EFFECT OF CALCIUM LEVEL IN THE SUBSTRATE ON THE DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.)

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Summary

Calcium level in the substrate was found to have a very marked effect on the distribution of ^{45}Ca in subterranean clover. Sites of initial accumulation of ^{45}Ca included the vein endings along the distal margins of the leaflets, the lateral veins, and the proximal halves of the petioles.

In plants with a normal calcium supply these sites were quickly saturated and the isotope became uniformly distributed in the lamina, which contained a higher concentration than its associated petiole. There was a positive correlation between leaf age and ^{45}Ca concentration. In the petiole a somewhat higher concentration of ^{45}Ca finally occurred in the distal when compared with the proximal half.

Calcium-deficient plants differed in that, with the exception of the vein endings, the initial sites of accumulation of ^{45}Ca were not rapidly saturated. In the lamina ^{45}Ca moved into the interveinal tissues adjacent to the vein endings. Provided acute calcium-deficiency symptoms did not occur, there was finally a build-up of ^{45}Ca in the distal half of the petiole. The concentration in the petiole was greater than in the lamina. Before runner-stem formation began there was not the same correlation in deficient plants between leaf age and ^{45}Ca concentration as in normal plants, indicating that much of the newly absorbed ^{45}Ca was acquired by young leaves of deficient plants at the expense of the oldest. This resulted in petiole collapse in old leaves due to the lack of build-up of calcium concentration in the distal halves of the petioles. The development of marginal necrosis of youngest leaves due to calcium deficiency was associated with a further disorganization of ^{45}Ca distribution. ^{45}Ca accumulated in epidermal hairs and flowers.

Translocation of ^{45}Ca into new leaves and flowers produced up to 9 weeks after transfer from radioactive low-calcium solutions to non-radioactive normal- or low-calcium solutions was demonstrated. This mobile calcium came mainly from root tissue.

Cold water, hot water, hot 0·5N HCl, and hot 0·5% ammonium oxalate extracts were made of parts of leaves of different maturities from normal- and low-calcium plants sampled at two stages of growth, the first before and the second after the onset of petiole collapse in the low-calcium plants. For all leaf parts, the cold water plus hot HCl extracts contained over 90% of the total ^{45}Ca activity. For plants under normal calcium nutrition, the ratio of ^{45}Ca soluble in cold water to that soluble in hot HCl was greater than 1, the ratio increasing with leaf age for all plant parts except the distal halves of the petioles. For plants under low-calcium nutrition, before the onset of deficiency symptoms, the above ratio, although lower in value, followed similar trends with leaf age as found in the normal-calcium tissues. After the onset of petiole collapse the ratio fell sharply to less than 1.

I. INTRODUCTION

Various workers have reported the results of experiments with ^{45}Ca which have established typical distribution patterns for this element in a number of plant

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species (Mallon and Urey 1946; Ririe and Toth 1952; Biddulph *et al.* 1958; Biddulph, Cory, and Biddulph 1959; and Rinne and Langston 1960). These experiments have been made with plants growing in solutions containing a normal supply of calcium and it has been concluded (e.g. Langston 1956) that the patterns bore a marked similarity to known deficiency symptoms.

However, it is known that subterranean clover (*Trifolium subterraneum* L.) may show either of two distinct symptoms of calcium deficiency, apparently depending on the rate of growth of the plants. These consist of a marginal necrosis of the youngest leaves, or a collapse of the distal portions of petioles (Millikan 1953). This latter symptom normally does not affect the youngest leaves and may even first affect the oldest leaf on the plant. This suggests that calcium distribution in plants growing under conditions of calcium deficiency may be different to that in plants receiving a sufficiency of this element. For this reason the experiments described herein were made. The possibility that calcium in calcium-deficient subterranean clover plants may be translocated into younger leaves was also investigated.

II. Methods

The plants used in these experiments were grown in water cultures. The composition of the complete nutrient solution was identical to that described by Millikan (1961). In calcium-deficient solutions calcium nitrate was replaced by an equivalent amount of sodium nitrate. However, to prevent premature death of plants growing in the calcium-deficient solutions two drops of 1M calcium nitrate solution were added as required.

Plastic pots (capacity 2 litres) painted on the outside with black paint followed by aluminium paint were used. To prevent them becoming radioactive, the pots were each lined on the inside with a thin polyethylene bag which could be disposed of after use.

The radioactive doses were dispensed into the solutions by means of micro-pipettes. Unless otherwise stated below, each dose consisted of a volume equivalent to 5 μc of original ^{45}Ca activity as $CaCl_2$ and containing 0·047 mg calcium. As the dose of ^{45}Ca given to the complete solution and calcium-deficient cultures was the same, the resultant specific activity of the available calcium supply to the plants was greatest in the deficient cultures. This is reflected in a greater radioactivity recorded in the calcium-deficient as compared with the complete-solution plants.

The distribution of the radioisotope in plant samples from the various experiments described below was determined by means of radioautographs and radioassays.

For radioautography, the plant samples, immediately after harvesting, were arranged on a sheet of blotting-paper of the same size as the film to be used, covered with another sheet of blotting-paper, and pressed lightly for approximately 30 sec. The plants were then transferred to a fresh sheet of blotting-paper. This was placed on a sheet of aluminium foil 0·001 in. thick and a similar sheet of foil was laid on top of the plant specimen and smoothed out to fit closely.

In the dark room a sheet of Kodirex No-screen X-ray film was placed on the upper sheet of aluminium foil and the whole lot was put between two sheets of $\frac{1}{2}$-in.

thick foam plastic, on the outsides of which were sheets of 10-gauge galvanized iron. The various layers were kept in position by several thick rubber bands and were then wrapped in a black plastic sheet to exclude light.

The package was next placed under weights in a cold room maintained at 30°F for the required exposure time, after which it was re-opened in the dark room and the X-ray film developed.

To determine whether the pressing of the specimens produced an artefact in the radioautograph of the calcium, selected leaves were harvested from Dwalganup and Mt. Barker subterranean clover plants and the three individual leaflets of each leaf were separated. They were then treated as follows: one leaflet was radioautographed without pressing; the second leaflet was first pressed and the third leaflet was dried between blotting-paper in a forced-air oven before being radioautographed. The results showed that neither pressing or drying affected the distribution of ^{45}Ca in the leaflet.

After being radioautographed the plant specimen was photographed. Before this could be done it was often necessary to rearrange the plant parts on the blotting-paper as they usually adhered to the aluminium foil covering them. Thus slight differences between the relative positions of plant parts in the photograph and in the corresponding radioautograph were sometimes apparent but this was not considered to be vital.

The radioautograph was then used to select portions of the plant for radioassay, the actual plant parts selected being recorded on a photograph of the specimen. The selected parts were cut up finely into aluminium planchets which were then placed in an oven and held at 105°C for 24 hr.

The samples were next radioassayed by means of an EHM 2/S Geiger tube (with an end-window thickness of 1·9 mg/cm^2) and associated scaling equipment. Counting time was normally 300 sec but this was increased to 1000 sec for samples with very low activity.

After counting, the samples were returned to the oven for 24 hr. The dry weight of each was then obtained to the nearest 0·01 mg. The radioactivity of the sample was finally expressed as the number of counts per minute per milligram of dry matter. All activities were corrected for decay of the isotope to the time of assay and were thus based on the original activity of the dose at the time of dispatch from the Australian Atomic Energy Research Establishment, Lucas Heights, N.S.W. These corrected activities were recorded on a print of the photograph or radioautograph of the specimen concerned. Details of the experiments made are given below.

(*a*) *Experiment* 1

Three pots of complete and of calcium-deficient solutions respectively were set up and two seedlings each of the Dwalganup and Mt. Barker varieties of subterranean clover were transferred to each pot 7 days after emergence in washed and steam-sterilized sand. The next day each pot was given a dose of 5 μc ^{45}Ca, and plants were harvested for radioactivity and radioassay 39 days later. This experiment was commenced on November 2, 1962.

(*b*) *Experiment* 2

Two pots of complete and of calcium-deficient solutions respectively were set up on November 2, 1962, and six seedlings of the Dwalganup variety of subterranean clover were established in each. After 5 weeks the solutions in all pots were renewed and each then received a dose of 5 μc of ^{45}Ca. This time was regarded as day 0, and plants were removed for radioautography and radioassay 7 and 27 days later.

(*c*) *Experiment* 3

This experiment, which was commenced on April 22, 1963, in an unheated glasshouse, was designed to determine:

(1) whether the slower rate of growth promoted by late autumn and winter conditions (Millikan 1961) was associated with a difference in calcium distribution when compared with the results of experiment 1 which was made during late spring and summer; and

(2) if any translocation of calcium could be demonstrated in subterranean clover.

Four pots each of Dwalganup and Mt. Barker varieties of subterranean clover (six plants per pot) were set up with normal and calcium-deficient solutions respectively. Two days later two pots of each variety at each calcium level were given the equivalent of an original 5 μc of ^{45}Ca. After growing in the radioactive solutions for 7 days all these plants were transferred to non-radioactive cultures of comparable calcium level. These pots were designated series A.

At the same time the non-radioactive plants of each variety in the other two pots at each calcium level were transferred to solutions of comparable calcium level but with the equivalent of an original 5 μc of ^{45}Ca added. These pots were designated series B. A full set of nutrients other than calcium was added to these pots on day 36.

Plants from each calcium level were removed for radioautography and radioassay at the following times after these transfers:

Series A: 2, 7, 15, 21, and 36 days.
Series B: 2, 7, 15, 36, and 73 days.

Samples were also taken at days 21, 28, and 50 from series B, but the results obtained from these are not presented in detail in this paper as all essential information is provided by the samples obtained on the five dates listed above.

On day 73 the remaining calcium-deficient plants of both varieties growing in the radioactive solutions in the series B pots were transferred to freshly prepared non-radioactive solutions which contained either complete nutrients or were deficient in calcium (each pot received only three drops of 1M calcium nitrate solution. These plants were designated series C.

Sections of roots or individual runner stems of the plants or both were harvested for radioautography and radioassay on days 16, 29, 37, 47, 55, and 64 after transfer to the non-radioactive solutions.

(*d*) *Experiment* 4

The object of this experiment was to determine the effect of calcium level in the substrate on the relative distribution of ^{45}Ca between certain constituents of different plant tissues. Two calcium levels were employed, i.e. complete solution and calcium deficiency. The pots containing the latter solution each received two drops of 1M calcium nitrate to prevent the premature death of the plants. Each calcium treatment was replicated six times and 12 seedlings of subterranean clover, cv. Dwalganup, were established in each pot.

On day 5, after the commencement of the experiment, 5 μc of ^{45}Ca was added to each pot containing complete solution, and 2·5 μc to each pot of calcium-deficient solution. The first sampling of plants was made on day 39 at which time no calcium-deficiency symptoms had developed in the plants in the low-calcium solutions.

Symptoms of petiole collapse first appeared in the plants growing in these solutions on day 46, and were severe when the second sampling was made on day 53. At each time of sampling one plant per treatment was taken for radioautography and radioassay and a further two plants per pot (12 per treatment) for extraction. The latter plant samples were subdivided as follows:

Sample 1 (day 39): Normal- and low-calcium plants—growing points; youngest fully expanded leaves; oldest leaves.

Sample 2 (day 53): Low-calcium plants—young fully expanded leaves without petiole collapse; young fully expanded leaves with petiole collapse; middle leaves with red lamina (Millikan 1953) and petiole collapse.
Normal-calcium plants—young fully expanded leaves; middle leaves.

Each of the above samples except the growing points in sample 1 was further subdivided into: distal leaf edge; remainder of lamina; proximal and distal halves of petioles.

Each of these subsamples was cut up finely and an aliquot of 200 mg fresh weight was added to a Thomas hand-homogenizer in which the tissue was thoroughly disrupted. This material was then transferred to a sintered-glass filter (porosity No. 2) by repeated washing with cold water. The residue was then thoroughly leached in succession with hot water, hot 0·05N HCl, and hot 0·5% ammonium oxalate according to the method described by Ordin, Cleland, and Bonner (1957). The leachate in each case was collected in a 50-ml beaker, evaporated to near dryness, and then transferred to aluminium planchets for final drying before radioassaying. The final residue was also transferred from the sintered-glass filter into a planchet for drying and radioassay of its ^{45}Ca content.

III. Results

(*a*) *Experiment* 1

Symptoms of sudden petiole collapse, which were identical in appearance with those of calcium deficiency described by Millikan (1953), first occurred in the calcium-deficient cultures on day 27 after the administration of the ^{45}Ca.

A selection of radioautographs and radioassays of plants sampled on day 39 are presented in Plate 1, Figures 1–4. These are typical of those of other plants subjected to comparable treatments which were also sampled at this time.

There were no important differences apparent between the results obtained with the Dwalganup and Mt. Barker varieties.

The results show that in the plants grown in the complete solution, the distribution of ^{45}Ca in the interveinal tissues of the oldest leaves was relatively uniform and comparable in concentration to that in the lateral veins. However, in progressively younger leaves, the concentration of the radioisotope in the veins relative to the interveinal tissues increased, although the overall activity of the lamina decreased. The lamina of the leaf normally contained a higher mean concentration of ^{45}Ca than its associated petiole. There was a small variation in ^{45}Ca concentration along the length of individual petioles, that in the proximal half usually being the highest.

In plants grown in calcium-deficient solutions, the relative differences in ^{45}Ca concentration between various tissues was markedly different to that described above. Thus, there was no decrease in the mean activity of young as compared with old leaves. The laminae of leaves with collapsed petioles usually were less radioactive than those of younger leaves on the same runner stem. In the younger leaves the ^{45}Ca was concentrated to a very marked degree in the veins, particularly in the endings around the margins of the leaflets, but there was evidence of a build-up of ^{45}Ca concentration in the interveinal tissues of the oldest leaves.

By comparison with the complete-solution plants, there was a much greater relative difference in concentration of ^{45}Ca between the proximal half of the petiole and its distal end. There were exceptions to this in the case of the petioles of the oldest leaves of the two varieties, in which calcium accumulation in the laminae had commenced, or evidently was about to do so. The collapsed portion of a petiole had a much lower activity than either the proximal portion of the same petiole or the associated lamina. The lamina often had a lower overall concentration of ^{45}Ca than the proximal portion of its petiole.

Where epidermal hairs occurred on leaves they showed a marked accumulation of ^{45}Ca, irrespective of the calcium level in the solution (Plate 9, Figs. 2 and 3).

(*b*) *Experiment* 2

On the day the ^{45}Ca doses were added to the culture solutions (designated day 0), the 5-week-old plants in the complete solution had initiated up to 10 runner stems, the oldest being approximately 5 in. long. The calcium-deficient plants had up to eight runner stems but little or no elongation of these had occurred, and the petioles of many leaves showed the typical calcium-deficiency collapse of the distal portion (Millikan 1953). Petiole collapse also occurred after the transfer to the radioactive solutions. The results of radioautographs and radioassays made on plants taken from these cultures on days 7 and 27 are shown in Plates 2 and 3.

On day 7 there was a relatively even distribution of the recently absorbed ^{45}Ca throughout the plants grown in the complete solution, although in the oldest leaves the petioles were somewhat more reactive than the laminae as a whole. However,

the most reactive tissues in the laminae of these leaves were the midribs, whereas in the youngest leaves they were the lateral veins (Plate 2, Figs. 1 and 3). Radioautographs and radioassays of the remaining five runner stems of this plant were made. These are not presented in this paper but they revealed an identical ^{45}Ca distribution to that described above.

On day 27 the runner stems of the plants growing in the complete solution were up to 12 in. long and the plants were flowering. A comparison of the results of the radioassays of plants sampled on days 7 and 27 shows that there had been a build-up in ^{45}Ca level in the leaves between these dates (Plate 2, Fig. 1; Plate 3, Fig. 1).

At day 27 there was also a progressive increase in the concentration of ^{45}Ca in the lamina with increasing age of the leaf (Plate 3, Figs. 1 and 3). The lamina was more reactive than its associated petiole and in the case of the oldest leaves the radioautograph shows that the distal halves of the petioles were more reactive than the proximal halves. Also, in the oldest leaves the ^{45}Ca was evenly distributed in the interveinal tissue which was more radioactive than the lateral veins, whereas these veins were the most reactive tissues in the younger laminae of the plant. There was also a notable concentration of ^{45}Ca in the basal tissue of each flower (Plate 9, Fig. 1).

In the calcium-deficient plants sampled on day 7 a marked concentration of the recently acquired ^{45}Ca occurred in the crown tissues and the bases of the petioles, particularly of the middle, and to a lesser extent, of the youngest leaves. The petioles of the oldest leaves were much less radioactive, especially of those leaves where petiole collapse had occurred. The laminae of all leaves were relatively low in ^{45}Ca, the greatest concentrations being in the midribs. There was no ^{45}Ca detected in the laminae of leaves whose petioles had collapsed before the transfer to the solution containing ^{45}Ca (Plate 2, Figs. 2 and 4).

The build-up of ^{45}Ca concentration in the petioles of the calcium-deficient plants continued between days 7 and 27 as is shown by the higher values for radioassays recorded in plants sampled on the latter date. The difference in radioactivity between young and old petioles and between the proximal and distal portions of the same petiole were less marked than on day 7 (Plate 2, Figs. 2 and 4; Plate 3, Figs. 2 and 4). However, leaves with collapsed petioles were found to have a very low concentration (where the collapse had occurred subsequent to transfer to the radioactive solution, i.e. in left-hand runner in Plate 3, Fig. 4), or to again be devoid of the isotope in the collapsed tissues.

By contrast with the plants grown in the complete solution, the laminae of the deficient plants were much less radioactive than their associated petioles, and irrespective of leaf age, the midrib was the most reactive, and the outer margin the least reactive, tissue of the lamina. Also, there was no consistent correlation between leaf age and ^{45}Ca concentration in the lamina of the deficient plants.

Although most of the recently acquired calcium had been retained by the petioles, there was evidence on day 27 that a limited amount had moved into the interveinal tissues of the laminae of leaves whose petioles had not collapsed. In

such cases there was a marked tendency for the distal margin to be lower in this new ^{45}Ca than the remainder of the lamina.

(c) *Experiment* 3

Due to the prevailing winter conditions, the general rate of growth of the plants in experiment 3 was much slower than that in experiments 1 and 2.

(i) *Series A*

After 7 days in radioactive solutions all plants at the time of transfer to non-radioactive solutions had a unifoliate and one partly expanded trifoliate leaf. To the time the experiment was terminated on day 36, no apparent difference in growth occurred between the normal- and low-calcium plants and no leaf symptoms of calcium deficiency were observed in the latter.

The radioautographs and radioassays made on plants removed from the cultures on days 2, 7, 15, 21, and 36 respectively are presented in Plates 4 and 5. Radioautographs of the normal-calcium plants sampled on day 36 are not included as they showed no difference in ^{45}Ca distribution from those sampled on day 21.

In the plants grown at the normal calcium level there was a rapid movement of ^{45}Ca out of the roots and hypocotyl, with the result that by day 7 their content of the isotope was very low. By day 2 the ^{45}Ca had entered into the cotyledons where it was higher in concentration than in the associated petioles. In contrast with the result described below for the low-calcium plants, no subsequent change in relative concentrations of ^{45}Ca in these tissues was recorded with time. Also by day 2 the ^{45}Ca in both the unifoliate and first trifoliate leaves was mainly located in the veins with the highest concentration in their endings along the distal margins. It became evident that the first calcium to enter these leaves was fixed in these sites, as this pattern of distribution did not change with time. A limited movement of ^{45}Ca into the vein endings of the second trifoliate leaf of the normal-calcium plant occurred, as indicated by the faint images of this leaf in the radioautographs of the 15- and 21-day samples (Plate 4, Figs. 5 and 6; Plate 5, Figs. 1 and 2). Although the second trifoliate leaf had not appeared when the plants were transferred to the non-radioactive solutions, it seems probable that the small amount of ^{45}Ca revealed in these radioautographs, had either already entered into the embryo leaf before this transfer, or had moved from the root and hypocotyl shortly afterwards. No ^{45}Ca was detected in the third or subsequently formed trifoliate leaves of the 15-, 21-, and 36-day samples.

In the series of plants sampled from the low-calcium cultures, ^{45}Ca activity in the roots was recorded up to day 7 for Dwalganup and day 21 for Mt. Barker varieties respectively. However, the radioautographs in Plates 4 and 5 reveal that considerably greater ^{45}Ca activity was initially located in the hypocotyl of each variety, but with time the ^{45}Ca content of this tissue relative to other parts of the plant diminished, although a limited amount of the isotope was still present in the hypocotyls of calcium-deficient plants sampled on day 36. This gradual movement of ^{45}Ca out of the hypocotyl coincided with the appearance of the isotope in successive trifoliate leaves as described below.

There is also evidence of movement of ^{45}Ca into the cotyledons after transference of the plants to the non-radioactive low-calcium solution. On day 2 the cotyledons of each variety contained considerably lower concentrations of ^{45}Ca than their petioles but by day 15 this was reversed. In the interim the concentrations of ^{45}Ca in the cotyledons had increased while that in the associated petioles had decreased.

There was a similar movement of ^{45}Ca into the lamina of the unifoliate leaf of the variety Mt. Barker, but not Dwalganup, subsequent to the transfer to the non-radioactive solution. Up to day 7 but not later, the petiole of this leaf contained a higher concentration of ^{45}Ca than the lamina. The ^{45}Ca concentration in the lamina increased to day 21 while that of the petiole increased to day 7 and then decreased.

At first, in the unifoliate leaves of both varieties, the highest concentration of ^{45}Ca in the lamina occurred in the veins, and in the vein endings in particular, but later the interveinal tissues of this leaf also acquired a relatively high concentration of ^{45}Ca. This leaf differed in this latter respect from the trifoliate leaves. The radioautographs and radioassays of the trifoliate leaves of the low-calcium plants shown in Plates 4 and 5 are of particular interest as they clearly show that up to day 36 ^{45}Ca in limited amount had been translocated into at least the first six of these leaves produced by each variety. This is in sharp contrast to the result obtained with plants grown with a normal calcium level. However, the distribution of the isotope in individual leaves of the calcium-deficient plants was similar to that described above for the normal-calcium plants, i.e. greatest concentration occurred in the vein endings with a lesser concentration in the remainder of the veins. Irrespective of relative age of the leaves, this pattern of distribution did not change with time.

(ii) *Series B*

At the time of transfer to solutions containing ^{45}Ca, all plants in this series had a unifoliate and one partly expanded trifoliate leaf. The plants in the normal and low-calcium solutions respectively made comparable growth until day 33 when the latter showed first symptoms of calcium deficiency in the form of a necrosis along the distal edges of the youngest leaves, similar in appearance to that depicted by Millikan (1953). This symptom occurred first in the Mt. Barker variety. Later, successive young leaves of both varieties failed to enlarge, and developed a necrosis which involved the whole of the lamina and extended into the distal end of the petiole. No petiole collapse as described above for experiments 1 and 2 occurred in the series B plants of experiment 3. The results of radioautographs and radioassays made on these plants are presented in Plates 6 and 7.

Although visual symptoms of calcium deficiency did not appear until day 33, it is clear from these results that, when compared with plants grown in normal calcium solutions, a low calcium level in the substrate affected ^{45}Ca distribution in the plant as early as day 2.

The day 2 samples from series A (Plate 4, Figs. 1 and 2) established the location of the calcium to enter the cotyledons and the unifoliate and first trifoliate leaves during the first 7 days of growth, while those from series B (Plate 6, Fig. 1) show that ^{45}Ca acquired during the next 2 days was distributed differently. In plants from both

the normal and low-calcium cultures this new ^{45}Ca moved principally towards the trifoliate leaf. In the normal-calcium plants it occurred in lower concentration in the petiole than in the lamina. In the lamina it was no longer selectively accumulated in the vein endings and also showed evidence of movement into the interveinal tissues. The pattern of distribution in the low-calcium plants differed in that the petiole had a higher concentration of ^{45}Ca than the lamina, and in the latter the midrib and veins of Dwalganup, and the vein endings in the Mt. Barker plants, were much more reactive than the interveinal tissues. From this stage, differences in calcium distribution in plants grown in normal and low-calcium substrates became marked.

In the normal-calcium plants a build-up in ^{45}Ca concentration in all tissues with time was recorded. The isotope continued to move into the cotyledons and unifoliate leaf up to day 36 when counts on these tissues were discontinued. From day 15 ^{45}Ca was higher in concentration in the lamina than in the associated petiole. In the latter it was relatively uniform along its length when compared with petioles of the low-calcium plants. With the trifoliate leaves the distribution of ^{45}Ca was similar to that in the normal-calcium plants of experiment 1 described above. A direct relationship between age of leaf and concentration of ^{45}Ca in the tissues was established from day 7. In each new leaf the isotope first occurred in greatest concentration in the veins, but it soon moved into the interveinal tissues. At this stage its concentration in the edge and the centre of the lamina was very uniform as shown by the radioassays of leaf edge and centre at days 36 and 73 presented in Plate 6, Figure 4, and Plate 7, Figures 1 and 3. From day 7 the midribs and trifoliate leaves of the normal-calcium plants were found to be low in ^{45}Ca, this being a feature whereby they differed notably from comparable leaves of low-calcium plants.

In the low-calcium plants there was a continual build-up of ^{45}Ca in the cotyledons and unifoliate leaf up to day 36, but in contrast with the normal plants the concentration was higher in the petiole than in the lamina (Plate 6). The trifoliate leaves of the low-calcium plants were initially also characterized by high concentrations of ^{45}Ca in the petiole when compared with the lamina of the same leaf. By day 15 this high concentration of ^{45}Ca was located in the proximal half of the petiole, and was in most cases considerably higher than that in the lamina (Plate 6, Fig. 3). By day 73, however, these oldest leaves of the plants all had a high ^{45}Ca concentration along the length of their petioles with the highest concentration in the distal end in many instances. With the Dwalganup variety, the lamina of these leaves also usually contained a higher concentration than the petiole (Plate 7, Figs. 2 and 4).

As early as day 7 the mean concentration of ^{45}Ca in the laminae of the trifoliate leaves of the low-calcium plants did not show the same degree of direct correlation with leaf age as in the normal-calcium plants and by day 15 there was actually a negative relationship between leaf age and ^{45}Ca concentration for the three oldest leaves of the Mt. Barker plant (Plate 6, Fig. 3).

In the lamina of the low-calcium plants, ^{45}Ca occurred first in high concentration in the vein endings, the lateral veins, and the midribs (Plate 6, Fig. 2). By day 15 there was evidence of movement of the isotope into the interveinal tissues adjacent to the vein endings (Plate 6, Fig. 3). This resulted in the characteristic marginal

accumulation of ^{45}Ca as shown in the 36- and 73-day samples (Plate 6, Fig. 5; Plate 7, Fig. 2).

The onset of marginal necrotic symptoms due to calcium deficiency disorganized the pattern of ^{45}Ca distribution in low-calcium leaves as described above. These symptoms first appeared on day 33 and the 36-day samples exhibited marginal necrosis in a young leaf of each variety as indicated in Plate 6, Figure 5. The occurrence of these symptoms in the youngest leaf was foreshadowed by the disruption of calcium distribution in the vascular tissues of the immediately preceding leaf. In the case of Mt. Barker, this leaf still showed the characteristic accumulation of ^{45}Ca in the vein endings, but the isotope was abnormally low in the rest of the vascular system when compared with the interveinal tissues. In the next youngest leaf in which the necrotic symptoms occurred, the disorganization of ^{45}Ca distribution was more marked, as there was virtually no accumulation of the isotope in the vein endings or lateral veins, although the midribs showed evidence of its presence. There was also very little ^{45}Ca accumulated in the proximal halves of the petioles of the affected leaves.

The Dwalganup leaf produced immediately prior to the affected leaf and also the latter, both showed a similar paucity of ^{45}Ca throughout the lateral veins including their endings and in the proximal portion of the petiole although, like Mt. Barker, they too showed some ^{45}Ca in the midrib. The disorganization in ^{45}Ca distribution associated with the onset of marginal leaf necrosis due to calcium deficiency is shown in Plate 8.

The plants from the low-calcium cultures of both varieties sampled on day 73 were showing very acute symptoms of calcium deficiency in their youngest leaves. The laminae of these leaves were found to be very low in ^{45}Ca (Plate 7, Figs. 2 and 4). The first affected leaves showed evidence of a much higher concentration in the proximal part of their petioles than in the laminae, but in the youngest affected leaves there was little evidence of such a difference. These severely affected leaves also showed a disruption in ^{45}Ca distribution similar to that which occurred in necrotic leaves on day 36 as described above.

(iii) *Series C*

At the time of transfer from the radioactive low-calcium solutions to non-radioactive solutions containing either a normal or low level of calcium, the plants were affected by very acute symptoms of calcium deficiency in the youngest leaves as depicted by Millikan (1953). They were typical of those plants the radioautographs of which are shown in Plate 7, Figures 2 and 4. The plants transferred to the normal solutions showed first signs of healthy new leaves after 7 days. By day 16 most runner stems had produced at least one new leaf. These first leaves remained relatively small in size, but leaves subsequently produced were larger and elongation of runner stems occurred.

Rate of growth was slow, however, as this experiment was made during the winter months. A striking feature of the plants transferred to the complete solution was that by day 29 all the leaves which were in existence at the time of the transfer had died. This was in marked contrast to the plants transferred to non-radioactive low-calcium nutrient solutions where the existing leaves did not die, although

many of them finally developed the petiole collapse characteristic of calcium deficiency. Before this collapse occurred, the small amount of calcium added to the solution caused the development of a limited number of new leaves and short runner stems in the Dwalganup, but not in the Mt. Barker variety. Most of these new leaves were ultimately affected either by marginal necrosis or petiole collapse.

Although root samples or individual runner stems or both were sampled for radioautography and radioassay on days 16, 29, 37, 47, 55, and 64 after transfer to the non-radioactive solution, only the results obtained from the samples on days 16, 29, and 64 are presented in Plate 10, Figures 1–4, as they indicate the relevant information to be gained from this experiment. The results from the 37-, 47-, and 55-day samples were in accord with the results described hereunder. The original ^{45}Ca contents in the leaves existing at the time of the transfer to the non-radioactive solutions would be comparable with those shown in the 73-day samples of series B (Plate 7, Figs. 2 and 4).

TABLE 1

EXPERIMENT 3, SERIES C: CHANGE IN CONCENTRATION OF ^{45}Ca IN ROOTS OF SUBTERRANEAN CLOVER AFTER TRANSFER FROM RADIOACTIVE LOW-CALCIUM SOLUTIONS TO NON-RADIOACTIVE SOLUTIONS CONTAINING EITHER NORMAL OR LOW LEVELS OF CALCIUM

Variety	Calcium Level	^{45}Ca in Roots (counts/min/mg dry matter)			
		At Transfer	Day 29	Day 55	Day 64
Dwalganup	Normal	65	8	5	3
	Low		—	8	3
Mt. Barker	Normal	62	5	4	5
	Low		—	7	—

Results of radioassays of root samples taken at the time of the transfer are presented in Table I. Before the death by day 29 of the original leaves of the plants transferred to the complete solutions, there was no evidence of any withdrawal of ^{45}Ca from their laminae as these contained levels of ^{45}Ca comparable to those shown in Plate 7, Figures 2 and 4, at each of the six times of sampling listed above. However, there had evidently been some movement of the isotope out of the petioles of these original leaves. Compared with the ^{45}Ca contents of comparable leaves sampled at the time of the transfer (Plate 7, Figs. 2 and 4) the petioles of the original leaves of the Dwalganup plants (Plate 10, Figs. 1, 2, and 3) generally contained lower absolute concentrations of ^{45}Ca and higher lamina/petiole ratios for ^{45}Ca concentration.

A similar change was indicated in some but not all of the original leaves of the Mt. Barker variety. This apparent withdrawl of ^{45}Ca from the petioles could be a cause of the death of these original leaves.

In the case of the plants transferred to non-radioactive low-calcium solutions, there appeared to be no significant change in ^{45}Ca distribution in the original leaves

(Plate 10, Figs. 1, 2, and 4) which remained alive for several weeks after those of the complete-solution plants had died. As shown in Table 1 there was an appreciable drop in ^{45}Ca concentration in the roots of the plants of both varieties in the first 29 days after transfer to non-radioactive normal or low-calcium solutions respectively. In the normal-calcium solutions there was a further slight fall in ^{45}Ca concentration in the roots of the Dwalganup but not of the Mt. Barker variety, between days 29 and 64 after the transfer.

There was definite evidence of movement of ^{45}Ca into new tissue formed by either variety after the transfer to normal solutions or to low-calcium solutions in the case of the Dwalganup variety (Plate 10).

The concentrations of ^{45}Ca in the laminae of the first of the new leaves on individual runner stems produced by the complete-solution plants increased between days 16 and 29, and finally were much higher than in the original youngest necrotic leaves on day 0 (Plate 7, Figs. 2 and 4) or on day 29 (Plate 10, Fig. 2).

Plants sampled on day 16 after transfer to low-calcium non-radioactive solutions had not produced any new growth and showed a similar ^{45}Ca distribution in the tops to that of plants sampled on day 0 (Plate 7, Figs. 2 and 4; Plate 10, Fig. 1). This pattern of distribution remained unchanged in the Mt. Barker plants which produced no new growth, but in the Dwalganup plants there was evidence of movement of ^{45}Ca into new growth, as shown by the radioautograph and radioassays of the plant sampled on day 64 (Plate 10, Fig. 4). Symptoms of petiole collapse were associated with a greater concentration of ^{45}Ca in the proximal than in the distal portion of the petiole or in the lamina.

(*d*) *Experiment* 4

The results of radioautographs and radioassays of plants sampled on days 39 and 53 are presented in Plate 11. The differences in relative ^{45}Ca concentration between various plant tissues due to difference in calcium level in the substrate are in general accord with results already described above.

The results of radioassays of the chemical fractions extracted from various plant parts of the samples obtained on days 39 and 53 are shown in Tables 2 and 3.

For each sample the relative differences in total activities between the various parts of the plant samples, grown at each calcium level, are in general accord with the radioautographs and radioassays of comparable tissues of plants sampled at the same time (Plate 11).

(i) *Sample* 1

The results of the first sample taken on day 39, before the onset of calcium-deficiency symptoms in the plants growing in the low-calcium cultures are set out in Table 2.

For each plant part, and irrespective of calcium level in the nutrient solution, over 90% of the ^{45}Ca was recovered from the fractions soluble in either cold water or hot HCl (Table 2). However, the ratio of ^{45}Ca content between these two fractions varied between plant tissues, and was affected by the level of calcium nutrition.

In the normal-calcium plants the ^{45}Ca activity in the extract soluble in cold water was consistently higher than in the hot HCl extract for all plant parts. However, there was an important difference due to the age of the tissues. In the case of the old leaf tissue the ratio of ^{45}Ca activity in cold water extracts to that in hot HCl extracts was many times greater for the lamina edge and centre tissues, and over twice as great for the proximal half of the petiole than in the corresponding tissues of the young leaves. The ratio in the distal halves of the petioles was not affected by leaf age while that of the growing point was comparable to the young leaf tissues.

By contrast with the normal-calcium plants, the ^{45}Ca activity in the cold water-soluble extracts was not consistently higher than that of the hot HCl extracts in all tissues of the low-calcium plants. In the young leaves, the ratio of ^{45}Ca activity in the cold water extracts to that in the hot HCl extracts of the leaf edge and centre was comparable with that of similar tissues of the complete-solution plants. However, the petioles of the low-calcium leaves differed from the normal petioles in that the greatest ^{45}Ca activity occurred in the hot HCl extract.

Also, the increase in the ratio of ^{45}Ca activity in the cold water extracts to that in the hot HCl extracts of the low-calcium plants due to age of the tissues was not as great as for the normal-calcium plants. As in the normal plants, leaf age did not affect the ratio of cold water-soluble to hot HCl-soluble ^{45}Ca in the distal halves of the petioles of the low-calcium plants. Thus, in the latter plants, this tissue in the the old leaves also had more ^{45}Ca which was soluble in hot HCl than cold water.

(ii) *Sample* 2

The results of the second sample, taken on day 53 after the onset of severe calcium-deficiency symptoms in the plants grown in the low-calcium cultures (Plate 11, Fig. 2), are set out in Table 3. Irrespective of calcium treatment, it was again found, as with sample 1, that the cold water-soluble plus the hot HCl-soluble fractions accounted for approximately 90% of the ^{45}Ca found in the various plant parts.

With the plants grown under normal calcium nutrition, when allowances are made for differences in maturity of the older leaves sampled at each harvest, the distribution and level of activity of ^{45}Ca in the leaf parts, and in the various chemical fractions after 53 days are in general agreement with the results recorded in Table 2 from the first sample. The main difference between the results of the two samples of normal-calcium plants was that, at the second sampling, the centres of the youngest leaves contained more ^{45}Ca soluble in hot HCl than in cold water. The increase in the ratio between these fractions due to leaf age was not as great for the young middle leaves sampled on day 53 as for the old leaves sampled on day 39.

With the plants grown under low-calcium nutrition, a comparison of the results in Tables 2 and 3 indicates that appreciable changes in the level and distribution of ^{45}Ca had taken place with the onset of severe deficiency symptoms between days 39 and 53. At the second sampling the ^{45}Ca activity in the leaf parts was greatly reduced and in contrast with sample 1, there was no increase in ^{45}Ca concentration with leaf age.

TABLE 2

EXPERIMENT 4: SAMPLE 1, DAY 39—RESULTS OF RADIOASSAYS OF ^{45}Ca IN VARIOUS EXTRACTS OF 200-MG (FRESH WEIGHT) SAMPLES OF SUBTERRANEAN CLOVER (CV. DWALGANUP) GROWN AT EITHER NORMAL- OR LOW-CALCIUM LEVELS

Percentage of ^{45}Ca in each extract shown in parenthesis

Plant Part	Normal Calcium: ^{45}Ca Activity (counts/min) in:						Ratio of Activity of Cold Water Extract to Hot HCl Extract	Low Calcium: ^{45}Ca Activity (counts/min) in:						Ratio of Activity of Cold Water Extract to Hot HCl Extract
	Cold Water Extract	Hot Water Extract	Hot 0·05N HCl Extract	Hot 0·5% Ammonium Oxalate Extract	Residue	Total Activity		Cold Water Extract	Hot Water Extract	Hot 0·05N HCl Extract	Hot 0·5% Ammonium Oxalate Extract	Residue	Total Activity	
Growing point	289 (62·9)	11 (2·5)	160 (34·7)	0	0	460	1·8	586 (61·8)	48 (5·0)	307 (32·4)	3 (0·4)	5 (0·5)	949	1·9
Young leaf														
Lamina edge	371 (55·8)	12 (1·8)	226 (33·9)	8 (1·2)	49 (7·3)	666	1·7	2193 (56·8)	103 (2·7)	1301 (33·7)	48 (1·2)	218 (5·7)	3864	1·7
Lamina centre	371 (50·9)	19 (2·6)	308 (42·3)	8 (1·1)	23 (3·2)	728	1·2	938 (60·1)	14 (0·9)	608 (39·0)	0	1	1561	1·5
Petiole distal	385 (68·8)	8 (1·3)	167 (29·8)	0	0	559	2·3	159 (44·0)	33 (9·2)	170 (46·8)	0	0	362	0·9
Petiole proximal	244 (61·1)	3 (0·8)	152 (37·9)	1	0	400	1·6	180 (37·1)	5 (1·0)	301 (61·9)	0	0	486	0·6
Old leaf														
Lamina edge	2776 (88·1)	70 (2·2)	265 (8·4)	10 (0·3)	31 (1·0)	3151	10·5	12070 (77·7)	385 (2·5)	3026 (19·5)	22 (0·1)	33 (0·2)	15536	4·0
Lamina centre	2742 (89·2)	19 (0·6)	309 (10·0)	4 (0·1)	0	3073	8·6	3829 (74·7)	77 (1·5)	1199 (23·4)	11 (0·2)	6 (0·1)	5123	3·2
Petiole distal	612 (65·9)	48 (5·1)	267 (28·8)	1 (0·2)	0	928	2·3	776 (44·8)	88 (5·1)	868 (50·1)	0	0	1732	0·9
Petiole proximal	571 (76·8)	18 (2·4)	147 (19·7)	5 (0·7)	3 (0·4)	744	3·9	616 (52·7)	53 (4·5)	483 (41·3)	6 (0·5)	12 (1·0)	1170	1·3

TABLE 3

EXPERIMENT 4: SAMPLE 2, DAY 53—RESULTS OF RADIOASSAYS OF ^{45}Ca IN VARIOUS EXTRACTS OF 200-MG (FRESH WEIGHT) SAMPLES OF SUBTERRANEAN CLOVER (CV. DWALGANUP) GROWN AT EITHER NORMAL- OR LOW-CALCIUM LEVELS

Percentage of ^{45}Ca in each extract shown in parenthesis

Plant Part	Normal Calcium: ^{45}Ca Activity (counts/min) in:						Ratio of Activity of Cold Water Extract to Hot HCl Extract	Low Calcium: ^{45}Ca Activity (counts/min) in:						Ratio of Activity of Cold Water Extract to Hot HCl Extract
	Cold Water Extract	Hot Water Extract	Hot 0·05N HCl Extract	Hot 0·5% Ammonium Oxalate Extract	Residue	Total Activity		Cold Water Extract	Hot Water Extract	Hot 0·05N HCl Extract	Hot 0·5% Ammonium Oxalate Extract	Residue	Total Activity	
Young leaf														
Lamina edge	483(52·1)	14(1·5)	346(37·3)	6(0·6)	78(8·5)	927	1·4	943(34·4)	109(4·0)	1688(61·6)	0	0	2739	0·6
Lamina centre	378(37·1)	21(2·0)	544(53·4)	9(0·8)	67(6·6)	1019	0·7	102(24·9)	0	295(72·3)	9(2·1)	3(0·7)	409	0·4
Petiole distal								53(20·6)	11(4·2)	194(75·2)	0	0	257	0·3
Petiole proximal								15(8·1)	0	155(86·0)	11(5·9)	0	180	0·1
Young leaf—petiole collapse														
Lamina edge								1012(48·1)	118(5·6)	964(45·8)	3(0·1)	9(0·4)	2105	1·1
Lamina centre								300(40·9)	21(2·9)	413(56·2)	0	0	734	0·7
Petiole distal								36(17·0)	0	174(83·0)	0	0	209	0·2
Petiole proximal								136(14·6)	11(1·2)	784(84·2)	0	0	931	0·2
Middle leaf														
Lamina edge	1628(81·7)	23(1·1)	317(15·9)	0	25(1·2)	1993	5·1							
Lamina centre	1721(81·2)	18(0·9)	365(17·2)	6(0·3)	10(0·5)	2120	4·7							
Petiole distal	659(68·4)	13(1·3)	291(30·2)	1(0·1)	0	964	2·3							
Petiole proximal	537(65·4)	12(1·5)	264(32·1)	9(1·0)	0	821	2·0							
Middle leaf—petiole collapse														
Lamina edge								1907(69·5)	65(2·5)	693(25·3)	0	79(2·9)	2745	2·8
Lamina centre								487(47·0)	56(5·4)	494(47·7)	0	0	1037	1·0
Petiole distal								59(19·2)	20(6·5)	225(73·0)	4(1·3)	0	308	0·3
Petiole proximal								287(33·3)	62(7·1)	508(58·9)	5(0·6)	0	862	0·6

In the young leaves with petiole collapse, the amount of ^{45}Ca was less in the lamina edge and more in the proximal part of the petiole than that present in young leaves without petiole collapse.

Under low-calcium supply, and without regard to leaf age or time of sampling, in no instance did the distal halves of the petioles contain more than 7·5% of the ^{45}Ca that was in the leaf as a whole. In comparable tissue under normal calcium supply, the corresponding value was between 12 and 24%.

The percentages of ^{45}Ca in the water-soluble extracts of the calcium-deficient plants were much lower, and in the hot HCl extracts correspondingly greater in the second sample than in the first. Even so, in both samples the ratio of ^{45}Ca soluble in cold water to that soluble in hot HCl followed a similar pattern of increase with maturity of all leaf parts, except in the case of the distal petiole sections. It appears that for the distal halves of the petioles this ratio is related solely to the level of calcium in the plant, rather than to the maturity of the tissue as is the case with the other leaf parts. In all leaves studied, at both harvests for plants in the normal-calcium solutions, the ratio for the distal petiole sections was 2·3, whereas in the low-calcium plants at the first harvest it was 0·9 and under severe calcium-deficiency conditions it fell to 0·3 at the time of the second sampling.

IV. Discussion

The experiments described herein have shown that the distribution of ^{45}Ca in plants is markedly affected by the level of calcium in the substrate.

Sites of initial accumulation of ^{45}Ca in subterranean clover have been demonstrated. These are the vein endings along the distal margins of the leaf, the veins themselves, the marginal interveinal tissue, and the proximal half of the petiole. In normal-calcium plants the sites in the vein endings are soon saturated and the element accumulates in the lateral veins and moves from them uniformly into all interveinal tissues. However, it has been clearly shown that the calcium which initially moves into the vein endings in both normal and low-calcium plants is permanently fixed therein, thus pointing to some special but unknown function related to calcium nutrition in these sites. In this regard it is significant that where marginal necrosis due to calcium deficiency occurred, there was no calcium accumulation in the vein endings.

In normal-calcium plants it is concluded that import of calcium into the tissues is continuous with little or no export as shown by the existence of a positive correlation between leaf age and ^{45}Ca concentration. This positive relationship was soon established even in plants which were first grown for 5 weeks in a non-radioactive normal calcium solution. Similar evidence of the accumulation of calcium in the oldest leaves of plants is presented by Mallon and Urey (1946), Ririe and Toth (1952), Biddulph *et al.* (1958), Biddulph, Cory, and Biddulph (1959), and Rinne and Langston (1960).

In low-calcium plants the initial sites of calcium accumulation described above, with the exception of the vein endings, were not rapidly saturated, with the result that a distinctly different pattern of distribution of ^{45}Ca to that in normal plants has been demonstrated. That the limited supply of calcium accumulates in

the petiole at the expense of the lamina has been clearly shown in the above experiments. This resulted in the petioles of calcium-deficient plants having at first higher concentrations of ^{45}Ca in their proximal ends. Later, the proximal sites were evidently saturated as there was a build-up of ^{45}Ca in the distal half of the petiole so that the concentration along its length became relatively uniform or even highest in the distal end.

It has been shown further in the present experiments that, before the onset of acute symptoms in the youngest leaves, calcium-deficient plants may not show the same positive correlation between leaf age and ^{45}Ca concentration, as normal plants. Under conditions of deficient supply it is thus evident that recently acquired calcium does not move uniformly into all leaves as in normal plants, but some of it may be preferentially acquired by younger leaves at the expense of older leaves.

Although this phenomenon has been demonstrated in both the summer and winter experiments described above, it seems that the most important effects resulting from it are likely to occur where temperature conditions are conducive to rapid growth. It is under such conditions that the symptom of collapse of the distal portion of the petiole due to calcium deficiency (Millikan 1953) is most evident. This symptom usually does not first appear in the youngest leaves and may even occur in the oldest, as is shown in Plate 1, Figure 2. It may also occur before any marginal necrosis is present in the youngest leaves. As the collapsed distal portion of the petiole has been shown to be very low in ^{45}Ca it is evident that the usual build-up of calcium in this tissue did not occur due to the preferential movement of newly acquired ^{45}Ca into the younger leaves, whose laminae, as a result, may contain concentrations of the isotope comparable to that in the oldest leaf.

Calcium deficiency is normally regarded as a condition which produces symptoms in the youngest tissues of plants. For this reason, petiole collapse of the older leaves of subterranean clover due to calcium deficiency is an unexpected phenomenon, but the present work has provided for the first time an explanation for its occurrence in these older leaves.

It is generally accepted that calcium is immobile in the plant. Recent reviews of the literature on this subject have been made by Williams (1955), Biddulph *et al.* (1958), Bollard (1960), and Zimmermann (1960). In the present experiments the positive correlation found between leaf age and calcium concentration in normal plants, and, in acutely deficient plants, the relatively high concentration of calcium which occurred in old leaves when compared with the very low concentration in young leaves showing severe necrotic symptoms, appear to support the view that calcium is immobile in the plant.

It has nevertheless been demonstrated by the experiments described herein, that some translocation of ^{45}Ca may occur when calcium-deficient plants are provided with sufficient non-radioactive calcium to induce new growth. It was found that a notable amount of the ^{45}Ca, which was previously unavailable to the young leaves while the plant was acutely calcium-deficient, was translocated to new leaves produced up to 9 weeks after the non-radioactive calcium was supplied. It appears that the mobile ^{45}Ca came mainly from the roots and, possibly also during the first 2–3 weeks, from the petioles of plants transferred to complete solutions.

Biddulph, Nakayama, and Cory (1961) and Bell and Biddulph (1962) have reported that the entry of ^{45}Ca into stem tissue showed two phases, namely a reversible "exchange" phase, followed by an irreversible accumulation phase. In their experiments the "exchange" phase was completed within 3 hr. Biddulph *et al.* (1958) also showed that the delivery of ^{45}Ca from roots to top in bean plants was largely completed in 6 hr.

Kislev (1961) has reported the results of experiments with various plants which showed that ^{45}Ca which had been absorbed earlier did move to other parts of the plant. Upward movement of the isotope was 4–10 times greater than downward movement. No translocation was observed in bean plants, this result being in accord with that of Biddulph *et al.* (1958). Earlier Ferrell and Johnson (1956) had observed substantial movement of previously deposited calcium into newly developed buds of western white pine.

Charles (1953) has suggested that the xylem elements in some plants may be negatively charged. In his review Epstein (1962) has concluded that cations moving in stems of plants may be retarded by attachment to negatively charged sites, and the presence of competing ions may accelerate their movement. It seems probable that this is the mechanism involved in the translocation of ^{45}Ca in subterranean clover described above. This entails an "exchange" phase in the conducting tissues of the calcium-deficient subterranean clover plants of considerably longer duration than that found by Biddulph, Nakayama, and Cory (1961) and Bell and Biddulph (1962).

The results obtained in experiment 4, from the serial extraction of ^{45}Ca in subterranean clover, suggest that with an adequate calcium supply there is sufficient calcium to saturate all available sites in the developing leaf tissue in addition to maintaining a high percentage of free or water-soluble calcium in the tissue. As the leaves mature, uptake of calcium continues and since there now appears to be fewer sites of absorption or fixation, this results in most of this additional calcium remaining water-soluble. Abutalybov (1956), studying the distribution of calcium in cotton plants, found that the largest percentage of water-soluble plus absorbed calcium is accumulated in the young plant parts, the high percentage of these two components being related to the high content of calcium in the protoplasm.

In apparent contrast with the results obtained for subterranean clover, Abutalybov found, in the older plant parts, the levels of acid-soluble forms of calcium were high, and attributed this to a decline in cellular activity, a lower protoplasmic calcium content, and to an increase in calcium cell sap concentration, probably as calcium oxalate crystals. A possible explanation to this discrepancy may be that cotton, by comparison with subterranean clover, has a high capacity for fixing calcium as oxalate, sulphate, or phosphate in the older tissue.

As described above, with the onset of acute calcium deficiency in subterranean clover, the distribution of newly acquired ^{45}Ca differed from normal in that the younger tissues received an increased proportion at the expense of the older tissues. In addition, there was insufficient calcium supply to saturate all sites of absorption and fixation, this resulting in a marked fall in the ratio of water-soluble to acid-soluble calcium in the tissues of the deficient plants, when compared with normal plants.

In leaves with petiole collapse, the activity of ^{45}Ca in the distal collapsed portion was very low, and approximately 75% of it was absorbed or fixed on to the sites present. This contrasts with the proximal portions of the same petioles in which the proportion of water-soluble ^{45}Ca increased slightly with leaf age. This increase, however, could be attributable to the blockage of further calcium transport through the collapsed distal tissue. Ordin, Cleland, and Bonner (1957) have suggested that the role of calcium in cell wall stability may be the formation of double salts involving two anhydrogalacturonic carboxyl groups which could cross-link galacturonic acid residues of different pectin molecules and might thus be expected to stiffen the cell wall. Also, Edgington, Corden, and Dimond (1962) have observed that the stems of calcium-deficient tomato plants contained more water-soluble pectin than normal plants. They attributed the increase to the lack of calcium bonding of the uronic carboxyl groups in pectin. Hydrolyses of pectic substances by pectic enzymes was faster in calcium-deficient than in normal tissue. These results provide an explanation for the occurrence of the petiole collapse symptom in subterranean clover tissues in which the normal build-up of calcium did not occur.

An accumulation of ^{45}Ca occurred in epidermal hairs on petioles and laminae of leaves of subterranean clover. In young leaves the hairs were much higher in ^{45}Ca than the epidermal cells from which they arose (Plate 9, Fig. 3).

Plummer (1962) has also reported that ^{45}Ca became particularly abundant in the epidermal hairs of sunflower leaves. The root hairs of several plant species were also found by Cormack, Lemay, and McLachlan (1963) to concentrate ^{45}Ca in a form which could not be extracted with hot water. Their suggestion that gradual calcification is essential for the normal growth and form of the hair is supported by the present experiments.

The basal tissues of the flowers of subterranean clover were also found to be higher in ^{45}Ca than the petals and peduncles. It was not determined which part of the flower was principally involved in this accumulation. The relative calcium contents of pollen and parts of the gynoecium have been given added interest by the suggestion made by Mascarenhas and Machlis (1962) that calcium may be a universal factor promoting the growth of the pollen tube to the ovule. Calcium deficiency was found by Steffensen (1955) to produce at least 17 times more chromosomal aberrations and micronuclei in pollen of *Tradescantia* than did plants receiving an optimal calcium supply.

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EXPLANATION OF PLATES 1–11

PLATE 1

Figs. 1–4.—Experiment 1: Radioassays (expressed as counts/min/mg dry matter) of individual runner stems of subterranean clover plants (cv. Dwalganup and Mt. Barker) after 39 days in nutrient solutions containing ^{45}Ca. Time of exposure 6 days.

PLATE 2

Figs. 1–4.—Experiment 2: Radioautographs and radioassays (expressed as counts/min/mg dry matter) of subterranean clover plants (cv. Dwalganup) grown for 5 weeks in non-radioactive solutions before transfer to solutions of comparable calcium level containing ^{45}Ca. Plants sampled 7 days after this transfer. Figures 1 and 2 photographs; Figures 3 and 4 corresponding radioautographs. Time of exposure: Figure 3, 12 days; Figure 4, 4 days.

PLATE 3

Figs. 1–4.—Experiment 2: As for Plate 2. Plants sampled 27 days after transfer to solutions containing ^{45}Ca. Time of exposure for Figures 3 and 4, 13 days.

PLATE 4

Figs. 1–6.—Experiment 3, series A: Radioassays (expressed as counts/min/mg dry matter) and radioautographs of subterranean clover (cv. Dwalganup and Mt. Barker) grown for 7 days in nutrient solutions containing ^{45}Ca and then transferred to non-radioactive solutions. Figures 1, 3, and 5, photographs; Figures 2, 4, and 6, corresponding radioautographs. Sampling times after transfer: Figures 1 and 2, 2 days; Figures 3 and 4, 7 days; Figures 5 and 6, 15 days. Times of exposure: Figures 2 and 4, 14 days; Figure 6, 37 days.

PLATE 5

Figs. 1–6.—Experiment 3, series A: As for Plate 4. Sampling times after transfer: Figures 1–4, 21 days; Figures 5 and 6, 36 days. Times of exposure: Figures 2 and 4, 37 days; Figure 6, 55 days.

PLATE 6

Figs. 1–5.—Experiment 3, series B: Radioautographs and radioassays (expressed as counts/min/mg dry matter) of subterranean clover (cv. Dwalganup and Mt. Barker) grown for 7 days in non-radioactive solutions and then transferred to solutions of comparable calcium level containing ^{45}Ca. Arrows in Figure 5 indicate leaves showing onset of marginal necrotic symptoms. Sampling times after transfer: Figure 1, 2 days; Figure 2, 7 days; Figure 3, 15 days; Figures 4 and 5, 36 days. Times of exposure; Figures 1 and 2, 14 days; Figure 3, 20 days; Figure 4, 17 days; Figure 5, 13 days.

PLATE 7

Figs. 1–4.—Experiment 3, series B: As for Plate 6, but sampled 73 days after transfer. Times of exposure: Figures 1 and 3, 19 days; Figures 2 and 4, 14 days.

PLATE 8

Radioautographs showing the disorganization in the distribution of ^{45}Ca associated with the onset of marginal necrosis in young leaves due to calcium deficiency

Fig. 1.—Typical accumulation of ^{45}Ca in the veins and their endings of a leaf produced before the onset of calcium-deficiency symptoms in the plant.

Fig. 2.—Movement of ^{45}Ca out of the veins, but not their endings, in a leaf produced immediately prior to the first leaf on the plant to show marginal necrosis.

Fig. 3.—First leaf on plant to show slight marginal necrotic symptoms. There was no accumulation of ^{45}Ca in the vein endings, but some increase in concentration occurred in the distal margins.

Fig. 4.—Leaf from plant with acute marginal necrotic symptoms. The concentration of ^{45}Ca in the marginal tissues was very low relative to the veins and the petiole.

PLATE 9

Figs. 1–3.—Radioautographs of subterranean clover showing the accumulation of ^{45}Ca in the flowers (Fig. 1), epidermal hairs, vein endings, and distal marginal interveinal tissue (Figs. 2 and 3). Fig. 1, normal-calcium solution; Figures 2 and 3, low-calcium solution.

PLATE 10

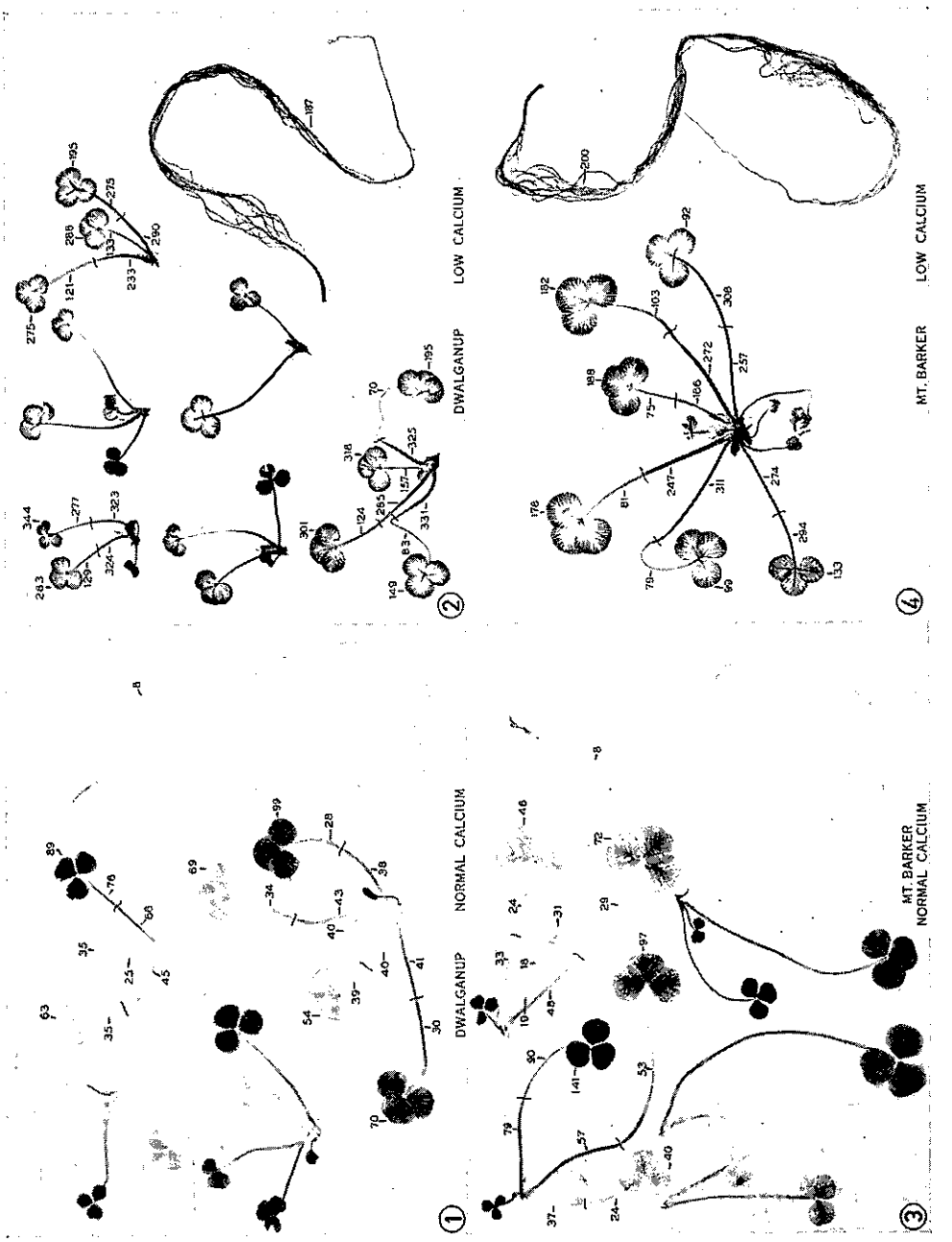
Figs. 1–4.—Experiment 3, series C. Radioassays (expressed as counts/min/mg dry matter) and radioautographs of subterranean clover (cv. Dwalganup and Mt. Barker) grown for 73 days in low-calcium nutrient solutions containing ^{45}Ca, and then transferred to non-radioactive solutions as follows:

Figure 1: Sampled 16 days after transfer to complete solution (top row) and low-calcium solution (bottom row). Arrows indicate new leaves produced since transfer. Time of exposure, 18 days. Figure 2: Sampled 29 days after transfer to complete solution. All the original leaves at time of transfer now dead. Time of exposure, 25 days. Figure 3: Sampled 64 days after transfer to complete solution. Time of exposure, 57 days. Figure 4: Sampled 64 days after transfer to low-calcium solution. Time of exposure, 57 days.

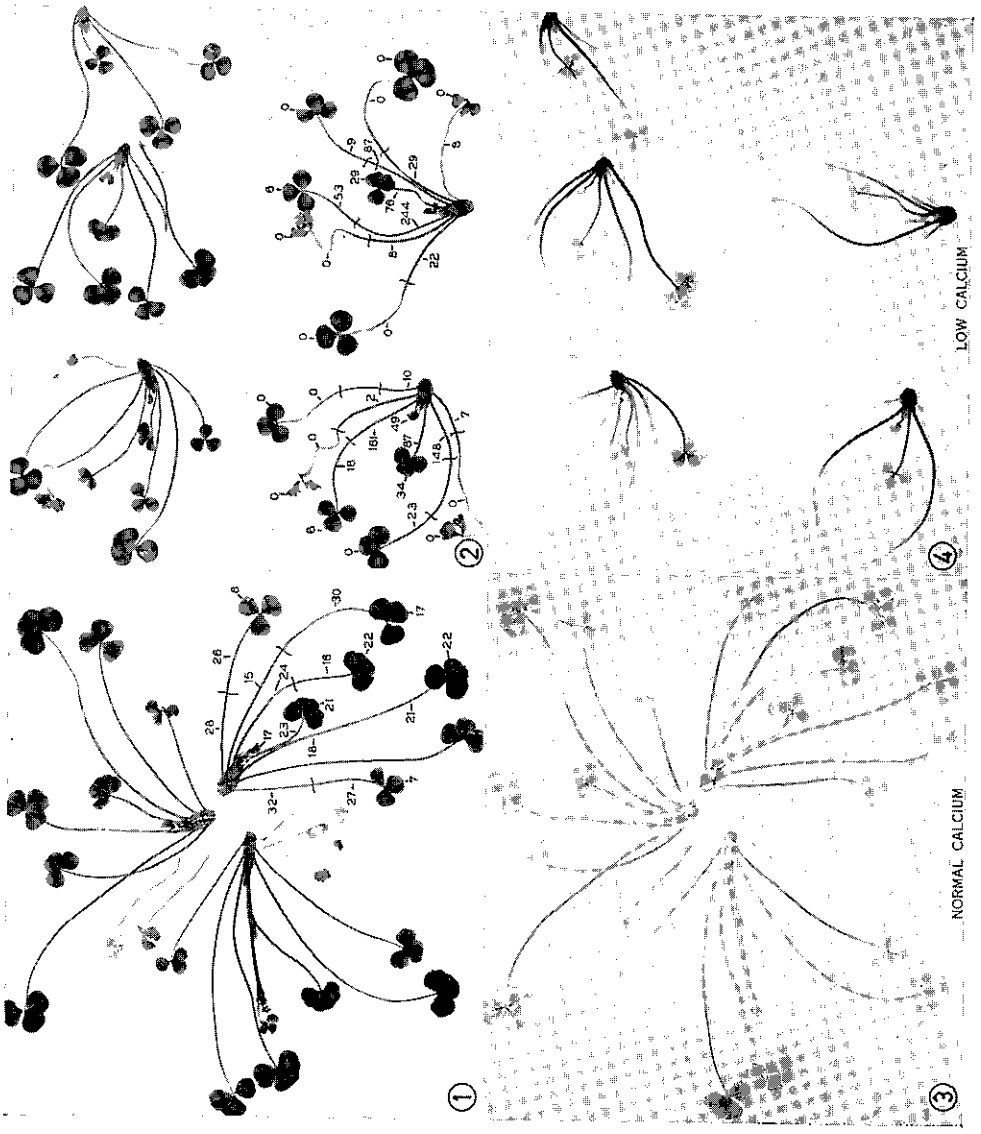
PLATE 11

Figs. 1 and 2.—Experiment 4: Radioautographs and radioassays (expressed as counts/min/mg dry weight) of subterranean clover plants (cv. Dwalganup). In Figure 1, plants harvested on day 39, before the onset of calcium deficiency in the low-calcium plants. In Figure 2, plants harvested on day 53, after the development of severe petiole collapse in the low-calcium plants. The arrows in each figure indicate leaves typical of those taken at each harvest for the serial extraction of calcium.

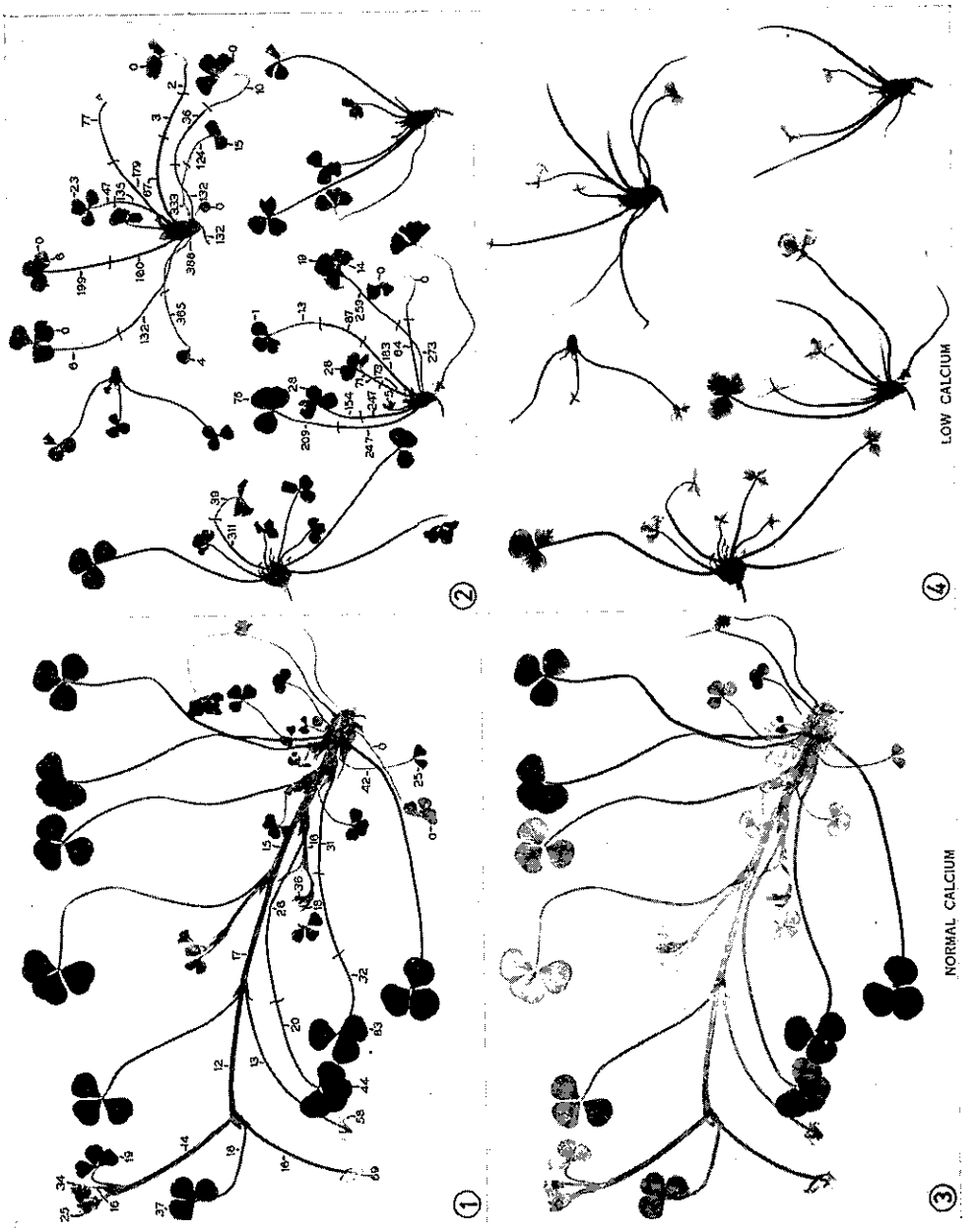
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



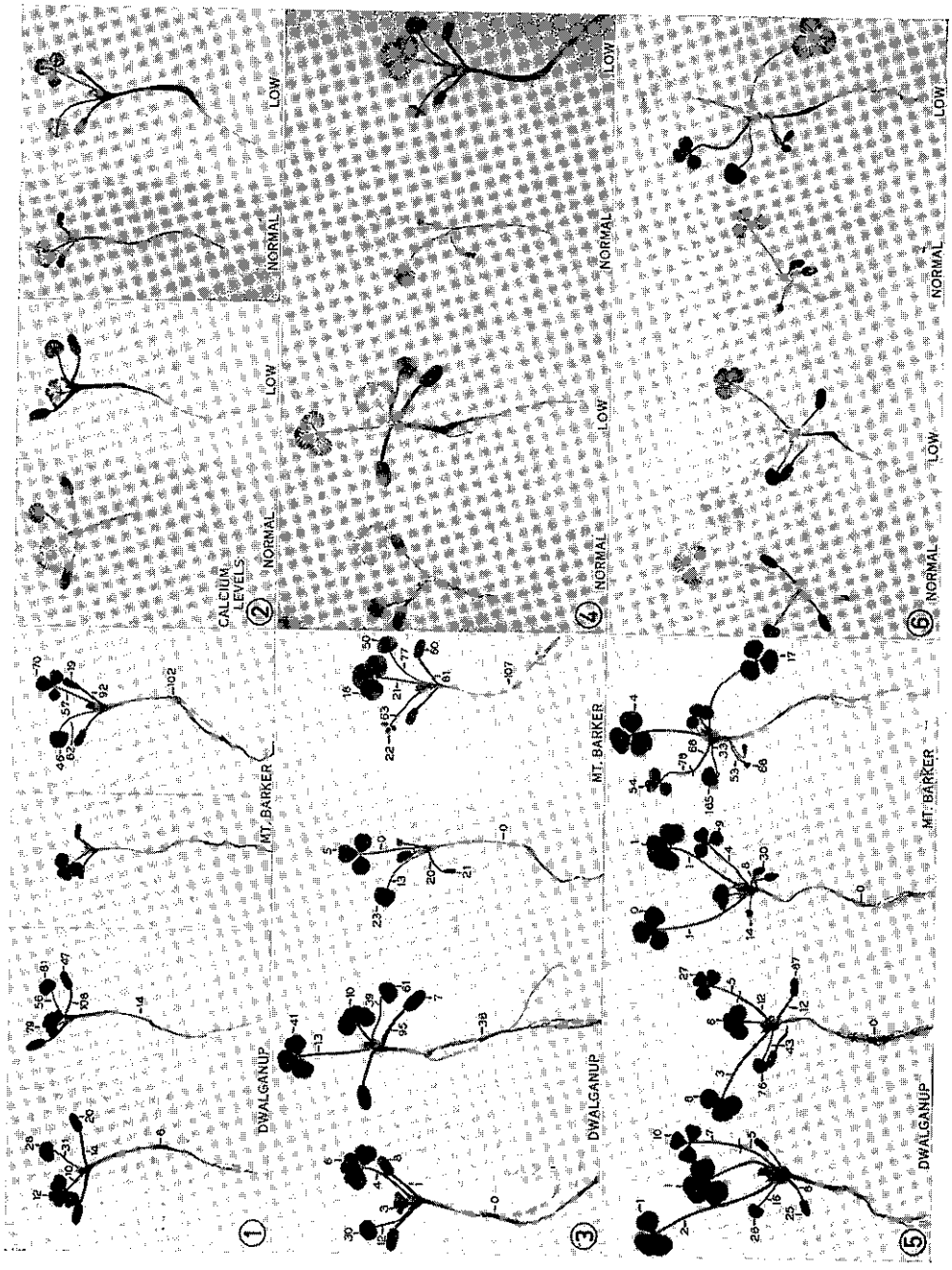
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



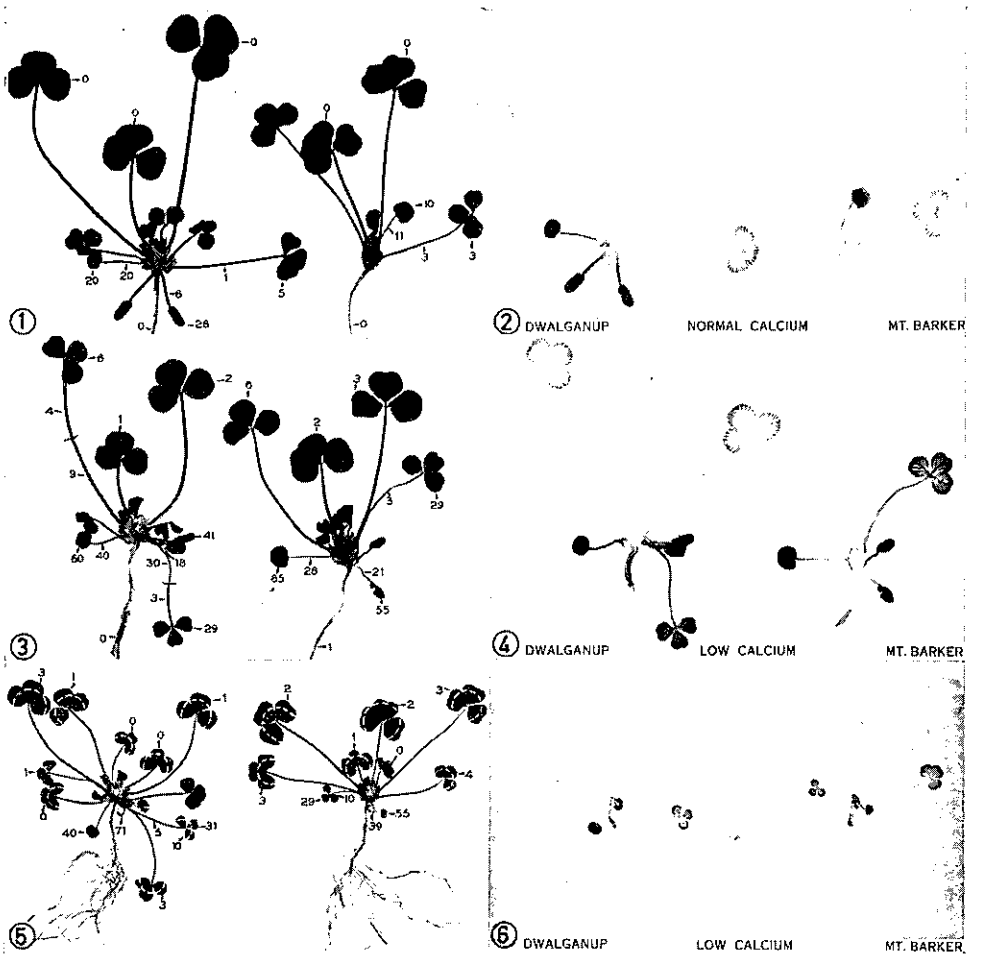
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



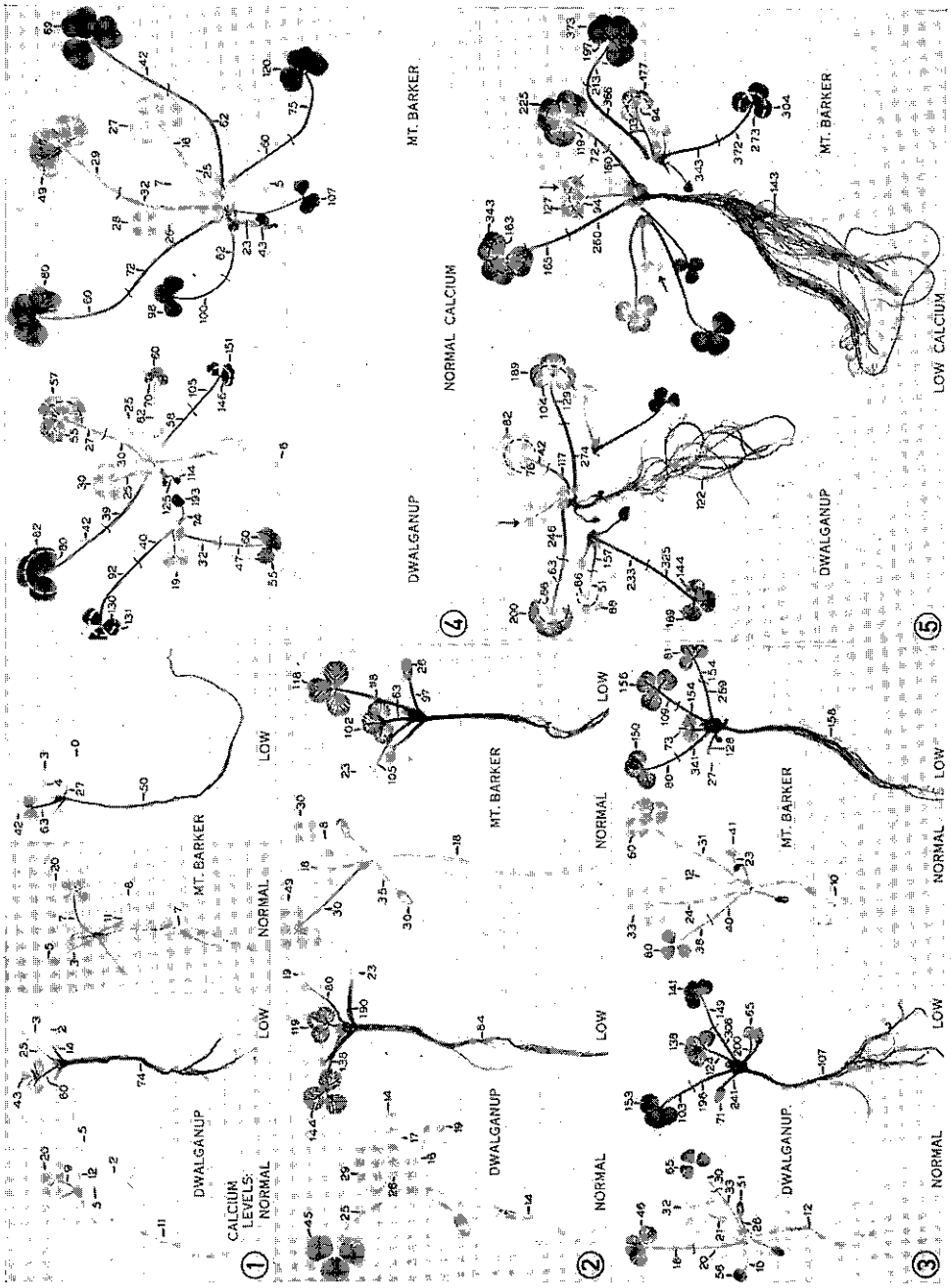
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



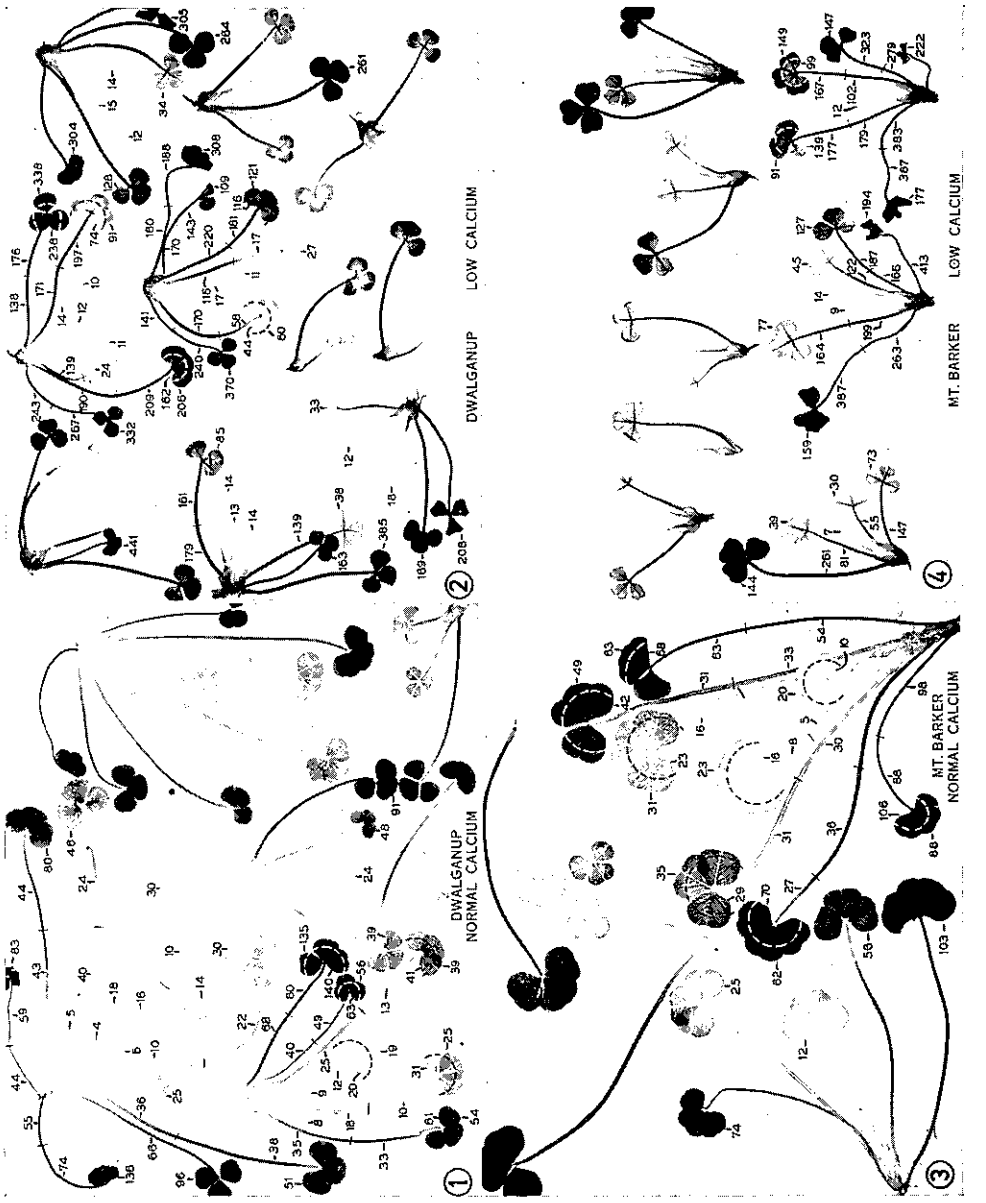
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



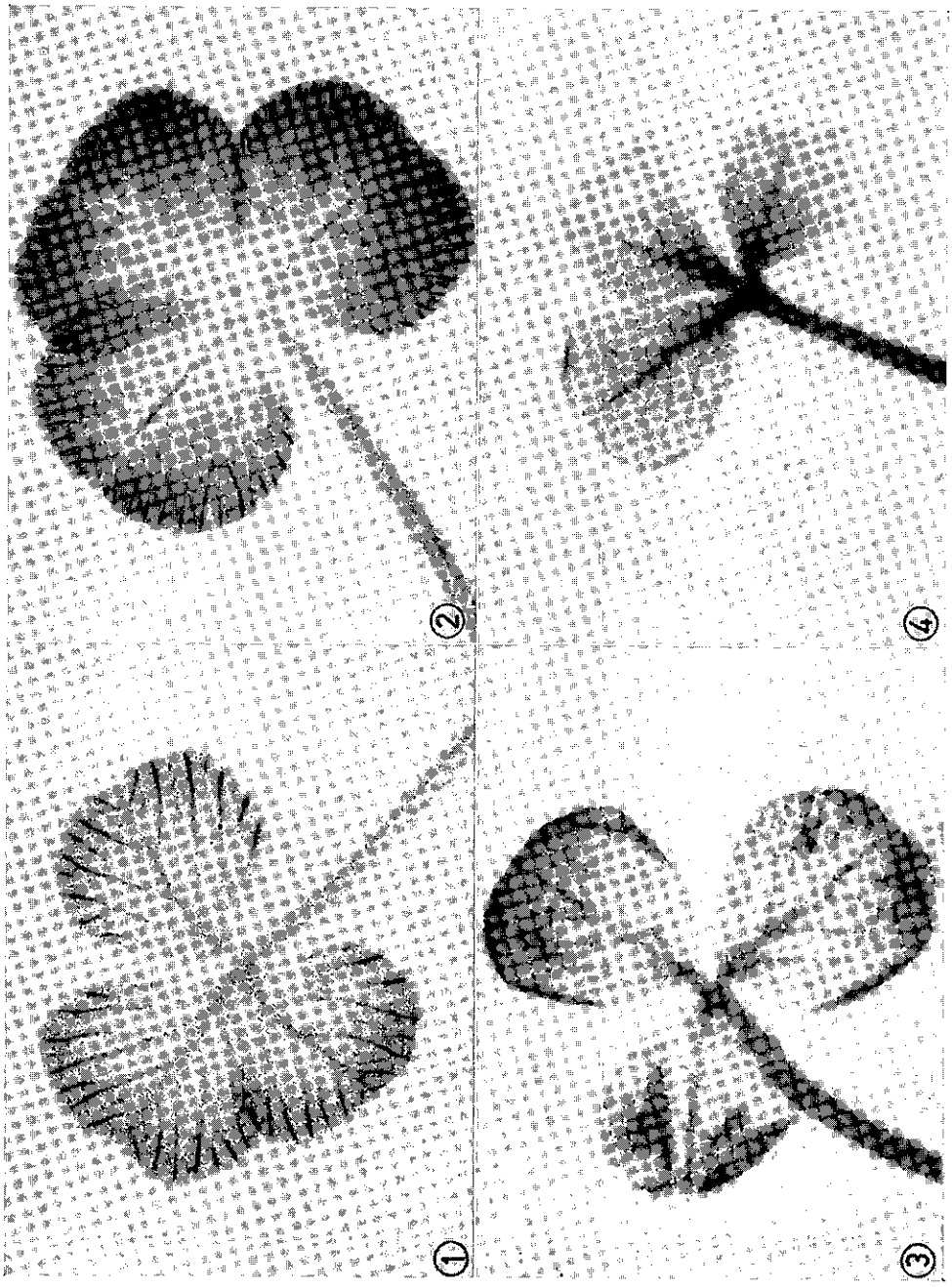
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



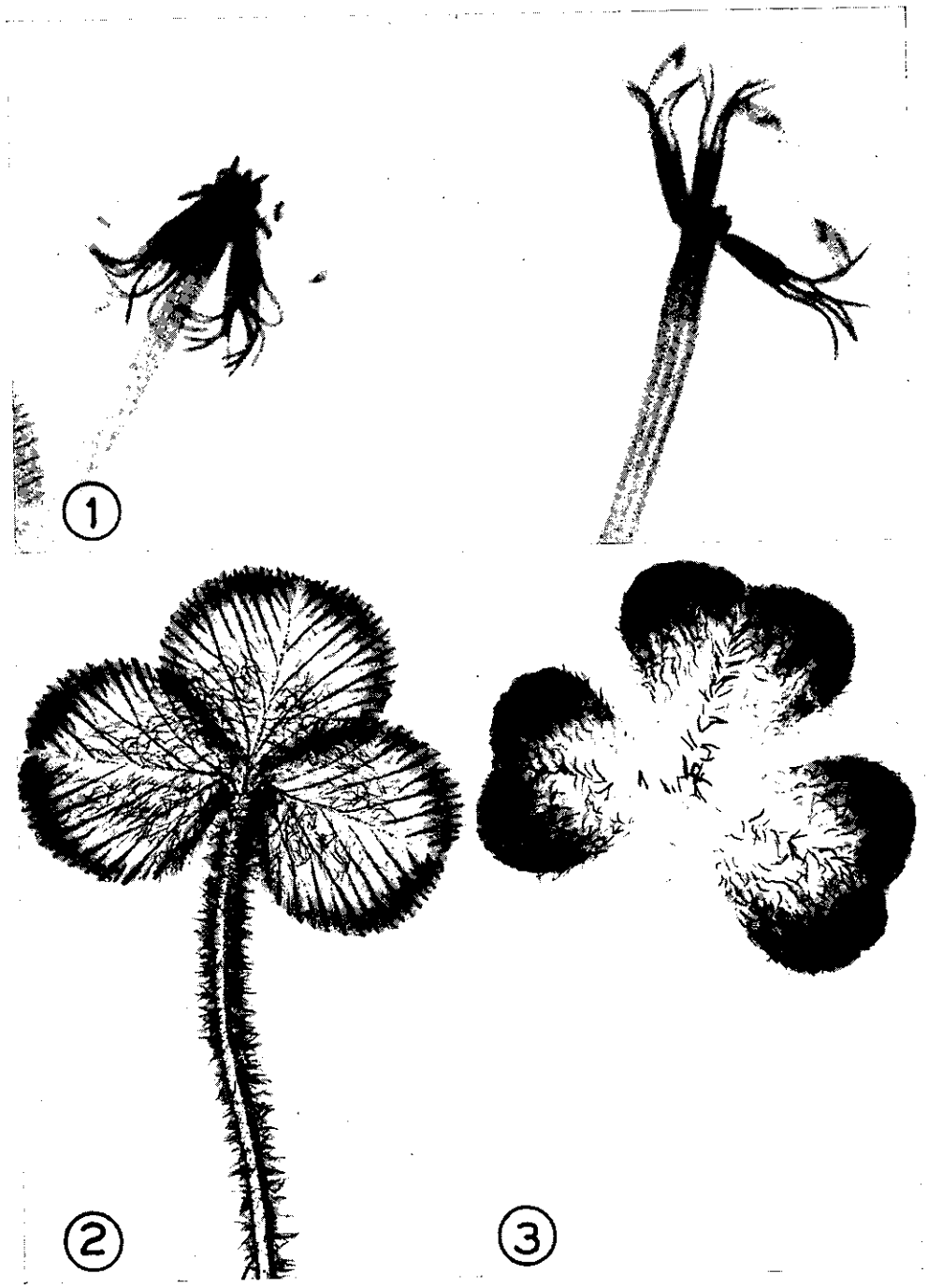
DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER



DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER

DISTRIBUTION OF ^{45}Ca IN SUBTERRANEAN CLOVER