INTERACTION BETWEEN CALCIUM LEVEL AND NITROGEN SOURCE ON GROWTH AND ^{45}Ca DISTRIBUTION IN SUBTERRANEAN CLOVER

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Summary

Using either ammonium or nitrate as the nitrogen sources, dry matter yields were highest from plants grown on a normal calcium nutrition, whereas plants supplied with urea grew best at low calcium level. When ammonium and nitrate were added together, yields were independent of the calcium concentration in the substrate. Plants fed solely ammonium nitrogen made extremely poor growth.

The main factor affecting ^{45}Ca distribution within subterranean clover was the calcium concentration in the substrate. The percentage of ^{45}Ca retained in the roots was greatest at the low calcium level. At this level it was also greatest in the plants fed ammonium and least in those fed ammonium plus nitrate. The concentration of ^{45}Ca in plant tops was equal in the presence of ammonium plus nitrate or urea, and higher than with nitrate or ammonium. Between nitrogen sources the lamina:petiole, leaf edge:leaf centre, and petiole distal:petiole proximal ratios of ^{45}Ca concentrations were different at the low, but not at the normal, calcium level.

I. Introduction

It is well known from nutrient culture studies that different nitrogen sources have a profound effect upon plant growth. The majority of plants, whilst making vigorous growth with nitrate, remain stunted and develop root and leaf abnormalities when supplied with ammonium as their sole nitrogen source. Their growth on either ammonium plus nitrate or urea is generally intermediate between the above two extremes (Pardo 1935; Bollard 1959). The plant's response to these different nitrogen sources can be further modified by any factor, either internal or external, that affects its rate of nitrogen absorption or assimilation (Pardo 1935; Street and Sheat 1958; Grasmanis and Leeper 1965).

Relatively little is known about the influence of a plant's calcium status on its subsequent response to various nitrogen sources. However, from the few reports that have been published there is evidence supporting the existence of calcium–nitrogen source interactions. Early work by Eckerson (1924) showed calcium to be essential for the reduction of nitrate in protein synthesis in plants. This was later confirmed by Nightingale *et al.* (1931) who clearly demonstrated that calcium-deficient tomato plants were incapable of absorbing and assimilating nitrate. Skok (1941) found that bean plants supplied with adequate calcium made better growth on

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nitrate than on urea nitrogen, whereas when grown at a low calcium status the urea then became the superior nitrogen source. In a study on the growth-depressing effect of a high-nitrate nutrition, Alexander and Stark (1959) showed with cauliflowers that such a depression could be eliminated by increasing the calcium concentration in the substrate.

The study described herewith was designed to investigate the effect of four nitrogen sources — ammonium, ammonium plus nitrate, nitrate, and urea — upon the growth and distribution of radioactive calcium (^{45}Ca) within subterranean clover grown in nutrient cultures with either a low or normal calcium supply. Subterranean clover was chosen as the test plant since the distribution pattern of ^{45}Ca in normal and calcium-deficient plants has been documented by Millikan and Hanger (1964).

II. Methods

Subterranean clover (*Trifolium subterraneum* L., cv. Dwalganup) seeds were germinated in sterilized coarse quartz sand, and when the seedlings had developed to the cotyledonary stage they were transferred to the nutrient cultures. High-density plastic pots (2-litre capacity) lined with a plastic bag were used.

Each culture had the basic composition of 6·5 m-equiv/l K^+, 2 m-equiv/l Mg^{2+}, 1·5 m-equiv/l PO_4^{3-}, and 0·001% Fe Na EDTA. Micronutrients were added at the rate described by Millikan (1961). The concentrations of the variable ions (m-equiv/l) in each treatment were as follows:

	Low-calcium Treatment				Normal-calcium Treatment			
Nitrogen source	NH_4^+	$(NH_4^+ + NO_3^-)$	NO_3^-	Urea	NH_4^+	$(NH_4^+ + NO_3^-)$	NO_3^-	Urea
Variable ions:								
Ca^{2+}	0·1	0·1	0·1	0·1	8	8	8	8
NH_4^+	14	7			14	7		
Na^+			14				14	
SO_4^{2-}	22·1	8·1	8·1	8·1	30	16	16	16
NO_3^-		7	14			7	14	

The concentration of urea in the culture was 7 mM.

Each treatment was replicated six times. To half was added aqueous $^{45}CaCl_2$ at the estimated activity of 5 μCi (containing 2·0 μg Ca^{2+}) for the normal- and 2·5 μCi for the low-calcium cultures. The remaining non-radioactive cultures were used to determine the volume of either 0·1N NaOH or 0·1N H_2SO_4 required for each pot in order to restore the pH values of all cultures to 6. Adjustments were made twice weekly. Throughout the experimental period the maximum fluctuations in the pH values recorded for the various cultures were: NH_4^+ 5·6–6·1, $NH_4^+ + NO_3^-$ 5·5–6·2, NO_3^- 5·9–6·3, and urea 6·0–6·4. All solutions were completely renewed on day 31.

Two plants from each pot were harvested on days 31, 42, and 51. The non-radioactive plants were immediately dried at 70°C in a forced-draught oven and later weighed, whereas the radioactive plants were firstly autoradiographed and later separated into parts and radioassayed. The techniques employed in autoradiography and radioassaying have been described elsewhere (Millikan and Hanger 1964).

III. Results

Dry matter production by subterranean clover was influenced by both the nitrogen source available to the plant and the calcium concentration in the substrate (Fig. 1). Ammonium by itself was an unsatisfactory nitrogen source. By day 31 plant growth had either been suppressed or completely halted, depending upon the calcium level, and many plants had chlorotic or necrotic leaves.

With a normal calcium supply, the plants produced their highest dry matter yields when either ammonium plus nitrate or nitrate were the nitrogen sources. However, with the limited calcium supply the plants responded differently to the two nitrogen sources. Dry matter yields remained unaltered where ammonium plus nitrate were supplied, but with solely nitrate growth was depressed and the symptoms of petiole collapse developed (Millikan 1953). This symptom appeared prior to day 31, and failed to progress beyond the unifoliate and first trifoliate leaves.

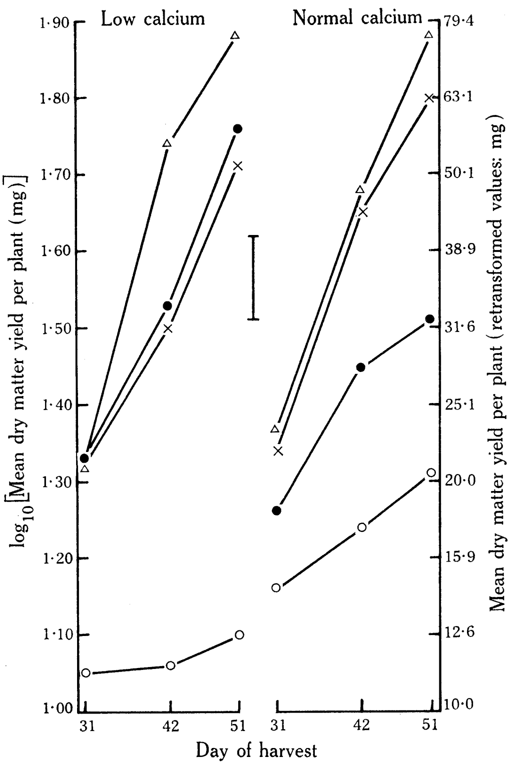

Fig. 1.—Effect of nitrogen source on the dry matter yield of subterranean clover grown in nutrient cultures with either a low or normal calcium content. Harvests were made on days 31, 42, and 51. The vertical bar indicates the least significant difference (5% level) between nitrogen sources, calcium levels, and harvest times. ○ NH_4^+; △ NH_4NO_3; × NO_3^-; ● urea.

The interaction between urea and calcium level on plant growth was different to those with the other three nitrogen sources. Best dry matter yield occurred at the low calcium level, particularly from between days 42 and 51.

The concentration of ^{45}Ca (as counts/min/mg dry matter) in subterranean clover plants (Fig. 2) was similar when either ammonium plus nitrate or urea were used as nitrogen sources. Where solely nitrate had been supplied, the ^{45}Ca concentration was much lower in the plants grown at the low calcium level, and this could account for the development of petiole collapse when grown in the low-calcium cultures. With the above three nitrogen sources, the calcium concentration within the plant did not vary significantly with time, indicating that there was a balance between calcium absorption by the roots and dry matter production.

The ammonium-fed plants had the lowest ^{45}Ca concentration. However, concentration did increase with plant age, and by day 51 it was similar to that found in plants fed nitrate.

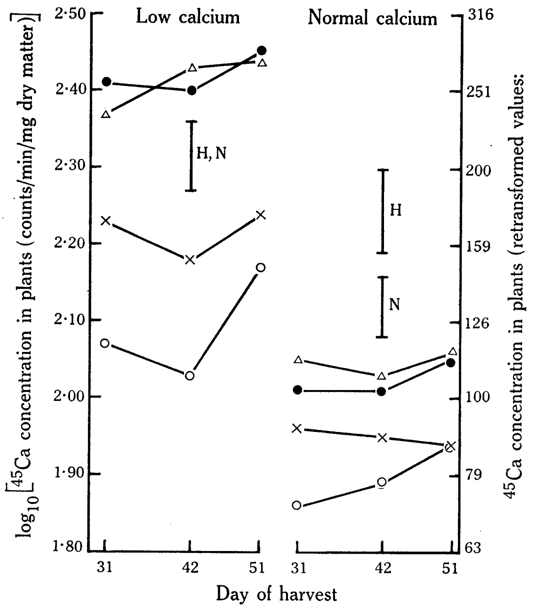

Fig. 2.—Effect of nitrogen source on the concentration of ^{45}Ca in subterranean clover plants grown in nutrient cultures with either a low or normal calcium content and harvested on days 31, 42, and 51. Vertical bars indicate least significant differences (5% level) between either nitrogen source (N) or harvest time (H) or both (H, N). ○ NH_4^+; △ NH_4NO_3; × NO_3^-; ● urea.

The percentage of the plants' ^{45}Ca content located in the top growth (Fig. 3) showed that neither nitrogen source nor time of harvest affected the distribution of

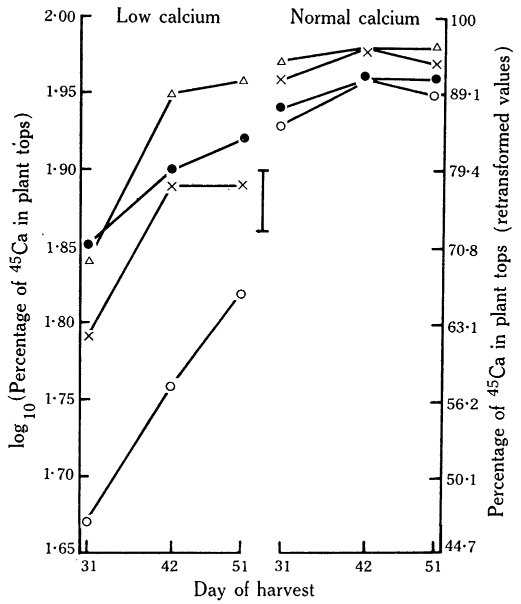

Fig. 3.—Effect of nitrogen source on the percentage of ^{45}Ca content found in the plant tops of plants grown under low- or normal-calcium conditions. Harvests were made on days 31, 42, and 51. Vertical bar as for Figure 1. ○ NH_4^+; △ NH_4NO_3; × NO_3^-; ● urea.

^{45}Ca between the tops or roots, providing there was an adequate calcium supply. By contrast, in plants low in calcium, there was a higher retention of ^{45}Ca in the roots, particularly at the time of the first harvest on day 31. However, by day 42 there had been a substantial increase in the percentage of ^{45}Ca located in the plant tops. In plants supplied solely with ammonium this rise continued until the termination of the experiment on day 51. In the low-calcium plants, the highest root retention of ^{45}Ca occurred with ammonium and least with ammonium plus nitrate as the respective nitrogen sources.

An examination of the between-treatment effects on the ^{45}Ca concentration in individual leaves was restricted to the cotyledon and the unifoliate and first two trifoliate leaves, since only these were common to all three harvests. Even so, the stunted ammonium-fed plants had to be omitted from the analysis. The results are presented in Figure 4.

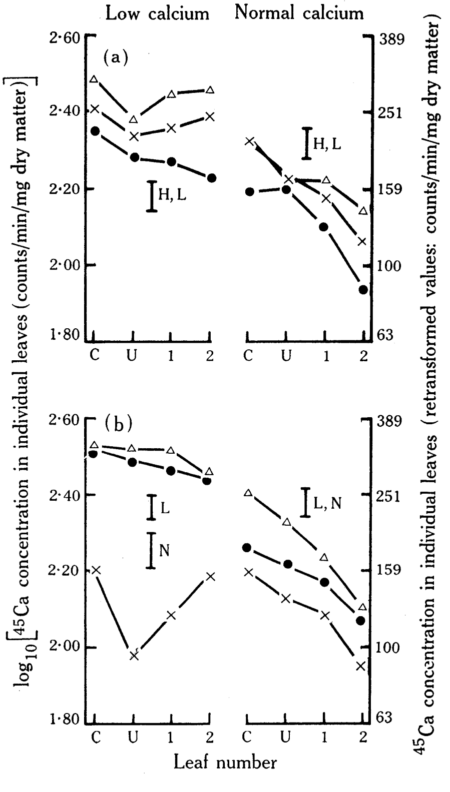

Fig. 4.—Concentration of ^{45}Ca found in individual leaves (lamina+petiole) of subterranean clover grown under normal- and low-calcium conditions, as influenced by (*a*) harvest time irrespective of nitrogen source (● day 31; × day 42; △ day 51); and (*b*) nitrogen source irrespective of harvest time (△ NH_4NO_3; × NO_3^-; ● urea). Vertical bars indicate least significant differences (5% level) between leaves (L) or nitrogen sources (N) or between both leaves and harvest time (H, L) or leaves and nitrogen sources (L, N). *C*, cotyledons; *U*, unifoliate leaves.

Averaging the results over the three nitrogen sources [Fig. 4(*a*)] the ^{45}Ca concentration found in the leaves on day 31 fell with decreasing leaf age, irrespective of the calcium status of the substrate. The subsequent harvests revealed that relative changes in the ^{45}Ca concentration was influenced by the calcium status of the substrate. At both normal and low calcium levels the ^{45}Ca concentration in each leaf (except the unifoliate) increased significantly between days 31 and 51. In normal-calcium plants the concentration gradient between the leaves was maintained over this period, whereas in those from low-calcium plants the concentrations were comparable in all leaves by day 51. This result supports an earlier finding by

Millikan and Hanger (1964) of a preferential routing of calcium to the younger leaves when the calcium supply is restricted.

The elimination of harvest time from the statistical analysis [Fig. 4(*b*)] showed that under the low-calcium nutrition, all leaves from the nitrate-fed plants had the lowest ^{45}Ca concentration, particularly the unifoliate and first trifoliate leaves, in which petiole collapse occurred. At the normal calcium level, the highest concentration of ^{45}Ca was found in leaves from plants fed ammonium plus nitrate, and lowest in those fed nitrate. There was a sharp drop in concentration with decreasing leaf age, a trend that was not apparent or significant in the low-calcium plants.

The results of radioassays (not presented) showed that the total ^{45}Ca content of the first two trifoliate leaves increased with time. In addition, since these two leaves were fully developed by day 31, it is unlikely that their dry weights altered significantly between days 31 and 51. Therefore, any changes in the ratios of the ^{45}Ca concentrations found in different parts of these leaves, presented in Figure 5 [lamina : petiole (L/P); lamina edge : lamina centre (LE/LC); and petiole distal : petiole proximal (PD/PP)], would be due to either a redistribution within the leaf of previously deposited ^{45}Ca, or to the preferential routing of recently acquired ^{45}Ca to a certain part of the leaf.

The three ratios for leaves from plants grown on a normal-calcium nutrition were not affected by nitrogen source [Fig. 5(*a*)].

The ratios from low-calcium leaves, however, were modified by nitrogen source. The L/P ratios, besides being considerably lower, were also less than 1, indicating a relative accumulation of ^{45}Ca in the petiole, this petiole accumulation being greatest with nitrate as nitrogen source. Within the lamina the LE/LC ratio suggests a routing of ^{45}Ca into the leaf edge, except where solely nitrate was supplied. The PD/PP ratios show that, where both urea and nitrate were used as nitrogen sources, the ^{45}Ca concentration was greatest in the proximal half. Thus, under a low calcium regime, the results confirm that the movement of ^{45}Ca within the plant was restricted, since there was a well-defined concentration drop along the petiole to the lamina.

Examining only the harvest effect [Fig. 5(*b*)], the low-calcium plants had their lowest L/P and PD/PP ratios at the time of the first harvest on day 31, indicating a retention of the leaf's ^{45}Ca in the base of the petiole. However, there were successive increases in the PD/PP ratio and by day 51 the ^{45}Ca concentration was fairly uniform throughout the length of the petiole. Nevertheless, it was not until the final harvest on day 51 that there was a significant increase in the L/P ratio indicating a preferential ^{45}Ca flow into the lamina. Within the lamina the LE/LC ratio increased strongly from day 31 to day 42, but not thereafter.

In contrast to the low-calcium leaves, those with an adequate calcium supply had their highest concentration in the lamina, the L/P ratio increasing sharply from day 31 to day 42. However, the distribution of the isotope within both the lamina and the petiole was relatively uniform and not influenced by time.

IV. Discussion

This study has demonstrated highly significant interactions between the calcium level and the nitrogen source upon the growth of subterranean clover plants, and the distribution of ^{45}Ca within the plant tissue.

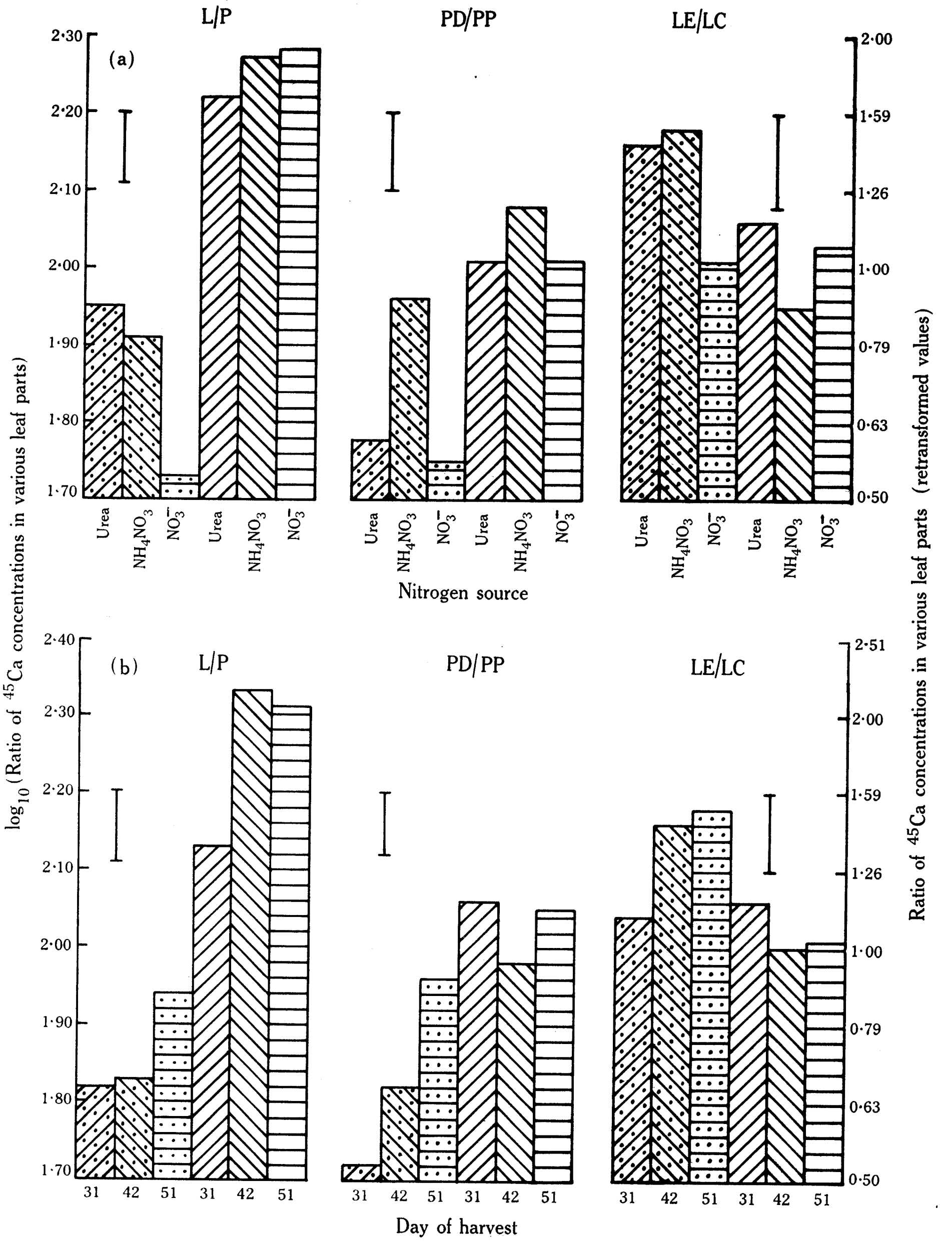

Fig. 5.—Mean ratios of the ⁴⁵Ca concentrations in the lamina and petiole (L/P), distal and proximal halves of the petiole (PD/PP), and the lamina edge and centre (LE/LC) for the trifoliate leaves 1 and 2 of subterranean clover. (*a*) Effect of nitrogen source irrespective of harvest time. Vertical bars indicate least significant differences (5% level) between nitrogen source and calcium level. (*b*) Effect of harvest time irrespective of nitrogen source. Vertical bars indicate least significant differences (5% level) between harvest time and calcium level. Dotted area: low-calcium plants. Cross-hatched area: normal-calcium plants.

Of the four nitrogen sources used, only ammonium nitrogen severely restricted plant growth. The severity of the stunting induced by the ammonium ion was unexpected since the pH of the nutrient cultures had been regularly adjusted to 6 so as to prevent the development of highly acidic conditions which are known to be conducive to ammonium toxicity within the plant. Both Davidson and Shive (1934) and Wallace, Biely, and Bhan (1959) successfully grew peach seedlings and citrus respectively on ammonium nutrition at a pH of 6.

Although the ammonium ion played the dominant role in restricting the development of subterranean clover, an adequate supply of calcium to the plant produced a slight but significant improvement in growth. It is unlikely that this response to calcium was merely the result of overcoming a calcium deficiency within the plants. Even though these plants had the lowest concentrations of ^{45}Ca compared with plants on other nitrogen sources, their growth rate was so slow that it is doubtful whether a critical shortage of calcium would occur under these conditions in susceptible tissue. Bolle-Jones (1955), following the growth of rubber tree seedlings in sand cultures supplied with different ratios of nitrate to ammonium, reported that at the higher ammonium levels the plant's calcium requirement appeared to be lower, even though there was a depressing effect of the ammonium ion on calcium uptake.

The necessity of calcium for ammonia assimilation by plants, particularly when grown in substrates with low pH values, has been demonstrated by Iwanova (1934) and Becking (1956). Lomander (1965) found that yeast was dependent upon calcium for growth if ammonium was the only nitrogen source available. Thus it is considered from the evidence available that the better growth of the subterranean clover plants grown in the normal-calcium solutions was brought about by a calcium-induced increase in ammonium assimilation by the plant.

The nitrate-fed plants also grew less under a low calcium nutrition. However, in this instance the reduction was associated with the onset of calcium deficiency, as indicated by the appearance of petiole collapse. This symptom did not appear in plants of comparable vigour grown on the other nitrogen sources under a low-calcium nutrition. It is of interest to note that the symptom of petiole collapse as described by Millikan (1953) and Millikan and Hanger (1964, 1966) occurred in plants grown in nutrient cultures in which nitrate was the sole nitrogen source.

A possible explanation for the development of calcium deficiency symptoms in the nitrate-fed plants is that there had been an inadequate uptake of calcium by the roots. Compared with plants supplied with either ammonium plus nitrate or urea, the nitrate-fed plants had the lowest calcium concentration thus making them prone to a calcium stress within the tissue. This result was unexpected since it is generally considered that plants fed solely nitrate nitrogen have a higher cation (including calcium) content when compared with plants grown on other nitrogen sources (Arnon 1939; Leukel and Franch 1941; Evans and Weeks 1947; Kirkby and Mengel 1967; Townsend 1967).

It was also found that nitrogen source affected the distribution of ^{45}Ca within the leaf petiole. In the low-calcium series, the plants fed ammonium plus nitrate had a more uniform distribution of ^{45}Ca along the length of the petioles than those fed nitrate or urea. In the plants from the latter two nitrogen treatments there had been a retention of ^{45}Ca in the proximal portion of the petioles, and, as previously reported by Millikan and Hanger (1964), petiole collapse in old subterranean clover leaves was characterized by the failure to accumulate calcium in the distal halves of the petioles. The absence of petiole collapse in plants supplied with urea may have been because of the higher calcium level in the tissue, which was sufficient to maintain the integrity of the cells. There is also evidence that when urea is the sole nitrogen source, the plant's basic calcium requirement is lower. Skok (1941) has demonstrated, with beans grown in calcium-deficient solutions, that the appearance of calcium deficiency symptoms was delayed and of reduced severity when urea was supplied to the solutions in place of nitrate.

In their review on calcium, Pierre and Allaway (1941) suggested that the reduction in a plant's calcium requirement by urea was by eliminating the necessity for calcium in the nitrate reduction process in the plant. Another function of calcium, proposed by Wallace, Frolich, and Lunt (1966) is that of alleviating the toxicities of other cations in plant tissue. Since a high nitrate intake by the plants would be accompanied by a corresponding high intake of cations in order to maintain electroneutrality, this function of calcium could be very important. Wallace, Frolich, and Lunt (1966) have also stated that the plant's specific requirement for calcium approaches that of a micronutrient.

The relative growth behaviour of plants supplied with urea at the two calcium levels was different to that found with the other nitrogen sources. The urea-fed plants made their most vigorous growth in solutions low in calcium. In earlier work Beaumont, Eisenmenger, and Moore (1933) reported that clover plants fed urea outyielded those fed either nitrate or ammonium plus nitrate. However, the calcium concentration in their cultures was less than half that of normal-calcium cultures used in the present experiment and this could account for the discrepancy between the two studies.

The cause of the reduced plant vigour of the urea-fed plants in the normal-calcium solutions is not known. The calcium concentration within the plants was similar to that found in those fed ammonium plus nitrate. Thus, it cannot be concluded that the retardation in growth was the result of an excessive calcium uptake, unless the assumption is made that the basal calcium requirement of these urea-fed plants had been greatly reduced. Another possible explanation is that a high plane of calcium nutrition is antagonistic to urea, either by affecting its uptake by the plant roots, or its assimilation into the plant's nitrogen cycle.

The calcium status of the nutrient solutions had no effect upon plant growth when ammonium and nitrate were supplied together. In addition, the presence of these two nitrogen ions in the nutrient solutions appear to have a synergistic effect, as these solutions supported the most vigorously growing plants in the experiment. Hanger (1967) has also reported this synergistic effect of ammonium plus nitrate on parsnips grown in nutrient solution when the pH was stabilized by the addition of either calcium carbonate or calcium hydrogen phosphate. It appears as though the uptake and utilization processes involved with each nitrogen ion are mutually beneficial to each other, especially under a low-calcium nutrition.

V. Acknowledgments

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