BIOLOGICAL NITROGEN FIXATION IN PLANTS OF *PHASEOLUS VULGARIS* AS AFFECTED BY PHOTOSYNTHATE SUPPLY.[1]

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1. INTRODUCTION

Nitrogenase activity in the nodules varies with legume species and with stage of development. Maximum activities were detected during vegetative growth (*11*), after flowering (*1, 5, 17*) and in the pod-filling stage (*10, 24, 26*). In *Phaseolus vulgaris*, a sharp drop of nitrogenase activity occurred just after flowering (*1, 5, 17*). BAIRD and WEBSTER (*1*) showed a coincidence of the flowering period with the beginning of senescence in the nodules; this fact was attributed to a shortage of carbohydrates in the nodules due to the senescence of the lower leaves, the main sources of assimilates for the nodules, and for the appearance of new sinks in the plant.

In order to gain additional information about this matter, the relationship

between biological nitrogen fixation and photosynthate translocation to the nodules of dry bean plants was studied.

2. MATERIALS AND METHODS

Seeds of *Phaseolus vulgaris,* cv. Carioca, were sterilized with absolute ethanol for 1 minute, with 15% H_2O_2 for 5 minutes, and then washed six times with sterile water. Following inoculation with a liquid culture of *Rhyzobium phaseoli* C-05 (CENA, Piracicaba), they were placed to germinate in the greenhouse in plastic trays containing washed and sterilized sand.

Nine days after, two seedlings with fully expanded primary leaves were transferred to 9-liter plastic pots containing HOAGLAND and ARNON culture solution (9), pH adjusted to 6,0, under continuous aeration. The solution only contained nitrogen (KNO_3 2.5 mM) in the first 13 days to act as a «starter dosis» for the initial plant growth before the nodules became active, according to LIMA (14). After that, the plants were grown in a nitrogen-free culture solution changed every 15 days with its pH being daily adjusted to 6.0.

Two groups of 32 pots each were randomly distributed in the greenhouse. In the first group the plants remained intact during development, while in the second one the flower buds were removed. Weekly, and until 73 days after seeding, 4 pots of each group were randomly selected and transported to the laboratory.

The plants were adapted to the laboratory conditions for 16 hours, where the diurnal ilumination was made with OSRAM mixed-fluorescent lamps WHL/C, 250N,220-270 V, yielding 5 klux of light intensity. Then, one of the plants of each pot had one of its trifoliates of the 3rd, 4th, 6th of 7th node, counted from the cotyledon one, according to the plant stage of development, introduced within an 865 ml plastic chamber where 4 ml of $^{14}CO_2$ (sp.act. 5uCi/ml) were injected with a gastight syringe. The labelled gas was externally generated by the reaction of ^{14}C-sodium bicarbonate and lactic acid in a device described by SILVA (23).

Following an exposure time of 1 hour to the $^{14}CO_2$, the air of the chamber was made to bubble into a Ba(0H)$_2$ solution to eliminate any residual radioactive gas and the treated trifoliate was removed from the chamber. The treated plants remained in the laboratory conditions for 23 hours more and then were separated into the following parts: leaves exposed to the $^{14}CO_2$, upper and lower trifoliates (with regards to the treated one), upper and lower stems, roots, nodules and, when present, flowers and fruits. The plant parts had their dry weights determined by oven drying at 70°C for 48 hours and then were ground in a WIG-L-BUG AMAL-GAMATOR to a texture equivalent to 200 mesh.

Aliquots of 20 mg of the ground material and 1.0 ml of TRITON X-100 were introduced into 20 ml glass counting vials. After homogenization in a Vortex mixer, the suspension was dispersed in 10 ml of a scintillation cocktail containing 30 g of Cab-0-Sil, 4 g of PPO and 250 mg of dimethyl POPOP per liter of toluene. The ^{14}C activity of the vials was computed in a Liquid Scintillation Counter, Beckman, model LS-233, using a ^{14}C full window isoset and maximal 2 sigma error of 1.5%. No corrections for the quenching effect were made since it remained reasonably constant.

The second plant of each pot (not exposed to $^{14}CO_2$) was cut at the height of the cotyledon node and had its roots placed inside 300 ml flasks of hermetical closure. After replacement of 13% of the flask air volume by acetylene, the flasks were incubated for 30 minutes and aliquots of 500 ul removed to estimate the ethylene content by GLC (6). A model 2100 Varian Gas Chromatograph was used,

together with a stainless steel column (L = 2 m; i.d. = 3.2 mm) packed with POROPACK-R (80-100 mesh) and employing nitrogen as the carrier gas. The temperature conditions were: injector (70ºC), column (50ºC) and detector (120ºC). The rate of acetylene reduction, expressed as umoles of C_2H_4/plant/hr was calculated by peak height measurements.

In the same plant material used for the test of nitrogenase activity, the nitrogen content of the plant parts and, by summation, of the whole plants, was determined by the Kjeldahl technique. The nitrogen increments in the whole plant were calculated by the difference between the nitrogen content in a given sampling time and in the first one.

3. RESULTS AND DISCUSSION

The two groups of plants showed a marked visual contrast. In the fruit development period (59 days after seeding) the upper leaves of the intact plants were light green and the lower ones yellow, suggesting nitrogen deficiency symptoms as described by EPSTEIN (4). This was confirmed by the low leaf nitrogen content of 1.66%, while the normal value is around 2.6% (3). The nodule tissue, by its turn, showed visible signs of degeneration. The plants from which the flower buds were removed, thereafter called «without flowers», presented, at the same time, a greater number of leaves with a dark green color (%N = 2.6). Signs of nodule degeneration appeared only 73 days after seeding when the plants initiated their senescence.

Time curves of nitrogenase activity, ^{14}C activity, nitrogen amount, and nodule dry weights behaved similarly in the intact plants (Figures 1 and 2). The values of these parameters increased sharply from almost zero at 24 days after seeding, reached maxima at the onset of the fruiting period and declined thereafter. This decline can be attributed both to the appearance of new sinks of assimilates in the plants, the fruits, and to the initiation of senescence in the lower leaves, the major sources of these compounds for the nodules (27), bringing about a shortage of photosynthates in the nodules which began to degenerate. The observed behavior of the nitrogenase activity agreed with those related by FRANCO *et al* (17) and BAIRD and WEBSTER (1) in *Phaseolus*. The relationship between nitrogenase and ^{14}C activities confirmed the great importance of the availability of readily formed photosynthates for an efficient nitrogen fixation, as previously reported by LAWRIE and WHEELER (11, 13) in peas and *Vicia faba*. Several other authors (7, 19,21,22,28,30) had also shown a direct dependence of nitrogenase activity on plant net photosynthesis.

The plants «without flowers» also showed shape similarity among the curves of the measured parameters (Figures 3 and 4), but quite distinct from those exhibited by the intact plants (Figures 1 and 2). Exception for the nodule weights curve, the other three had a first point of maximum 45 days after seeding, higher than the correspondent values for the intact plants. Then followed a phase of decline until the beginning of the fruiting period when these parameters were maximal in the intact plants. The low values of nitrogenase activity in this phase (Figure 3) could be related to the enzyme activity dependence on the plant nitrogen demand as suggested by HARDY *et al* (6). With the removal of the flower buds, the absence of these potential sinks in this period may have caused a drop in the nitrogen demand by the plant and a consequent decrease of the enzyme activity. LAWRIE and WHEELER (12) also reported a drop of the nitrogenase activity of pea plants upon removal of the flower buds but not a concomitant
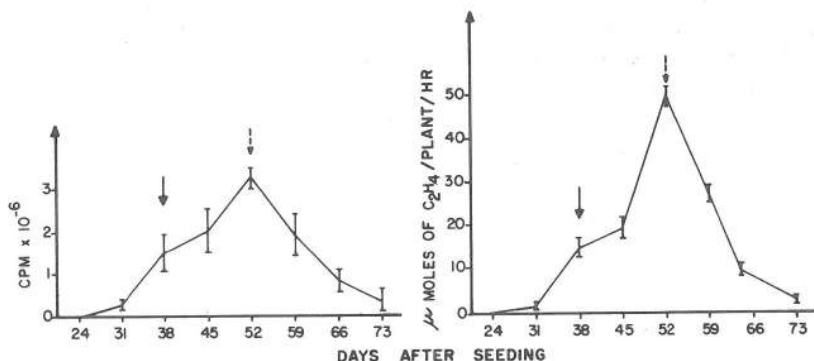
FIGURE 1 - ^{14}C and nitrogenase activities in root nodules of intact bean plants in several stages of development. Vertical lines mean standard deviations of 4 replications. ▼= beginning of flowering; ▽=beginning of fruiting.
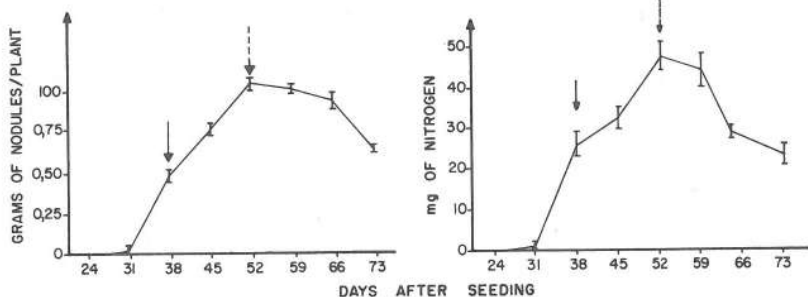

FIGURE 2 - Dry matter weights and nitrogen amounts in root nodules of intact bean plants in several stages of development.

decrease of nodule ^{14}C activity. LOVEL *et al* (15), however, showed a reduced flow of assimilates to the nodules of pea plants when the apical buds were removed.

Although the nodules are considered as metabolic sinks (16, 18) it is quite possible that the decrease of nodule radioactivity (Figure 3) had resulted from a preferential photosynthate partition benefiting the shoots mostly as a response to the stimulation of the vegetative growth. This stimulus was probably caused by a change in the plant hormonal balance induced by the removal of the flower buds (25).

From 52 days after seeding on, the three parameters began to increase again
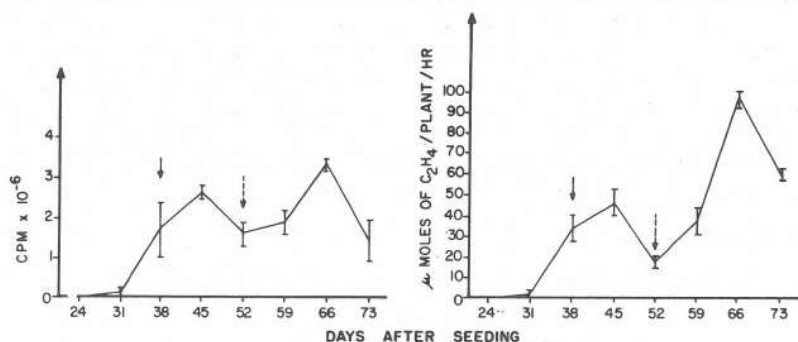
FIGURE 3 - ^{14}C and nitrogenase activities in root nodules of bean plants "without flowers" in several stages of development. Vertical lines indicate standard deviations of 4 replications. ↓ = beginning of flowering; ⌄̇ = beginning of fruiting.
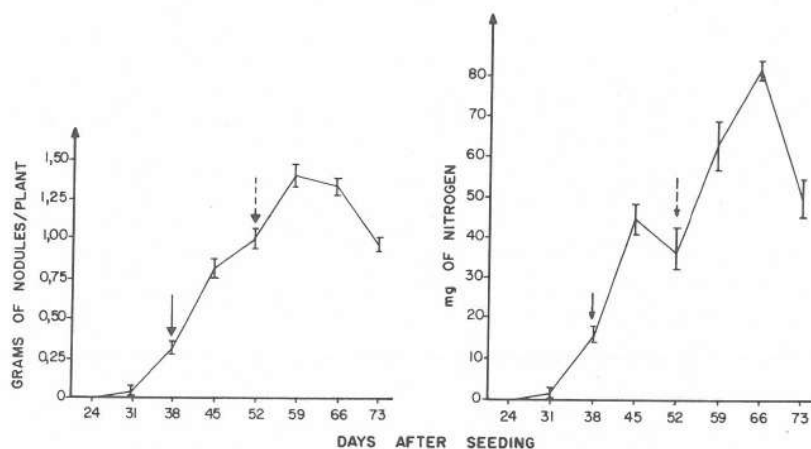

FIGURE 4 - Dry matter weights and nitrogen amounts in root nodules of bean plants "without flowers" in several stages of development.

(Figures 3 and 4) in coincidence with the period of formation of new leaves. A second point of maximum, higher than the first one, was reached in 66 days while the corresponding situation in the intact plants was an abrupt decline (Figures 1 and 2). It could be inferred that despite the strong draining of assimilates produced by the stimulus of the vegetative growth, the new leaves, acting as sources,

fed the nodule system properly. The same kind of behavior was found by ROPO-NEM and VIRTANEM (*20*) in pea plants in which the removal of the flower buds stimulated vegetative growth and increased both nitrogenase activity and nodule growth.

It should be stressed that the plants «without flowers», at the end of the observation period, accumulated 70% more nitrogen than the intact ones (Figure 5). The drop of the nitrogen increments beginning 59 days after seeding in the intact plants was probably a result of leaf fall in this period.
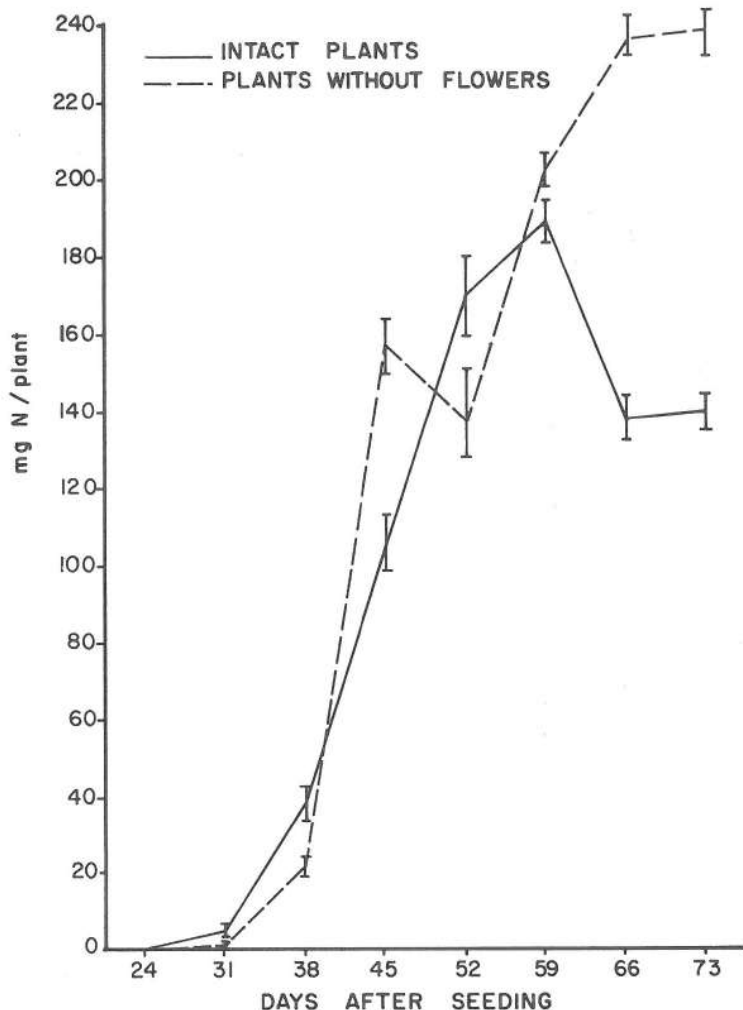

FIGURE 5 – Nitrogen increments of bean plants in several stages of development. Vertical lines indicate standard deviations of 4 replications.

Partition of the ^{14}C-assimilates among the various plant parts, except for the exposed trifoliates, was about the same for both groups of plants in the pre-flowering stage (Figures 6 and 7) when the lower stems and roots contributed more than 70% of the total radioactivity. A similar behavior was found in *Phaseolus* by several authors (2,27,29).

In the flowering stage, when the nodules were active metabolic sinks, the fraction of nodule ^{14}C activity increased about 8.5 and 15 times in the intact and in the plants «without flowers» respectively, the greatest reduction being found in the roots (Figures 6 and 7). Until the beginning of the fruiting period, the nodules of the intact plants continued to accumulate assimilates, though less intensively, while in the plants «without flowers» the trend was a slight decline. The roots of both groups of plants had an equal gain of accumulated radioactivity on a percent basis as a result of a greater flow of carbohydrates coming from the shoots.

The most striking difference in the ^{14}C activity partition occurred in the pod-filling stage. About 74% of the total radioactivity was in the fruits of the intact plants, with the lower stems, roots and nodules presenting the greatest reductions. This behavior was similar to those reported by HERRIDGE and PATE (8) in *Vigna unguiculata*. The high flow of assimilates to the fruits, reducing drastically their availability to the nodules, seemed to be the major cause of nodule degeneration and of the sharp drop of biological nitrogen fixation in the bean plant.

Yet because of the absence of fruits, the nodules of the plants «without flowers» contained on a percent basis about 5 times more assimilates than in the
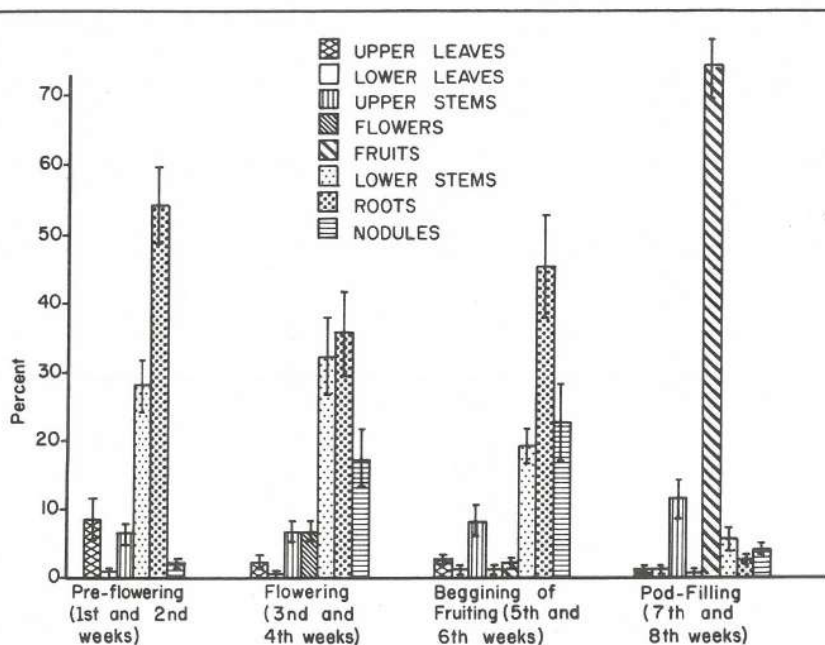

FIGURE 6 - Percent of plant ^{14}C activity in the parts of intact bean plants. Vertical lines indicate standard deviations of 4 replications.

intact plants (Figures 6 and 7). This explains why the nodules of those plants remained active in this stage. The greater percentage of ^{14}C activity in the upper stems of the plants «without flowers» as compared with the intact ones, could be justified by the stimulus to the vegetative growth induced by the removal of the flower buds in those plants.

4. SUMMARY

The effect of photosynthate availability on biological nitrogen fixation in root nodules of *Phaseolus vulgaris,* growing in nitrogen free culture solution in the greenhouse, was studied by leaf exposure to $^{14}CO_2$ in several stages of development.

The plants that remained intact during development showed highest ^{14}C activity, nitrogenase activity and nitrogen amount at the onset of fruiting, declining thereafter. About 74% of the total radioactivity was found in the developing seeds, which acted as strong sinks of assimilates, causing nodule degeneration and nitrogen deficiency in these plants.

In the plants from which flower buds were removed, the maximum values of the studied parameters coincided with the pod-filling stage, when the nodules accumulated 5 times more radioactivity than in the intact plants. This kept nodules active and obviated nitrogen deficiency.
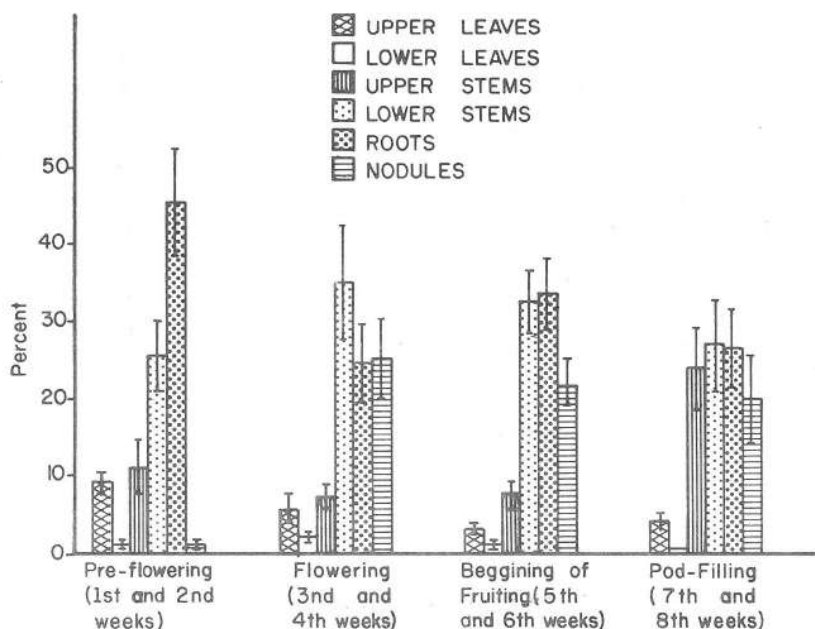

FIGURE 7 - Percent of plant ^{14}C activity in the parts of bean plants "without flowers".

The similar behavior of the curves of the measured parameters revealed a high dependence of biological nitrogen fixation on photosynthate supply to the nodules.

5. RESUMO

Estudou-se o efeito da disponibilidade de fotoassimilados sobre a fixação biológica do nitrogênio em nódulos de *Phaseolus vulgaris*. Para tal fim, as plantas foram cultivadas em solução nutritiva isenta de nitrogênio, em casa de vegetação, sofrendo exposição foliar ao $^{14}CO_2$ em diversos estádios de crescimento.

Nas plantas que permaneceram intactas durante o desenvolvimento, as atividades da nitrogenase e do ^{14}C, bem como a quantidade de nitrogênio nos nódulos, tiveram valores máximos no início da frutificação, declinando nos períodos subseqüentes. No período de enchimento dos grãos, cerca de 74% da radioatividade localizaram-se nas sementes em formação, que atuaram como fortes drenos de fotoassimilados, causando degenerescência nodular e deficiência de nitrogênio nas plantas.

Nas plantas que tiveram os botões florais removidos, os valores máximos dos parâmetros estudados ocorreram no período de enchimento dos grãos, tendo os seus nódulos acumulado cinco vezes mais radioatividade do que os das plantas intactas. Isto manteve os nódulos ativos e a deficiência de nitrogênio ausente.

A similaridade de comportamento dos parâmetros medidos confirmou a grande dependência da fixação biológica do nitrogênio para com o suprimento nodular de fotoassimilados.

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