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Calcium and Nodulation in Subterranean Clover (Trifolium subterraneum L.)[1]

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Abstract. **From a study of the effects of Ca ions on the nodulation of subterranean clover in flowing culture solutions it is concluded that root infection or nodule initiation has a higher Ca requirement than either nodule development or host plant growth in the presence of fixed nitrogen.**

Increasing Ca concentration from 246 to 720 μM had no effect on growth of the host plant but increased the number of nodules from 7 to 24 per plant. Decreasing Ca concentration from 246 to 4 μM progressively decreased both plant growth and nodule numbers.

It is suggested that nodule infection or initiation required higher solution concentration of Ca than nodule development since transferring plants after 10 days in lower Ca treatments to 720 μM Ca for 7 days did not increase nodule numbers above those on plants treated continuously at low Ca. Similarly transferring plants from 720 μM Ca to lower Ca treatments did not decrease nodule numbers much below those on plants grown continuously at 720 μM Ca even though growth was depressed. Once initiated, nodule development proceeded at concentrations of Ca too low for plant growth.

The high Ca requirement for root infection or nodule initiation was not thought to be due to effects on survival or growth of *Rhizobium* and could not be related to effects of Ca on a number of relevant processes:—tap root length, root hair development, or lateral root initiation.

Ca has been thought for many years to have a specific role in legume nodulation. However, early reports (1) failed to distinguish between the Ca requirements for host plant growth and for nodulation (12). Moreover, a more recent claim that the Ca requirement for nodulation of subterranean clover is greater than that required for growth of the host plant (5) has been challenged (10).

Since rapid depletion of Ca concentrations may accompany growth of plants in standard culture solutions, we have re-examined the effect of Ca concentration in solution on the nodulation and growth of subterranean clover using a flowing culture apparatus (2) in which specified Ca concentrations are maintained constant. Additional treatments were included to distinguish among the various phases of nodulation which might be affected by Ca. In this paper, the convenient grouping of nodulation phases into 3 major stages—root infection, nodule initiation, and nodule development—has been followed (3, 4, 8). The stages of infection and initiation include all phases prior to the appearance of the nodule.

Materials and Methods

Plant Culture. Seeds of Mt. Barker strain of subterranean clover (*Trifolium subterraneum*, L.) were sterilized with 50:50 ethanol: 30 % hydrogen peroxide and washed with de-ionized water. After 24 hours in aerated de-ionized water, germinating seeds were placed on cheesecloth over basal nutrient solution of the same composition as used in the main experiment with the exception that it contained 3 μM $CaSO_4$. The solution was aerated, was maintained at 20° at pH 5.0 to 5.5 and was changed daily. When 7 days old, 12 seedlings were transferred to each of 32 containers in each of 6 culture units of specified Ca concentrations.

Details of design and performance of the nutrient solution units have been published (2). In each unit a total volume of 2800 liters of nutrient solution was continuously recirculated through 32 containers in parallel at a rate of 1300 liters container^{-1} day^{-1}. Filtered air was bubbled through the solution in each pot and the solutions were maintained at 20 ± 2°. The pH was maintained at 5.0 ± 0.3 by titrating twice daily with 0.5 M KOH; 0.25 M K_2SO_4 was added to maintain equal K concentrations in all units.

$CaSO_4$ was added to give the following Ca concentrations in μM: 4 ± 0.2, 9 ± 0.5, 30 ± 2, 81 ± 1, 246 ± 6, 720 ± 10. Ca concentrations

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were determined every third day by atomic absorption spectrometry after addition of Sr to 0.25 % (w/v) to prevent interference from other elements (11) and maintained at the above concentrations throughout the experiment. The basal nutrient concentrations were as follows (μM) :—Mg 100: N (as NH_4^+) 200: N (as NO_3^-) 450: Na 22.5: P 2.5: S 100: Zn 0.475: Mo 0.02: B 3: Cu 0.1: Co 0.045: Mn 0.5: Fe (as sequestrene 138) 3. The initial K level in the basal nutrient solution was 250 μM and the addition of KOH and K_2SO_4 gave a final level of 310 μM. To ensure an adequate supply of basal nutrients one quarter of each tank was drained after 12 days and refilled with fresh solution.

Two days (D2) after transplanting seedlings into treatments, suspensions of *Rhizobium trifolii* strain TA1 were added to give about 4000 viable *Rhizobium* cells per ml of nutrient solution. The plants were thinned to 10 per container on D9. On D10, 4 replicates were transferred from the high Ca level (720 μM) to each of the 5 lower levels and 4 replicates from each of these lower levels were transferred to the high Ca level. Extra containers were maintained at the 5 lower levels of Ca so that the number of plants at each Ca treatment was identical.

Harvesting. At planting (D0) duplicate subsamples of 50 seedlings were divided into tops and roots and weighed fresh after blotting excess water from roots. On D2, 7 plants were selected at random from each Ca level, removed, photographed, and replanted. Transferring seedlings from the seedling solution to the experimental solution resulted in a check in root hair growth which left a zone 0.5 to 1.0 cm before new root hairs appeared. Because of this it was possible to measure, on the photographs, the effect of Ca on the rate of taproot growth as well as root hair length.

The first nodules were visible under the microscope by D7 (microscopic nodules) and to the naked eye by D9 (macroscopic nodules). On D10, 4 replicates were harvested, fresh weights of tops and roots were measured and the roots stored in formalin for root growth assessment. Root hair length at 1 cm from the taproot on the second and fourth laterals was measured using a binocular microscope. The length of taproot, the number of primary laterals, and the number of secondary laterals on the primary laterals were recorded.

On D17 the 4 replicates that had been transplanted and the 4 that had remained were harvested. Fresh weights of tops and roots were obtained and the roots were stored in formalin until nodulation was recorded. Nodules were rated as follows: 1) small white nodules, 2) medium sized nodules, 3) large pink nodules. The distribution of nodules on roots was recorded as those within 2 cm from the base of the taproot and those further than 2 cm.

Results

Effects of Continuous Ca Concentrations. Plants grew vigorously with relative growth rates of 18 to 19 % per day at the 2 highest levels of Ca (246 and 720 μM). At Ca concentrations below 246 μM plant fresh weight decreased (fig 1A) and at the 2 lowest Ca levels (4 and 9 μM) Ca deficiency symptoms similar to those described by Millikan (6) were induced. Ca affected plant growth in a similar way at both harvests.

By contrast with its lack of effect on growth, Ca at 720 μM trebled the number of nodules per plant at 246 μM Ca (fig 1A) although virtually all plants were nodulated at 246 μM Ca (table I). At Ca concentrations below 246 μM nodule numbers

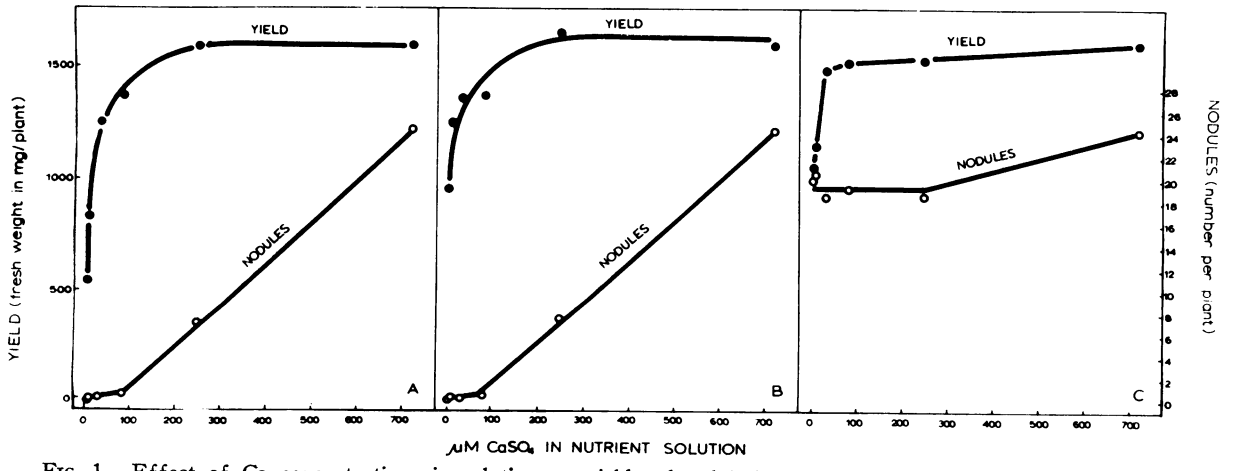

FIG. 1. Effect of Ca concentrations in solution on yield and nodulation of subterranean clover. A) Plants grown at the Ca concentrations shown in the figure continuously for 17 days. B) Plants grown at the Ca concentrations shown in the figure for 10 days then transferred to 720 μM Ca for a further 7 days. C) Plants grown at 720 μM calcium for 10 days then transferred to the lower Ca concentrations shown in the figure for a further 7 days.

Table I. *Effect of Ca Concentration in Solution on Nodulation of Subterranean Clover*

Ca conc (μM)	4	9	30	81	246	720
Percent plants nodulated[3] D17	0[1]	5	12	27	98	100
Mean nodule size D17	...	2.5	2.3	2.3	2.2	2.4
Nodules <2 cm from base of taproot[2] D17	0.0	0.1	0.2	0.6	6.0	11.6
Nodules >2 cm from base of taproot[2] D17	0.0	0.0	0.0	0.0	1 0	12.8

[1] Values not joined by the same horizontal line differ significantly ($P<0.05$) by Duncan's range test.
[2] $\sqrt{n} + 1$ transformation used for analysis.
[3] Arcsin transformation used for analysis.

progressively decreased until at 4 μM Ca there were none. Apart from a few nodules on the distal taproot of plants at 720 μM Ca all nodules occurred on the primary laterals. As well as increasing the number of nodules, 720 μM Ca had a striking effect on increasing the distribution of nodules over the root system. At all lower Ca levels nodules were largely confined to a zone near the base of the root (table I).

Although Ca increased the number of nodules it did not affect the growth of individual nodules (table I).

The striking effects of 720 μM Ca on nodulation of subterranean clover plants compared with 246 μM could not be explained by changes in relevant processes of root development which were measured. It had no effect on the rate of taproot development over the initial 48 hours and little if any effect on taproot length at the first harvest (table II). It also had no effect on the number of secondary laterals, but increased the number of primary laterals to a small extent (table II).

Similarly, increasing Ca from 246 to 720 μM had no effect on length of root hairs on either taproots or primary laterals. However, as the Ca level was decreased below 246 μM root hair length progressively decreased on both taproot and lateral roots (table II). At all Ca levels the root hairs on the taproot were longer than those on the primary laterals. Thus the effect of Ca on root hair length did not parallel its effect on nodulation. Nor was there any observable difference in root hair density at 720 μM compared with 246 μM Ca.

Effect of Transplanting From Lower Concentrations to 720 μM Ca. Transplanting subterranean

Table II. *Effect of Ca Concentration in Solution on Root Development of Subterranean Clover*

Ca conc (μM)		4	9	30	81	246	720
Rate of taproot development (mm/24 hr)	D2	4.2[1]	4.0	5.4	7.4	10.3	10.1
Length taproot (cm/plant)	D10	6.7	6.9	5.8	8.9	12.7	13.6
No. primary laterals per plant	D10	28.0	26 5	25.1	29.1	39.8	47.5
No. primary laterals per cm taproot	D10	4.2	3.9	4.3	3.3	3.2	3.5
No. secondary laterals per plant	D10	553	551	823	822	734	807
Length root hairs on taproot (μ)	D2	...	270	340	360	430	440
Length root hairs on laterals (μ)	D10	51	74	77	120	177	177

[1] Values not joined by the same horizontal line differ significantly ($P<0.05$) by Duncan's range test.

Table III. *Effect of Transplanting From Lower Concentrations of Ca (At D10) to 720 μM Ca on Yield and Nodulation of Subterranean Clover*

	Conc of Ca (μM)										
D0 to D10	4		9		30		81		246		720
D10 to D17	4	720	9	720	30	720	81	720	246	720	720
Percent plants nodulated[2] D17	0[1]	3	5	15	12	10	27	30	98	100	100
Mean nodule size D17	...	2.0	2.5	2.2	2.3	2.5	2.3	2.2	2.2	2.4	2.4

[1] Values under each initial (D0-D10) Ca level not joined by the same horizontal line differ significantly ($P<0.05$) by Duncan's range test.
[2] Arcsin transformation used for analysis.

clover plants after 10 days' treatment at lower Ca concentrations to 720 μM Ca did not increase nodule numbers per plant (fig 1B) or number of plants nodulated (table III) in a subsequent 7 day period. Since the first nodules became visible in continuous 720 μM Ca on the seventh day after innoculation, this subsequent 7 day period should have been long enough to have permitted any nodules initiated at the time of transfer (D10) to have developed macroscopic nodules by final harvest (D17). Hence the result indicates that the Ca-sensitive process is one of root infection or nodule initiation rather than nodule development. Further support for an effect of Ca on infection or initiation rather than development is seen in the absence of any effect of transplanting on nodule size (table III). It is noteworthy that this same treatment produced sharp responses in the fresh weights of plants transferred from the 3 lowest Ca treatments (fig 1B).

Effect of Transplanting From 720 μM Ca to Lower Concentrations. The effects on nodulation of transplanting young plants from 720 μM to lower Ca concentrations confirms the conclusion that root infection or nodule initiation rather than nodule development is a Ca sensitive process. Transplanting had no effect on the number of plants nodulated (98–100 %) or the size of each nodule (2.3–2.4) and little effect on the number of nodules per plant (fig 1C). The slightly lower number of nodules on these plants requires explanation since evidence above indicates that the period after transfer is too short for development of nodules *de novo* in 720 μM Ca. Therefore it is suggested that the critical Ca-sensitive stage may not be completed for 1 or 2 days after the first stage of infection and that the small proportion of nodules which had not completed this Ca-sensitive stage at time of transplanting from 720 μM to lower Ca concentrations would not develop to completion within 7 days as they did if left in 720 μM Ca for the whole period. Since transplanting to the lowest Ca concentrations sharply decreased fresh weight of plants (fig 1C) it appears that once initiated at high Ca, nodule development is even less sensitive to low Ca levels than is plant growth.

Discussion

The results presented in this paper confirm previous reports (5) that nodulation requires a higher Ca concentration in the environment than host plant growth when fixed nitrogen is supplied. The results are also in agreement with those of White (10) for subterranean clover at pH 5.0. However, White (10) has emphasized the parallel effect of Ca on both nodulation and host plant growth at pH 6.0. The reason for his failure to show any discriminatory effect at pH 6.0 probably lies in the small range of Ca levels used.

The present work indicates that infection or initiation phases of nodulation are particularly sensitive to Ca. Once the nodule is initiated development proceeds unhindered even by concentrations of Ca too low for plant growth. How root infection or initiation is increased by Ca is not apparent. However, a suggested causal relationship between root hair development and nodulation (10) is rejected since in the present work the greatly increased nodule number at 720 μM compared with 246 μM Ca was not accompanied by any increase in root hair length or density. Moreover, at the lower Ca concentrations where effects on root hair development paralleled nodulation, plant growth was also affected. Nor was increasing Ca at these levels accompanied by any increase in secondary lateral root initiation as would be expected if the Ca-sensitive process was related to the number of foci for nodule initiation present, on the assumption that both nodule and lateral root initiations share common foci (8).

The larger number of nodules per plant at 720 μM compared with 246 μM Ca was accompanied by a change in the distribution of nodules, and it was only at 720 μM Ca that nodules were distributed widely over the root system. At 246 μM Ca they were largely restricted to a zone near the base of the root. As microscopic nodules were spread widely over the roots 5 days after inoculation at 720 μM Ca, infection must have occurred shortly after inoculation. Therefore the greater number of nodules at 720 μM Ca can not be attributed to a longer period of infection due to enhanced *Rhizobium* survival. Contamination prevented the determination of *Rhizobium* numbers by direct counts but no differences in *Rhizobium* survival or multiplication would be expected as their Ca requirement is small (7, 9) and less than that for plant growth (5).

Apart from the indication that *Rhizobium* numbers are probably not involved, the present experiment does not distinguish which phase in the complex series of interactions between *Rhizobium* and the host plant (8) in nodule initiation is specifically affected by Ca.

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