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Action of Day-length on Nodule Formation and Chlorophyll Content in Soybean

By

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Introduction

It is well known in agricultural practice that the formation of root nodules by a specific rhizobium strain has a great importance in regard to the production of leguminous plants.

In this respect, not only the suitable bacteria must be present, but nodule formation also requires adequate conditions of the environment. Different data prove for instance that, by some species, the nodules are made in great quantity only in a definite daylength (from Leonard, 1926, to Cajlahjan, 1945; see the discussion). Our own recent experiments gave the same result for soybean (Sironval and Bonnier, 1956): in long day, the nodules were heavy, numerous and red coloured, but in short day, they remained rare and very small, and their inside colour was pink only in the largest of them. This last character corresponds evidently in short day to the lack of leghaemoglobin, whose quantity is under the normal. It indicates that the daylength given to the plant controls, at least partially, the leghaemoglobin synthesis in the root nodules.

The interpretation of such a fact is difficult, but it seems interesting to consider in this purpose some recent experimental data showing that daylength affects profoundly the leaf metabolism. It is possible that there is an unknown relation between the functioning of the leaves submitted to precise

photoperiodic conditions and the capacity of producing effective root nodules containing leghaemoglobin.

In particular, several data one of us has been collecting during the last years (Sironval, 1954—1957) prove that chlorophyll metabolism — a substance in evident chemical connection with leghaemoglobin prosthetic group — is greatly dependent on daylength. Not only the total amount of chlorophyll varies in relation to the photoperiodic treatment, but the ratio between the two forms a and b is affected by photoperiodism. In different cases it is possible to promote in the leaves abnormal chlorophyll formation using photoperiodic conditions.

Therefore, it appears us entirely justified to investigate if chlorophyll metabolism in the leaf is related in some way to the daylength effect on the presence of red nodules on the roots.

The present paper exposes a preliminary essay to measure simultaneously, on one hand the action of photoperiod on the functional effectiveness and the number of the nodules, and on the other the correspondent chlorophyll content of the leaves.

Material and Methods

Soybean seeds are sown on a special soil mixture which is very poor in N (sterile loamy soil mixed with ordinary garden soil in proportion 3 : 1). The culture boxes are 60 cm. long, 22 cm. wide, and 24 cm. high. They have 45 plants each. They are placed in a Phytotron room, the temperature of which is maintained constant at 20°C during the whole culture, the air humidity being fixed at 80 per cent. The light is provided with special "Phytor" fluorescent lamps (intensity of approximately 4,500 lux at the soil surface). In these conditions the plants grow perfectly.

In each experiment one part of the sowing is grown during long day (16 hours) and the other during short day (8 hours). As well in long as in short day two series are made: one is inoculated with a specific rhizobium strain S. 3012, the other not (control; in this case, the soybean roots do not have any nodules). Altogether, four series are formed:

- 1) plants in 16 hours, inoculated with rhizobium;
- 2) plants in 16 hours, not inoculated (control);
- 3) plants in 8 hours, inoculated with rhizobium;
- 4) plants in 8 hours, not inoculated (control).

The inoculation is performed as follows:

holes 2—3 cm. deep are made in the culture soil. Each of them receives one seed on which $\frac{1}{2}$ cm.³ of a rhizobium suspension is dropped. After that,

the seed is covered. The same process is employed for the control, only that the suspension drop consists of pure culture medium without rhizobium.

Two different soybean varieties are used, "Capitole" and "Illini", distinct by the type of growth and the photoperiodic reaction. Soybean "Capitole" is rapidly flowering in 16 hours; it does not grow very high; the fruits are normally produced in 16 hours only. On the contrary, soybean "Illini" is rapidly flowering in 8 hours; it grows higher than the "Capitole", and its fruits are found normal exclusively in 8 hours.

Results

I. Effect of daylength on nodulation

Our results are concerned with four successive experiments, the two first of which are restricted to "Capitole" variety. We expose them separately.

Experiment 1. The sowing of "Capitole" seeds took place the 28/4/1953. Four months later the plants were extracted carefully out of the soil, the roots were washed, and the nodules counted and examined.

The inoculated 16 hours' soybeans had 17—50 nodules, the average being approximately 30 per plant. The inoculated 8 hours' soybeans, 3—21, the average being 9. Table 1 presents the figures for three separate series of 10 plants, both in long and short day. The difference is great; the inoculated long day plants have three times more nodules than the short day ones. (In the same time, the not inoculated control had them in neither daylength).

The examination of the 16 hours' nodules showed that they were normally heavy (5 to 7 mm in diameter), wrinkled, and that their internal tissue was dark red; whereas the 8 hours nodules remained small, their inside tissue varying from yellow to pink, or, in one or two of them, to more near the red. None gave a green colour.

Experiment 2. The second experiment repeated essentially the first, but the nodules were counted separately on the secondary roots and on the

Table 1.

Series nr.	Number of nodules after 4 months culture																					
	in 16 hours day, plant nr.										in 8 hours day, plant nr.											
	1	2	3	4	5	6	7	8	9	10	M	11	12	13	14	15	16	17	18	19	20	M
1	40	25	50	26	30	25	49	25	35	45	35.0	7	19	21	4	13	6	16	13	13	5	10.7
2	25	19	24	50	31	21	26	23	27	35	28.1	3	7	6	8	8	8	15	13	12	9	8.9
3	25	33	25	31	30	17	23	34	25	18	26.1	11	9	7	12	7	5	2	9	7	9	7.8

Effect of daylength on nodulation of "Capitole" soybean; experiment 1; M=mean.

Table 2.

Series nr.	Number of nodules after 2 months culture																					
	in 16 hours day, plant nr.										in 8 hours day, plant nr.											
	1	2	3	4	5	6	7	8	9	10	M	11	12	13	14	15	16	17	18	19	20	M
1 on the crown	5	7	5	8	7	9	4	8	6	8	6.7	5	3	2	4	3	7	4	2	7	0	3.7
1 on the roots	7	4	4	0	0	8	5	3	3	5	3.9	1	3	0	0	0	0	0	1	2	2	0.9
total	12	11	9	8	7	17	9	11	9	13	10.6	6	6	2	4	3	7	4	3	9	2	4.6
2 on the crown	9	6	5	3	11	10	8	9	7	5	7.3	2	4	2	3	0	1	0	3	1	1	1.6
2 on the roots	10	4	2	3	7	4	16	4	4	1	5.5	0	0	0	2	1	4	1	0	4	4	1.6
total	19	10	7	6	18	14	24	13	11	6	12.8	2	4	2	5	1	5	1	3	5	5	3.2

Effect of daylength on nodulation of "Capitole" soybean; experiment 2; the figures are given separately for crown and lateral roots; M=mean.

crown of the soybeans, in the region of the connection primary root-stem (Table 2).

On the crown, the nodules were found bigger and a little more numerous than on the lateral roots. Nevertheless, in both cases, there were always significantly more nodules in long than in short day. Table 2 gives the results after 2 months' culture for two series of 10 plants in each day length. Naturally, the controls showed no nodules.

As in the first experiment, the 16 hours' nodules were heavy and their surface wrinkled; they had a beautiful red colour. On the contrary, the 8 hours' nodules were small and uncoloured.

Experiments 3 and 4. The third experiment was concerned with the two varieties "Capitole" and "Illini". The number of nodules was counted on the roots after 56 days.

At this moment, the inoculated "Capitole" plants had 1—8 nodules in long day (average 3.7 per plant), against none in short day (Table 3, part A).

The inoculated "Illini" plants showed a similar situation; they had 8—25 nodules — nearly all of them being perfectly red coloured — in long day (average 11.9; see Table 3, part A), against only 1—4 white nodules in short day (average 1.6 per plant). In no cases the control showed any nodules.

The fourth experiment, which covered 77 days of culture, confirmed entirely the preceding result. Once more it was seen that, by the two experimented varieties, the nodules grow easier in 16 than in 8 hours day (Table 3, part B).

This shows that the studied phenomenon is distinct from the photoperiodical reaction concerning flowering. Photoperiodically, soybean "Capitole" reacts differently than "Illini": it flowers easier in long day, while "Illini"

Table 3.

A. Experiment 3; figures after 56 days.

Variety	Average number of nodules	
	in 16 hours day	in 8 hours day
"Capitole"	3.7 (1—8)	0 (0)
"Illini"	11.9 (8—25)	1.6 (1—4)

B. Experiment 4; figures after 77 days.

Variety	Average number of nodules	
	in 16 hours day	in 8 hours day
"Capitole"	13.6 (9—15)	3.9 (2—8)
"Illini"	23.0 (10—35)	6.1 (4—9)

Effect of daylength on nodulation of the two varieties "Capitole" and "Illini"; experiments 3 and 4; in parenthesis, the extreme numbers found in a series of 50 plants.

in short day. Nevertheless, they both behave exactly in the same way in regard to the effect of daylength upon nodule formation by the specific rhizobium strain.

It is noteworthy that this identical behaviour answers to a similar growth response to daylength. Table 4 gives the average height of the plants in the

Table 4.

A. Experiment 3; figures after 56 days.

Variety	Average height of the plants (cm)			
	inoculated series		not inoculated series (control)	
	in 16 hours day	in 8 hours day	in 16 hours day	in 8 hours day
"Capitole"	33.1 (28—40)	22.7 (21—25)	31.0 (28—37)	23.0 (19—28)
"Illini"	65.2 (50—88)	37.9 (29—52)	78.0 (55—100)	33.0 (13—62)

B. Experiment 4; figures after 77 days.

Variety	Average height of the plants (cm)			
	inoculated series		not inoculated series (control)	
	in 16 hours day	in 8 hours day	in 16 hours day	in 8 hours day
"Capitole"	33.4 (29—41)	24.2 (22—27)	31.2 (28—37)	22.5 (20—26)
"Illini"	74.8 (55—100)	39.2 (28—55)	86.5 (60—110)	35.3 (28—39)

Effect of daylength on growth of the two soybean varieties "Capitole" and "Illini"; experiments 3 and 4; in parenthesis, the extreme numbers found in a series of 50 plants.

experiments 3 and 4. It appears that, in these experiments, "Capitole" and "Illini" were both regularly bigger in long day. This is true as well in the presence (inoculated series) as in the absence of nodules (not inoculated series, control).

This feature supports the conclusion that *normal nodulation occurs only in the daylength permitting a certain sufficient rate of growth*. In other words, it seems that the formation of effective red nodules not only acts — as it is well known — upon plant vigour, *but also depends on it in some degree*.

II. The chlorophyll content of the leaves

The chlorophyll content has been measured as follows:

samples 0.25 cm.² were taken of 10 homologous leaves, each of them being chosen on a different plant; they were extracted together with aqueous acetone in a small mortar. After suitable preparation and dissolution in ether the extract was examined in a Beckman DU spectrophotometer and the chlorophyll quantities calculated by Comar's method (1942).

1. *Chlorophyll content of soybean "Illini"*. We have measured the chlorophyll content in the first leaves of the 8 and 16 hours' soybeans, 25, 35, and 45 days after germination.

In general, in short as in long day, the chlorophyll level of the control plants, relatively low in the young leaf, increased with the blade size (for instance, it went from near 1.5 mg./100 cm.² till 4.0 mg./100 cm.²).

Table 5.

Date of the dosage	25th day			35th day			45th day		
	S.D.	L.D.	Diff.	S.D.	L.D.	Diff.	S.D.	L.D.	Diff.
Order of the leaves:									
1st leaf	2.46	3.24	+0.78	3.31	3.53	+0.22	—	—	—
2nd leaf	1.56	2.94	+1.38	2.93	3.92	+0.99	<u>4.14</u>	<u>2.73</u>	-1.41
3rd leaf	—	—	—	3.30	3.46	+0.16	<u>4.44</u>	<u>3.52</u>	-0.92
4th leaf	—	—	—	—	—	—	<u>3.44</u>	<u>3.39</u>	-0.05

Effect of daylength upon chlorophyll content of the control "Illini" soybean (cultivated without rhizobium); S.D.=short day; L.D.=long day; chlorophyll in mg/100cm.²; Diff.=difference: (L.D.)-(S.D.).

However, the pigment formation was not exactly the same in both daylengths. In the beginning, the 25th day, the chlorophyll quantity was lower in short day. But, after 10 days, the difference had diminished and it was completely reversed at the 45th day dosage (Table 5). At this moment, the long day leaves were no more accumulating chlorophyll, while the short day ones continued to augment their pigment content. This reflects a differentiation

Table 6.

Daylength	Measures	Young leaves			Adult leaves			Old leaves		
		+Rh	-Rh	Diff.	+Rh	-Rh	Diff.	+Rh	-Rh	Diff.
8 hours day	1	2.52	2.46	+0.06	2.97	2.93	+0.04	3.28	3.31	-0.03
	2	1.65	1.56	+0.09	2.90	2.57	+0.33	4.28	4.14	+0.14
	3	—	—	—	3.06	3.30	-0.24	4.27	4.44	-0.17
	4	—	—	—	3.70	3.44	+0.26	—	—	—
16 hours day	1	3.08	3.24	-0.16	4.31	3.92	+0.39	3.10	3.53	-0.43
	2	3.04	2.94	+0.10	4.12	3.92	+0.20	3.16	3.52	-0.36
	3	2.28	2.25	+0.03	3.64	3.46	+0.18	—	—	—
	4	3.16	2.66	+0.50	3.97	3.39	+0.58	—	—	—

Effect of the inoculation by rhizobium (+Rh) on the chlorophyll content of the leaves of "Illini" soybean in short and long day; not inoculated control=(-Rh); Diff.=difference (+Rh)-(-Rh); chlorophyll in mg/100 cm.².

in the properties of the leaves ageing in long and in short day (see Table 5, underlined figures).

As a rule, the type of pigment formation did not essentially change when the soybeans were inoculated with rhizobium, but the absolute pigment quantities were a little modified.

Table 6 compares some measures we have made in young, adult and old leaves, in short and long day, on one hand in presence of rhizobium (+Rh), and on the other in its absence (control; -Rh).

It is easily seen that, by young and adult leaves (14 measures), the chlorophyll content was generally a little higher in the inoculated soybeans. Only two cases on 14 showed more chlorophyll in the control.

The augmentation induced by inoculation has been found in short as well as in long day. However, it must be emphasised that, in several cases, it was more marked in long day (underlined figures in Table 6); such increases were not recorded in short day. It is possible that these particular data are in relation with the previously demonstrated fact that the nodules are more numerous and effective in a 16 hours day.

A very curious thing is that the major chlorophyll content of the inoculated plants has been observed only in the young and adult leaves, but not in the old ones. Here, the inoculation induced generally a lower pigment level. The fact was noted in many measurements outside the figures of Table 6, so that it is not a hazard (see also Table 8 on "Capitole" variety). Still more, as seen in Table 6, the decrease of the pigment content in the old leaves seems to be more evident in long day, in the presence of numerous rhizobium efficient nodules.

2. *Chlorophyll content of soybean "Capitole"*. Considering first the control plants, "Capitole" accumulated more than "Illini", specially in the old

Table 7.

Date of the dosage	25th day			35th day			45th day		
	S.D.	L.D.	Diff.	S.D.	L.D.	Diff.	S.D.	L.D.	Diff.
Order of the leaves:									
1st leaf	2.46	3.26	+0.80	3.58	4.01	+0.43	5.30	4.58	-0.72
2nd leaf	1.71	2.99	+1.28	3.22	3.96	+0.74	5.84	6.25	+0.41
3rd leaf	—	—	—	2.19	4.21	+2.02	5.82	7.51	+1.69

Effect of daylength upon chlorophyll content of the control "Capitole" soybean (cultivated without rhizobium); S.D.=short day; L.D.=long day; chlorophyll in mg/100 cm.²; Diff.=difference: (L.D.)-(S.D.).

leaves. The comparison of Table 7 with Table 5 indicates, for instance, that the third old leaf in long day contained two times more chlorophyll in "Capitole" than in "Illini".

Besides this, "Capitole" control plants differed from "Illini" ones in two other respects (Table 7):

1) Chlorophyll accumulation in "Capitole" leaves appeared regularly as a long persisting process. Even in the old leaf, we never found a decrease of the content. On the contrary, very much pigment continued to accumulate in long as well as in short day.

2) In "Capitole", the 16 hours figures remained continuously higher than the 8 hours ones (only one exception for the first leaf).

As indicated by other facts (Sironval, 1957), the recorded differences are probably in relation to the distinct photoperiodic behaviour of the experimented varieties.

In spite of this, the effect of inoculation by specific rhizobium was approximately the same for "Capitole" as for "Illini".

Table 8.

Daylength	Measures	Young leaves			Adult leaves			Old leaves		
		+Rh	-Rh	Diff.	+Rh	-Rh	Diff.	+Rh	-Rh	Diff.
8 hours day	1	2.48	2.46	+0.02	3.69	3.58	+0.11	5.11	5.30	-0.19
	2	1.85	1.71	+0.14	3.26	3.22	+0.04	5.61	5.84	-0.23
	3	2.29	2.19	+0.10	—	—	—	6.30	5.82	+0.48
16 hours day	1	3.47	3.26	+0.21	3.91	4.01	-0.10	4.08	4.58	-0.50
	2	3.37	2.99	+0.38	4.47	3.96	+0.51	6.01	6.25	-0.24
	3	2.72	2.45	+0.27	3.81	4.21	-0.40	7.32	7.51	-0.19

Effect of the inoculation by rhizobium (+Rh) on the chlorophyll content of the leaves of "Capitole" soybean in short and long day; not inoculated control=(-Rh); Diff.= difference (+Rh)-(-Rh); chlorophyll in mg/100 cm.².

Table 8 shows that the young "Capitole" leaves had regularly a little more pigment in the presence of nodules than in their absence. But, in the adult leaves, the picture changed: in several cases, there was more pigment in the control plants without rhizobium. Finally, as the leaves became older, a lower chlorophyll level in the inoculated soybeans was found as a rule. This evolution was particularly clear in long day, when the nodules are just functioning effectively.

Also, the facts concerning "Capitole" soybean correspond exactly to those presented as regard to the "Illini" variety. In both cases, *the presence of effective nodules, especially in long day, acts on chlorophyll accumulation, tending to increase its level in the young leaves and to decrease it in the old ones.* Although existing, the effect does not seem so marked in short day, when the inoculation does not induce the formation of numerous effective red nodules.

Discussion

The idea that nodule formation in Leguminosae could be affected by daylength arose from observations upon relations existing between carbohydrate synthesis and nitrogen fixation. It was supposed that, in diminishing carbohydrate synthesis in the leaves, short day treatment or exposure to low light intensity could act against nodulation. (Leonard, 1926; Rosenfels, 1927; Orcutt and Fred, 1935). However, 1) in some cases, the expected positive correlation between carbohydrate level and nodulation has been found completely reversed (see Orcutt and Fred, 1935), and 2) the results varied greatly from one experimenter to another. Also, Rosenfels found in soybean a better nodulation in short day, while Eaton in long day.

Starting from another point of view, Cajlahjan and Megrabjan (1945) performed more recently extensive experiments concerning different species. They regularly found — in opposition to other data of Moshkov (1939) — a better nodulation in long day. The described effect is entirely distinct from photoperiodic reaction, since long and short day plants react in the same manner, but it is related to growth rate. The authors think that it can be partially due to the higher content of growth hormones in the root parenchyme in long day. This opinion is near to a previous suggestion of Thimann (1936) that nodule formation is incited by the indoleacetic acid liberated by the rhizobium.

Our own results, — obtained in many repetitions and in strictly controlled environmental conditions —, prove that, according to Eaton and to Cajlahjan and Megrabjan daylength affects nodulation in soybean in such a way that the number and effectivity of the nodules decreases strongly in short day.

We observe — as Cajlahjan and Megrabjan do — that on one hand, the photoperiodic reaction and on the other, the action of daylength on nodulation are distinct phenomena. "Capitole" and "Illini" soybeans are photoperiodically different, but they behave in the same way for nodulation.

We find also that nodulation is related to growth rate. Daylength regulates soybean growth and at the same time the ability of the rhizobium to produce numerous nodules containing leghaemoglobin.

However, the formation and the functioning of the nodules appears us as probably related, not only to the growth rate, but also to the properties of the leaves affected by daylength, in particular to their chlorophyll metabolism. We demonstrate, for instance, that specially in long day, the effectivity of the nodules tends to modify the chlorophyll content of the leaf blade, increasing it in the young leaves and decreasing it in the old ones. The fact cannot be explained simply. Naturally, one can propose that a greater nitrogen fixation by red nodules favours chlorophyll synthesis in the young leaves. But, how explain the decrease observed in the old ones?

Probably, very complex interactions, involving many metabolic processes, are here concerned with. In particular, from our data, one cannot exclude the possibility of some unknown physiological connection between leghaemoglobin and chlorophyll metabolisms.

Summary

The authors study the effect of daylength upon nodulation and chlorophyll content in soybean. Nodules are found heavier, more numerous and more effective in long day than in short day. The effect is distinct from the photoperiodic reaction; it is related to the control of growth rate by daylength. The chlorophyll content of the leaves is affected simultaneously by the photoperiodic treatment and by the presence of effective red nodules, following certain rules which are described.

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