The Rôle of the Young Lucerne Plant in Determining the Infection of the Root by the Nodule-forming Bacteria.

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[Plate 31.]

Observations have been made at Rothamsted over a period of about five years upon the development of nodules on young seedlings of lucerne (*Medicago sativa*, L.). Some thousands of seedlings have been examined in various experiments and it was found to be the rule that the first appearance of nodules coincided with that of the expansion of the first true leaf. When lucerne is sown under summer glasshouse conditions, in pots of soil or sand, the seedlings are up in from 3 to 5 days, and in 6 to 8 days the first true leaf becomes visible. This is at first closed, but in 8 to 12 days from the date of sowing it opens out (fig. 1).

The following experiment illustrates the relationship between the opening

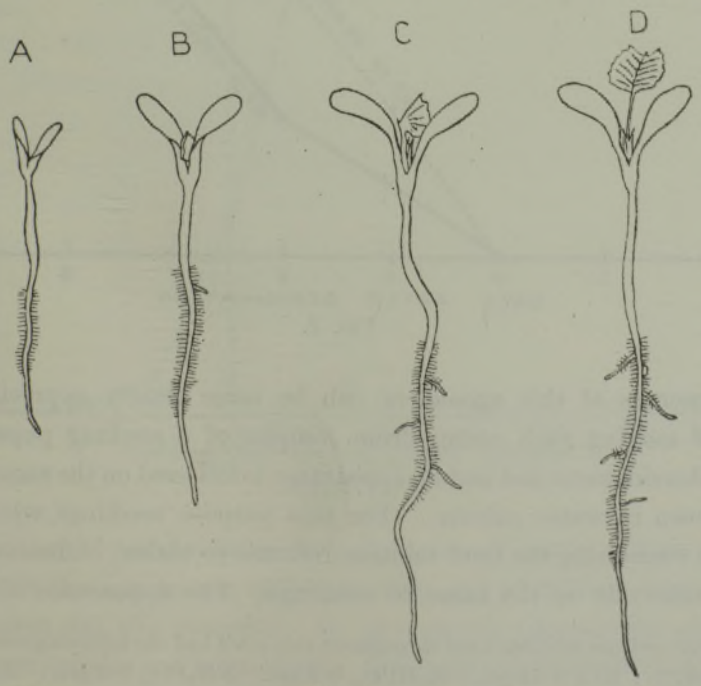

Fig. 1.

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of the first leaf and the appearance of nodules. Lucerne seed, inoculated with nodule bacteria was sown in 12 pots, each containing about 8 pounds of sand, and these were watered with a plant-culture solution free from nitrogen.* Five days after sowing the seedlings were up, and in another 4 the first true leaf, still closed, could be seen on most of them. The appearance of nodules and the opening of the first true leaves is shown in fig. 2, where each point represents observations made upon 20 seedlings, 10 from each of duplicate pots. The general agreement in the time of appearance of nodules and in the opening of the first true leaves is evident.

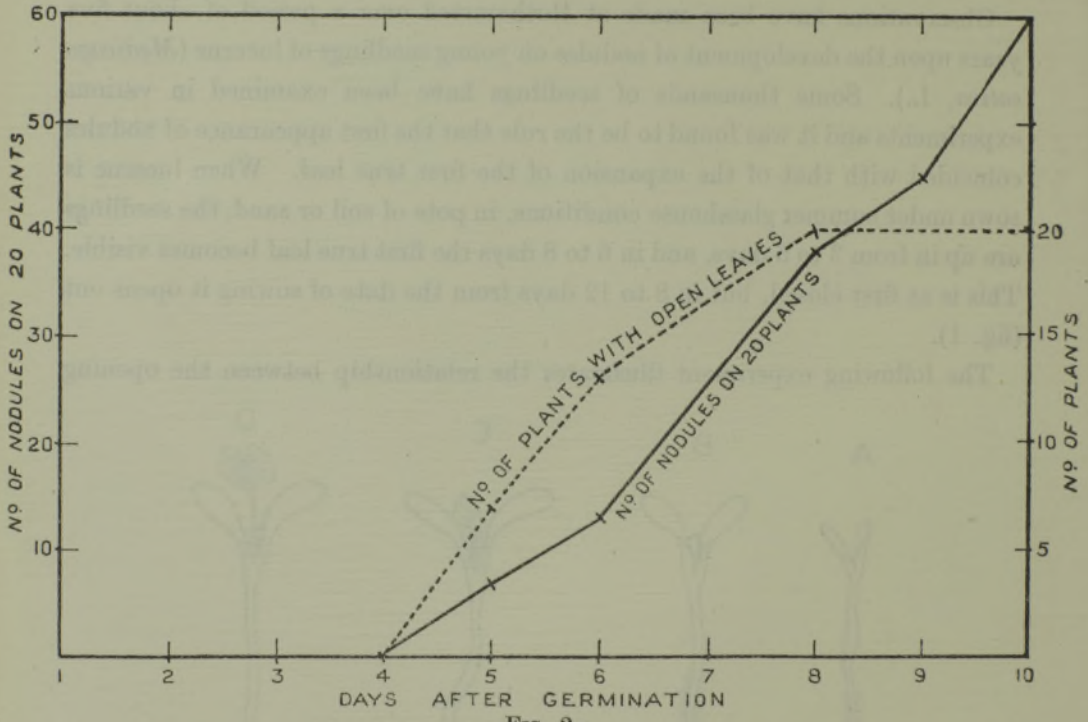

Fig. 2.

The closeness of this agreement can be more clearly appreciated when, instead of making such counts from samples of a seedling population, the course of development and nodule appearance is followed on the same individual plants grown in water culture. For this purpose seedlings were grown in test-tubes containing the food solution referred to above. Observations were made at intervals on the same 20 seedlings. The appearance of nodules is

* The plant-culture solution used throughout this work had the following composition :— Water, 1000 c.c. ; KCl, 0·74 grs. ; K_2HPO_4, 0·3 grs. ; KH_2PO_4, 0·3 grs. ; $MgSO_4 . 7H_2O$, 0·5 grs. ; NaCl, 0·5 grs. ; $CaSO_4$, 0·5 grs. ; $FeCl_3$, 0·04 grs.

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shown in fig. 3, where the age of each seedling is taken from the date of the opening of the first true leaf. Only one nodule had appeared on the day before this opening, although the roots had been in contact with a suspension of the bacteria for 9 days. Four more nodules appeared on plants the same day that the leaf opened and 8 more the following day.

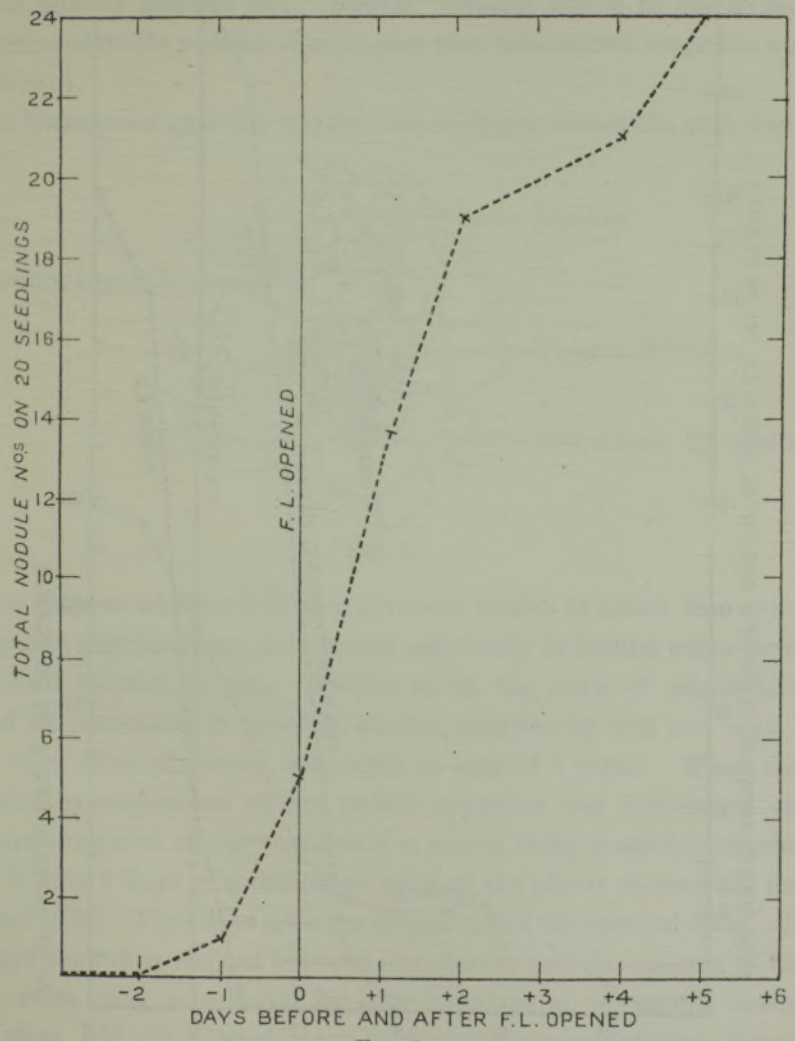

Fig. 3.

The appearance of nodules in another similar set of water cultures is shown in fig. 4, where the appearance of lateral roots and of the first leaf still in a closed condition are also recorded. In view of the comparisons sometimes drawn between nodules and lateral roots—it is interesting to note that the time of first appearance of the two do not coincide. The experiment also shows

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that appearance of the first nodules corresponds with the *opening* of the first leaf and not with its emergence from the growing point.

In the last set of cultures the roots had been in contact with a suspension of nodule bacteria for 10 days before the opening of the first leaves and the

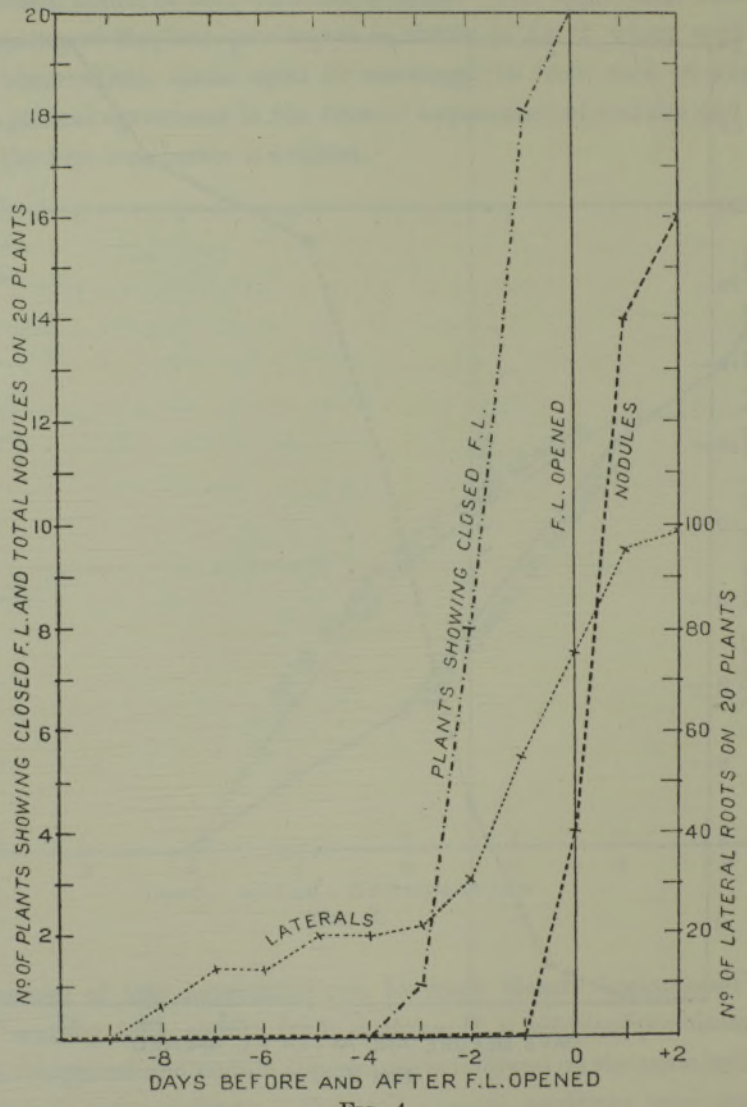

Fig. 4.

appearance of nodules. In seeking an explanation of this lag it seemed necessary to discover first whether the organisms fail to enter the root hairs during the "cotyledon stage" or whether, having entered the plant, they are unable to induce the formation of a nodule. Owing to the transparency of the root

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cortex, it is possible to see a nodule in the very early stages of its growth. When a root hair has been infected the infection strand passes through the cortical cells as far as the endodermis and, along its course, the cortical cells become more densely protoplasmic and commence to divide (fig. 5). In such a very young stage the cells affected are only about 25 in number but their increased opacity renders the "nodule" clearly visible in the living root. The absence ofvisible nodules thus implies that this earliest stage has not even been reached.

It was therefore a question whether the organism enters the root during the

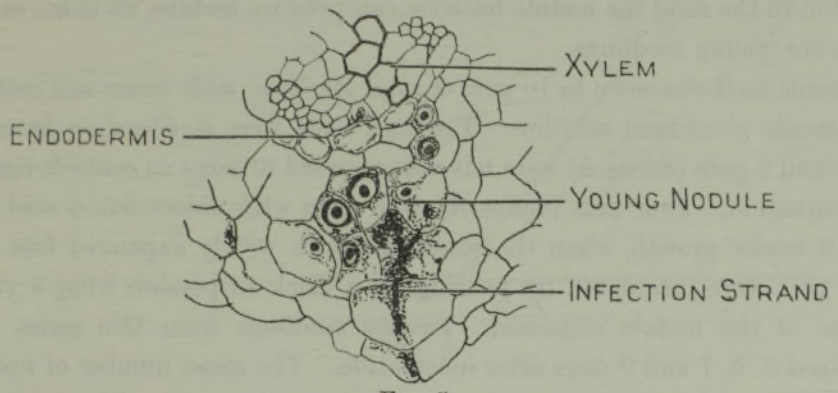

FIG. 5.

cotyledon stage or whether it is then normally unable to infect the root hairs. To determine this, seedlings were grown aseptically in boiling tubes containing a plant-food solution in agar. Twelve seeds, the coats of which had been sterilised by immersion in absolute alcohol followed by 0·2 per cent. $HgCl_2$ washed off with sterile water, were sown in each of 4 tubes. When they had germinated, a suspension of the nodule organism was introduced and the roots were examined at daily intervals, a search being made for infected root hairs. Within 4 days of germination most of the plants showed the first leaf in a closed state. Four days later the majority had the first leaf open. During the 8 days that thus elapsed between inoculation and the opening of the first leaf the roots were surrounded by large numbers of organisms, many in a motile state, but not a single root hair containing an infection thread was seen. The absence of the bacteria other than the nodule organism was shown by plating at the end of the experiment. One day after the opening of the first leaf on the majority of plants about 2 per cent. of the root hairs contained infection threads, which were easily seen owing to their high refractive index.*

* Previous observations on slightly more advanced seedlings showed 4 per cent. of the hairs with infection threads (Thornton (1)). Pierce (2) however, claims to have obtained a much heavier infection on roots placed between layers of filter paper.

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The evidence thus indicates that the bacteria do not normally enter the root before the first leaf opens.

This delay in the infection of the root until the opening of the first leaf may be wholly or partially due to some factor in the plant's physiology or may represent a period of development of the nodule organisms outside the plant necessary to enable them to reach an infective condition (*cf.* Thornton and Gangulee (3)). If the delay were solely due to the organisms, the time taken for nodules to appear should be independent of the age of the seedlings. The following experiment was therefore made to determine how soon after their addition to the sand the nodule bacteria can produce nodules on older, as well as on the young seedlings.

Lucerne seed was sown in 10 pots of sand sterilised with steam and watered with sterile plant-food solution. The seed coats were sterilised as described above and 2 pots (Series A) were left uninoculated to serve as controls against contamination. Four pots (Series B) were sown with uninoculated seed and, after 3 weeks' growth, when the seedlings had a widely expanded first leaf, these pots were inoculated by pouring on a thick suspension from a young culture of the nodule organism. Twenty seedlings from this series were examined 3, 5, 7 and 9 days after inoculation. The mean number of nodules per plant on each occasion is shown in fig. 6. A considerable number of nodules had appeared on the fifth day after inoculation.

That these nodules were produced by the culture added was shown by the almost complete absence of nodules on the control plants, examined at the end of the trial. On the day on which Series B was inoculated another 4 pots (Series C) were sown with seed and similarly inoculated. Seven days later the first leaves of the series were showing but had not opened. On the seventh, ninth, eleventh and thirteenth days after sowing and inoculation 20 seedlings from these pots were examined. The course of nodule appearance is shown in fig. 6. The appearance of the nodules was delayed until the true leaves opened after 9 days although Series B showed that the culture was capable of producing nodules within 5 days from the date of inoculation. The delayed appearance of nodules must therefore be due to the plants.

The rule that no nodules appear before the expansion of the true leaves is not absolute. When a large number of seedlings are grown together in sand culture there is a period during which the first leaf has opened on some plants but not on others. At this time nodules appear on the former plants but also on a few of the plants whose first leaves have not yet opened. Thus in the pot experiment illustrated in fig. 2 the examination made on the fifth day

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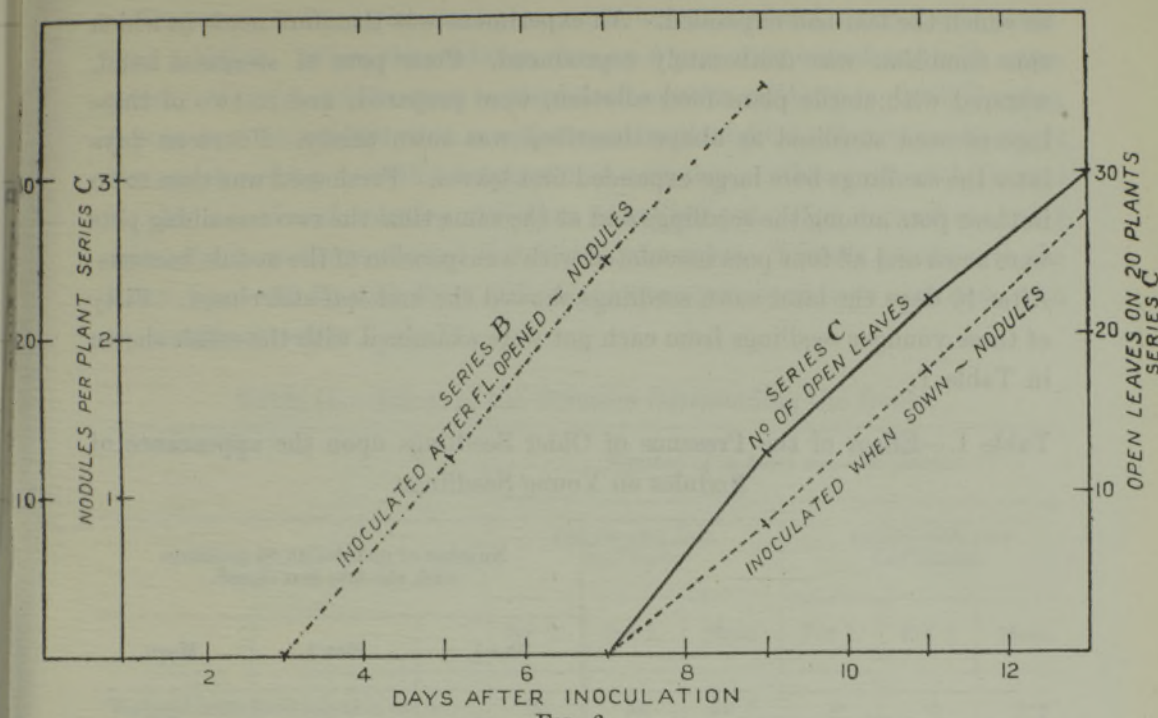

FIG. 6.

after germination showed the following distribution of nodule-bearing plants :—

Nodule-bearing plants,	with leaf open	5
,, ,,	without open leaf	2
Plants without nodules,	with leaf open	3
,, ,,	without open leaf	10

Other examples are shown in the control series shown in Tables I and II. In normal cases only a small proportion of such younger plants bear nodules. In one pot experiment, however, a wholly abnormal percentage of nodules appeared on plants whose first leaf had not yet opened. In this experiment the inoculated seed had been sown thickly in sand watered with plant-food solution. The seed germinated unevenly, some plants showing open leaves after 9 days, while 12 days after sowing only about half the plants had the first leaf open. On the twelfth day 20 seedlings with open leaves and 20 with leaves still closed were taken at random from 4 pots and examined. The former had a total of 96 nodules while the latter had 64.

This experiment was abnormal in that, owing to uneven germination, young plants with the first leaf unopen grew for some days mingled with older plants

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in which the leaf had expanded. An experiment was therefore made in which this condition was deliberately reproduced. Four pots of sterilised sand, watered with sterile plant-food solution, were prepared, and in two of these lucerne seed sterilised as above described was sown thinly. Fourteen days later the seedlings bore large expanded first leaves. Fresh seed was then sown in these pots among the seedlings and at the same time the two remaining pots were sown and all four pots inoculated with a suspension of the nodule bacteria. After 10 days the later sown seedlings showed the first leaf still closed. Fifty of these younger seedlings from each pot were examined with the result shown in Table I.

Table I.—Effect of the Presence of Older Seedlings upon the appearance of Nodules on Young Seedlings.

	Number of nodules on 50 seedlings with the first leaf closed.		
	Plot 1.	Plot 2.	Mean.
Only young seedlings present	6	5	5·5
Young seedlings growing amongst older.....	38	35	36·5

The presence of the older plants had induced the formation of a considerable number of nodules on the young plants growing amongst them at the stage when only occasional nodules would normally have appeared. This influence of the older plants upon their surroundings suggests the extrusion of some substance from the roots. To investigate this possibility an attempt was made to extract the solution from sand cultures in which lucerne seedlings had been growing. Pyrex glass troughs, 10 × 5 inches in area and 2½ inches deep, were filled with sand watered with equal volumes of plant-food solution and sown thickly with lucerne seed. When the seedlings were in the cotyledon stage the contents of one trough was carefully packed into a glass tube, 2 inches in diameter and 3 feet 6 inches long, clamped in a vertical position and fitted at the lower extremity with a perforated rubber bung and a glass delivery tube. The solution was extracted with the pressure of a head of distilled water, about 1 litre of the solution being thus obtained (Extract A). When the seedlings in the second trough had well-expanded first leaves the solution from this culture was extracted in a similar manner (Extract B). The two extracts were sterilised in the autoclave. Their action was tested in the following manner.

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Six pots were filled with sand, sown with lucerne seed and watered with plant-food solution. When the seedlings were up 2 pots were inoculated with a suspension of the nodule bacteria in plant-food solution made up in distilled water, 2 pots were inoculated with a suspension in plant-food solution made up in Extract A and 2 pots with a suspension in a similar solution made with Extract B. 15° c.c. of each solution were thus added per pot. Ten days after sowing some of the seedlings showed first leaves just open. Twenty plants with the leaves open and 20 with them still closed were examined from each pot, with the results shown in Table II.

Table II.—Effect of the Solution Surrounding the Roots.

	Number of nodules upon 20 plants.					
	Plants with first leaf open.			Plants with first leaf closed.		
	Pot 1.	Pot 2.	Mean.	Pot 1.	Pot 2.	Mean.
Watered with food solution alone	25	34	29·5	0	5	2·5
Watered with food solution + Extract A	20	20	20·0	0	7	3·5
Watered with food solution + Extract B	32	34	33·0	18	13	15·5

The extracts have produced no effect on the nodule numbers on such plants as had the leaves already open. The Extract A produced no significant effect at all, but Extract B, prepared from sand in which older seedlings were growing, caused a marked development of nodules on seedlings whose first leaf had not yet opened, increasing their numbers six-fold as compared with the control plants in the same stage. It would thus appear that, when the lucerne seedlings have attained a stage of development marked by the opening of the first true leaf, some substance is extruded from the roots which stimulates the infection of the root hairs by the nodule bacteria.

In order to observe whether the extracts had any influence upon the growth of the nodule organism, the plant-food solution plus 1·5 per cent. agar was made up with distilled water, with Extract A and with Extract B. A similar set of media were made up containing 1 per cent. saccharose. The media were sterilised in the autoclave, poured into sterile petri dishes and streaked with a 2-day old culture of the lucerne nodule organism. After one week's incubation

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at 25° C. scarcely any growth appeared on the media without saccharose. In the presence of saccharose no growth showed on the medium made up with distilled water, a slight opaque growth on the medium made up with Extract A and a considerable slimy growth on the medium containing Extract B. (See Plate 31.)

The substance formed by the older seedlings thus has an effect upon the growth of the nodule bacteria, but this effect would seem not to consist in supplying energy material since it showed itself only when sugar was added. The extracts did not reduce Fehling's solution and tests for sucrose and for pentoses were also negative. A pot experiment was made in order to see whether the presence of an amino acid would induce nodule formation on seedlings before the first leaves opened. Inoculated seedlings were given food solution alone and food solution containing 0·01 per cent. and 1·0 per cent. asparagine. In neither case were the nodule numbers increased by asparagine.

The manner in which the extract stimulates infection is not at present clear. Its effect in increasing the number of bacteria may be of some importance but observations of root hairs in plants grown on agar, referred to above, tend to show that this cannot be a limiting factor since seedlings in the cotyledon stage do not show infected root hairs even in the presence of considerable numbers of the bacteria. It is observable that in this case the bacteria exist either as individuals or else in loose clumps among the root hairs, whereas the infection of a root hair is preceded by the formation of a minute colony of bacteria imbedded in slime, showing as a bright refractile spot on the wall of the root hair. Such spots are illustrated by Marshall Ward (4). The fact that a slimy growth of the bacteria was induced when these were growing on agar containing Extract B may therefore be relevant.

The coincidence in time of nodule appearance with the opening of the first leaf raises the question as to whether these two events are causally connected or whether they are both the effect of some other physiological change in the seedling. To answer this question the course of nodule development was followed on seedlings from which the true leaves were removed. Lucerne seedlings were grown in test-tubes containing plant-food solution inoculated with a suspension of the nodule bacteria. In one series the true leaves were cut off as soon as they appeared; in the second series the cotyledons were cut off but the true leaves allowed to develop, and in the third, control series, the true leaves and cotyledons were left. The course of nodule development is shown in fig. 7.

On the control plants the appearance of the nodules followed the opening

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FIG. 1.—Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts and Saccharose.

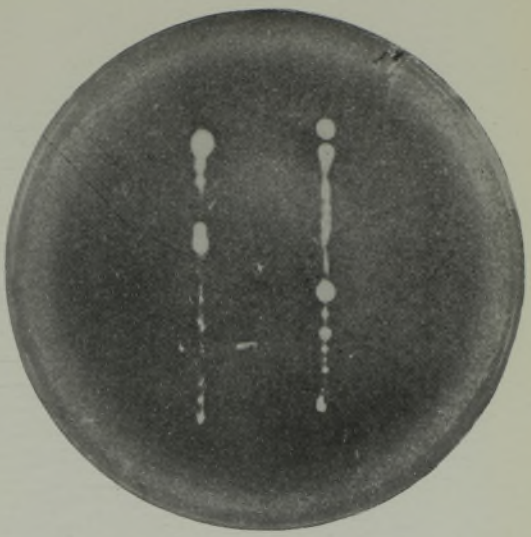

FIG. 2.—Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts, Saccharose and Extract A.

FIG. 3.—Lucerne Nodule Bacteria, growing on Agar with Nutrient Salts, Saccharose and Extract B.

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of the true leaves. In the series with the cotyledons cut, the opening of the true leaves followed a course similar to that shown for the control plants, but

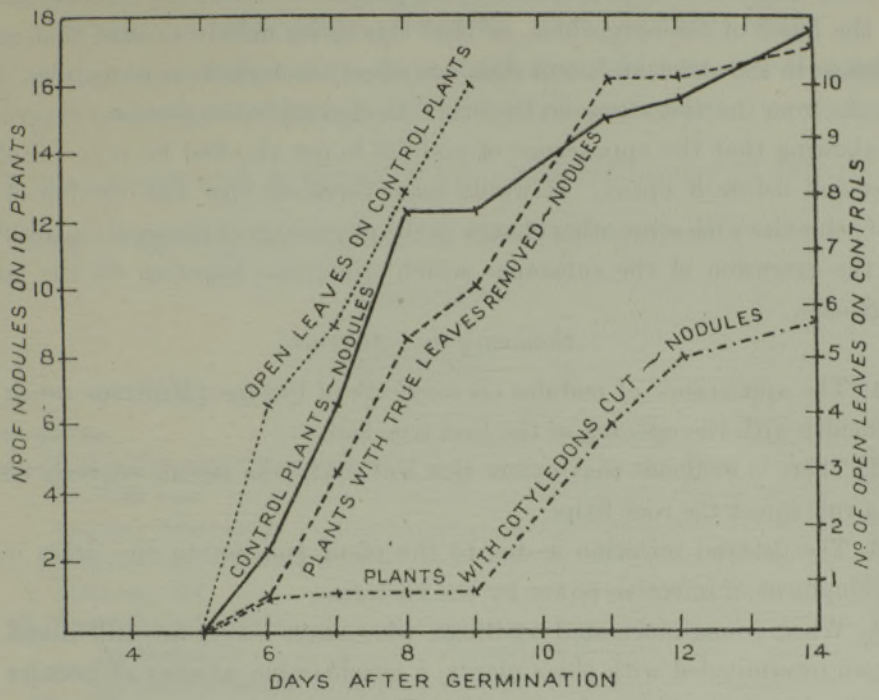

Fig. 7.

the development of nodules was delayed until considerable growth of the true leaves had occurred. This indicates that the passage into the root of substances from the cotyledons is one of the factors controlling the early appearance of nodules. Where the true leaves were removed, however, the normal course of nodule appearance was not significantly altered.

The substance affecting nodule formation is therefore not produced in the true leaf at the time of its expansion since its action is unaffected by the removal of the leaf. It seemed possible that it is formed in the growing point of the top. In the hope of testing this possibility, a sand culture experiment was made, in which seedlings were grown in three series of duplicate pots, those in the first series having the leaves cut before expansion, those in the second series having the terminal bud cut out and those in the third control series being untouched. Three weeks after sowing the control plants had one expanded leaf and, in the majority, a second closed leaf just appearing. Fifteen plants from one pot of each series were then washed and their nodules counted. Twenty days later a second similar examination was made. There were no significant

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differences in nodule numbers between any of the series. The removal of the terminal bud therefore did not affect the number of nodules formed. It was found, however, that in most of the plants two fresh buds were regenerated at the bases of the cotyledons, so that this series merely showed that severe damage to the shoot meristem does not affect the formation of nodules. The results from the first series, on the other hand, confirm the previous experiment in showing that the appearance of nodules is not checked by removal of the first leaf before it opens. It would seem therefore that the opening of this leaf coincides with some other change in the physiology of the seedling, resulting in the extrusion of the substance which stimulates infection by the nodule organism.

Summary and Abstract.

1. The appearance of nodules on seedlings of lucerne (*Medicago sativa*, L.) coincides with the opening of the first true leaf.

2. There is evidence that before this leaf opens the nodule bacteria do not as a rule infect the root hairs.

3. The delayed infection is due to the plant and not to any delay in the development of infective power by the bacteria.

4. When young inoculated seedlings, whose first leaves are still closed, are grown intermingled with older plants, a considerable number of nodules will develop on them, although scarcely any are formed on control seedlings of the same age, grown by themselves.

5. The solution surrounding the roots of seedlings whose first leaves are expanded has an influence in stimulating the appearance of nodules on younger seedlings, and increases the growth of the nodule organism on agar. The solution surrounding the roots of younger seedlings has no such effect.

6. The active substance inducing nodule appearance when the first leaf opens is not formed in this leaf, since the removal of the leaves while still closed has no effect on nodule appearance.

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