948) are in some respects similar to the effects of moderate calcium deficiency and of molybdenum deficiency. Boron deficiency reduced nitrogen fixation in *Vicia faba* (Brenchley and Thornton 1925). Percentage nitrogen was reduced and the plants showed symptoms of nitrogen deficiency. However, the plants showed symptoms of boron deficiency together with those of nitrogen deficiency, and did not respond much in yield to addition of combined nitrogen unless boron was also added. The effect of boron on nitrogen fixation was therefore largely due to its effect on the growth of the host plant. Mulder (1948) obtained similar results with pea plants grown in water culture.

Nitrogen deficiency induced by low calcium or molybdenum supply was associated with an increase in the number of nodules. Application of nitrogen to plants deficient in either calcium or molybdenum increased the percentage nitrogen and decreased the number of nodules. The same effect was obtained also where the plants were deficient in both calcium and molybdenum. Effects of molybdenum deficiency on nodulation were reported and discussed fully by Anderson and Spencer (1950a). By contrast with molybdenum, calcium, and nitrogen deficiencies, which decreased the percentage nitrogen and increased the number of nodules, sulphur deficiency reduced both the percentage nitrogen content of subterranean clover plants and the nodule numbers (Anderson and Spencer 1950b). This result led Anderson and Spencer to reject the carbon-nitrogen hypothesis (Wilson 1940) as an explanation of the effects of sulphur on nodulation. They pointed out that in all cases investigated by them, decrease in the number of nodules was associated with an increase in the percentage non-protein nitrogen. The increase in nodule numbers in moderately calcium-deficient plants reported in the present experiments was also accompanied by a decrease in the percentage non-protein nitrogen.

Anderson and Spencer (1950b) also drew attention to a correlation between nodule numbers and nitrogen requirement of the host plant: response to nitrogen was accompanied by decrease in the number of nodules. The effect of moderate

calcium deficiency in increasing nodule numbers is consistent with the claim that growth of these plants was limited by supply of available nitrogen and that calcium functions specifically in nitrogen fixation.

The increase in nodule numbers associated with calcium-deficient plants of low nitrogen status was therefore a secondary and not a direct effect of the calcium deficiency. In fact, where secondary effects of plant growth and nitrogen deficiency were eliminated by supply of adequate combined nitrogen, decreasing calcium supply decreased the number of nodules on young subterranean clover plants (Loneragan and Dowling 1958). Calcium supply had no effect on the growth of *Rhizobium* so that the extra calcium was required for infection or nodule development. The premature degeneration of the nodules of plants grown with low calcium in the present experiments indicates that the extra calcium was required for nodule development.

The degeneration of nodules in calcium-deficient plants is not a secondary effect of nitrogen deficiency. Even where ammonium nitrate was applied the number of degenerating nodules was higher on calcium-deficient than on calcium-sufficient plants. Moreover, the high percentage of degenerating nodules present on the crowns of moderately calcium-deficient plants is in striking contrast to their complete absence on molybdenum-deficient plants. In addition, there was a very high proportion of degenerating nodules on the severely calcium-deficient plants which showed no signs of nitrogen deficiency. This suggests that calcium deficiency induced premature nodule degeneration before it had any effect on nitrogen fixation.

The low calcium content of the nodules compared with that of the tops suggests that the nodules have a lower overall requirement for calcium. Jensen (1947) has also reported lower calcium levels in the nodules of several legumes, including subterranean clover, than in the tops. It is possible that a specific part of the nodule has a high calcium requirement. However, it seems unlikely that nodule development was directly influenced by the level of calcium in the nodule, since there was no difference between the percentage calcium contents of the nodules of the intermediate and high calcium treatments of the present experiments.

On the other hand, calcium could affect nitrogen fixation in the nodules through an effect on the supply of metabolites from the roots or tops. It has been suggested (Brenchley and Thornton 1925) that boron deficiency affects nitrogen fixation in this way. Under conditions of boron deficiency vascular strands failed to develop in the nodules of *Vicia faba* plants and nitrogen fixation was reduced. Nodules of plants grown without boron presented a curious burst appearance. The degeneration of nodular tissue of plants without boron was attributed by these authors to an inadequate carbohydrate supply. Mulder (1948) also reported that the nodules of pea plants not given boron and showing symptoms of nitrogen deficiency were black and shrivelled at harvest.

The similarity in the effects of calcium and boron deficiencies on nodule degeneration suggests that calcium may also be involved in supply of carbohydrates to the nodule.

There is also a strong similarity between the effects of low calcium supply on the nodules of subterranean clover plants and of reduced carbohydrate supply on

the nodules of lucerne plants placed in the dark (Thornton 1930). In darkened lucerne plants, as in low calcium subterranean clover plants (Loneragan and Dowling 1958) new nodules were not formed and existing nodules degenerated prematurely.

There is abundant evidence that calcium deficiency interferes with carbohydrate metabolism. According to Miller (1938), Boehm as early as 1875 observed an abnormal accumulation of starch in *Phaseolus vulgaris* grown without calcium; this has been confirmed for numerous plants by many investigators. In the same experiments in which organic nitrogen was not affected, Nightingale *et al.* (1931) and Hibbard and Grigsby (1934) found marked accumulation of carbohydrates in calcium-deficient tomato stems and pea plants. Joham (1957) found an accumulation of carbohydrates in the tops and depletion in the roots of calcium-deficient cotton plants, indicating that calcium has a role in the translocation of carbohydrates. The evidence therefore suggests that the premature degeneration of the nodules of calcium-deficient plants may well have resulted from such an effect of calcium deficiency on translocation of carbohydrates to the nodule. The effect of calcium deficiency on nitrogen fixation may also have resulted from its effects on the supply of carbohydrates or some other metabolite to the nodule.

Of the many species and strains of *Azotobacter* investigated, only one has been shown to have a specific requirement for calcium in nitrogen fixation (Burk and Horner 1939; Esposito and Wilson 1956; Norris and Jensen 1957). Allen and Arnon (1955) found no difference in the calcium requirement between plants of the blue-green alga *Anabaena cylindrica* grown on molecular nitrogen and on nitrate. These results are compatible with the suggestion that calcium affects nitrogen fixation by subterranean clover through its effect on the supply of metabolites to the nodule rather than through direct participation in the biochemical reactions of fixation.

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