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Non-photosynthetic CO_2 Fixation by the Nodulated Roots of Legumes: *In situ* Measurement with $^{14}CO_2$ during the Growth Cycle of Soybean*

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Abstract

CO_2 fixation in nodules and roots has been estimated during the whole cycle of soybeans grown on sand and in soil by exposing the root system to $^{14}CO_2$. In sand culture, CO_2 fixation by roots was measured on denodulated plants. It accounted for more than 50% of the total CO_2 fixation. The strong correlation found between nodule CO_2 fixation and nitrogenase activity fixation (as indicated by acetylene reduction) throughout the growth cycle has been used to determine the magnitude of nodule CO_2 fixation of soybean grown in soil. Root contribution was assessed by difference. Maximum rate for the roots was observed during the vegetative period (322 μgC h^{-1} pl^{-1}), while for the nodules it occurred during the pod filling stage (186 μgC h^{-1} pl^{-1}).

Keywords: $^{14}CO_2$, *Glycine max.*, phosphoenolpyruvate carboxylase, roots and nodules CO_2 fixation.

Introduction

In legume-*Rhizobium* associations, nitrogen reduction requires a large amount of energy, which is supplied by the host plant. This is mainly supported by concurrent photosynthesis. However a certain quantity of carbon originates from the phosphoenolpyruvate carboxylase activity (PEP Case) of nodules. Many workers have suggested that the carbon compounds thus produced help replenish the tricarboxylic acid cycle at a level adequate to support high rates of NH_4^+ assimilation, amino acids biosynthesis, cations transport, and energy-yielding metabolism (Christeller et al., 1977; Vance et al., 1985; King et al., 1986).

Carbon dioxide is fixed by the roots as well as by the nodules of many legumes

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(Christeller et al., 1977, Coker and Schubert, 1981). Therefore, the relationship between CO_2 and N_2 fixation can only be estimated after determining the contribution of nodules to total CO_2 fixation. This experimental constraint can partly explain why quantitative estimation of CO_2 fixation has often been made on excised organs. However, such artificial conditions induce strong perturbations of both root and nodule metabolism (Coker and Schubert 1981; Maxwell et al., 1984; Vance et al., 1985), and lead to difficulties in interpreting data and extrapolating experimental results to natural conditions. In situ measurements in sand do allow comparison of nodulated and denodulated plants, something which cannot be achieved in soil without considerable disturbances.

The objective of this study was to estimate CO_2 fixation by the root systems of soybeans grown in soil. The relative contribution of roots and nodules to total CO_2 fixation was first investigated on nodulated and denodulated soybeans grown on sand.

Material and Methods

Plant culture. Soybean (*Glycine max.* (L.) Merr. cv Hodgson), inoculated with *Rhizobium japonicum* strain G49, were grown outside and in a greenhouse. Outside, seeds were planted in soil in PVC containers and irrigated when necessary. The soil was originally devoid of *R. japonicum.* In the greenhouse, soybeans were sown in sand-filled pots and supplied every three hours with a nutrient solution free of nitrogen (Matsumoto et al., 1975). This was achieved automatically by raising the level of the solution into the bath containing the pots. The plants were exposed to natural daylight, and the photoperiod was extended to 16 hours with artificial lighting.

Labelling. The principle of the method was to expose the nodulated root system to an atmosphere enriched in $^{14}CO_2$. CO_2 fixation was then determined by the ratio between ^{14}C incorporated in plant and nodule material and the specific activity of the root atmosphere. This required that external and internal specific activities should be identical, which can only be achieved by maintaining high external CO_2 concentration (Christeller et al., 1977). Furthermore, high CO_2 concentration decreased the dilution effect of the respired CO_2 on specific activity. For these reasons, each labelling had been done with 5% of CO_2 (external), a level which can be found in the rhizosphere (Coker and Schubert 1981).

At different times during growth, (vegetative V3 and V5, reproductive R2, R3 and R5 in sand, R2, R3, R5, R6, R7 and R8 in soil according to the classification of Fehr et al., 1971) several plants were chosen and placed in the laboratory under artificial light at a quantum flux density of 600 μmol m^{-2} s^{-1}. The temperature was maintained at 25°C. Root and shoot atmospheres were carefully separated, using a physiological molding material (Prestik) placed around the cover of the root container and at the base of the stem. Root atmosphere was circulated with a pump in a closed system, wherein CO_2 and ^{14}C were generated by injection of non-labelled and labelled sodium carbonate into a vessel containing H_2SO_4. Aliquotes of root atmosphere were periodically sampled and analysed for ^{14}C content by scintillation counting. At the end of each labelling period, the system was flushed with CO_2-free air or water, and radioactivity was trapped on soda lime. The containers were then opened.

The slow diffusion of gases in soil required that, labelling periods be extended to 3 hours (Warembourg and Roumet 1989). To prevent dilution of $^{14}CO_2$ with the respiratory efflux of roots, nodules, and rhizosphere microflora, CO_2 and ^{14}C were regulated using an infrared gas analyser and an ionization chamber respectively operating solenoid valves in order to direct the air stream though soda lime or to add $^{14}CO_2$ (Warembourg and Roumet 1989). The specific activity was 1 kBq mgC^{-1} during each exposure. Oxygen concentration was also regulated to avoid any change in the various respiratory processes.

In the sand culture, 3 plants were denodulated in water 4 hours before each exposure to labelled gases. Together with 3 nodulated plants uprooted and freed of sand, they were placed in a 3 liter container and their root systems exposed to $^{14}CO_2$ at 50 kBq mgC^{-1}. In this case, diffusion of gases was immediate and labelling was only maintained during 5 minutes, without any regulation of CO_2, O_2, or ^{14}C concentration.

Plant sampling. It has been shown that part of the fixed C is used in metabolic processes, and that most of it is released as CO_2 soon after incorporation (Coker and Schubert 1981; King et al., 1986). In soil experiments, this process has already occurred due to the length of the labelling period. Therefore it is only the remaining C that can be used as an estimate of CO_2 fixation. For comparative purposes, plants were only harvested 12 hours after labelling in both soil and sand experiments. They were then analysed for C and ^{14}C content of roots, shoots and eventually nodules.

Nitrogenase activity. Nitrogenase activity of individual plants was estimated 3 hours prior labelling using the C_2H_2 reduction technique. Exposure of the nodulated roots lasted 1 hour at 10% C_2H_2 after which the plant containers were thoroughly flushed with outside air.

Results and Discussion

Carbon fixation by both roots and nodules of soybean grown in soil is shown in Fig. 1, together with C_2H_2 reduction. During vegetative growth, high quantities of $^{14}CO_2$ were fixed, in spite of low C_2H_2 reduction. Maximum rate of $^{14}CO_2$ fixation occurred during flowering, prior to maximum level of C_2H_2 reduction, which occured at the end of flowering. From pod formation to maturity, both activities decreased in a similar manner.

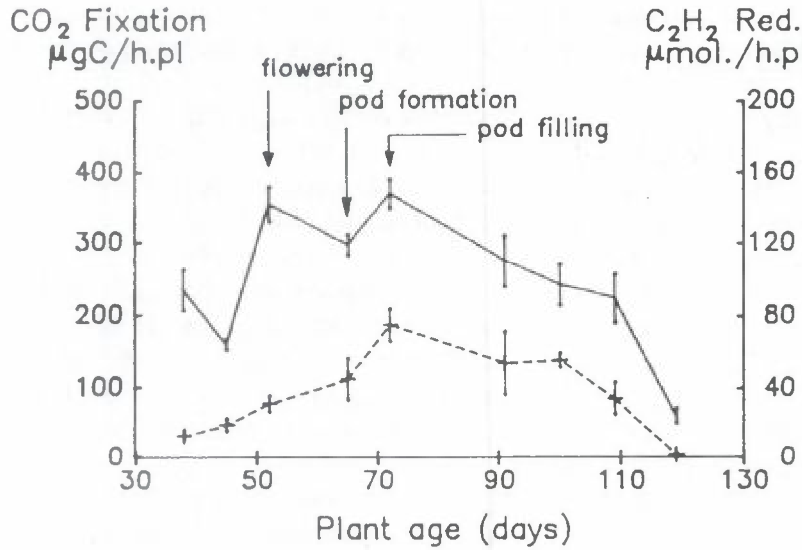

Figure 1. Hourly rates of CO_2 fixation (—) and C_2H_2 reduction (– –) obtained during the growth cycle of soybeans grown in soil. Bars are the ±SEM for 4 replicates.

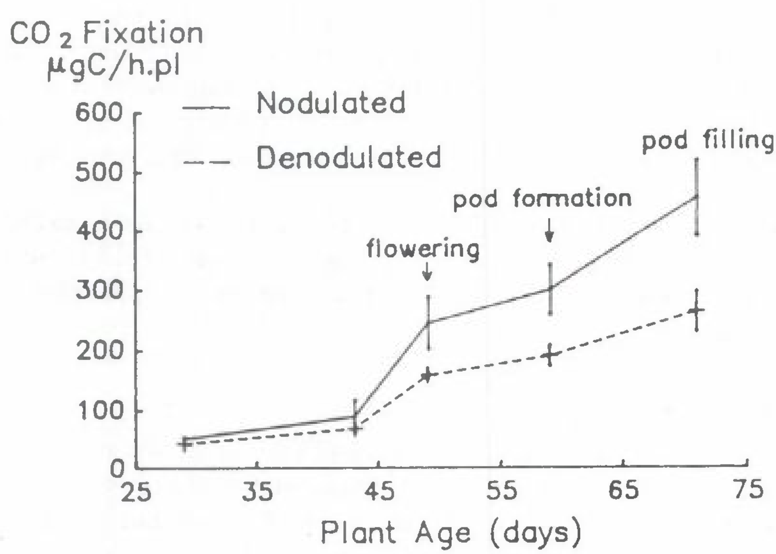

Figure 2. Rates of $^{14}CO_2$ fixation by nodulated and denodulated soybeans grown on sand. Bars are ±SEM for 3 replicates.

In sand culture, total CO_2 fixation was low, compared to measurements obtained in soil, during most of the vegetative growth, and increased thereafter (Fig. 2). As indicated by the activity of denodulated plants, 60 to 83% of the C fixed by nodulated plants originated from roots. The contribution of nodules to total CO_2 fixation was measured in sand cultures by comparing CO_2 fixation of nodulated and denodulated soybeans. The magnitude of CO_2 fixation by nodules is indicated in Table 1, together with C_2H_2 reduction. Throughout the growth period, there was a significant correlation between nitrogenase activity and nodule CO_2 fixation ($r = 0.985$, $n = 6$ according to the data of Table 1). Similar results have been observed during the growth cycle of both lupin (Christeller et al., 1977) and alfalfa (Vance et al., 1983).

Table 1. Sand culture: Root and nodule CO_2 fixation and nitrogenase activity during the growth cycle of soybean. ($, stages of development: V: vegetative, R: reproductive, as defined by Fehr et al., 1971)

Growth stage $	Age of plants days	CO_2 Fixation					C_2H_2 Reduction
		Root		Nodule		Root/ Root + Nodule	μmol h^{-1} pl^{-1}
		Total μgC h^{-1} pl^{-1}	Specific μgC h^{-1} $mgDW^{-1}$	Total μgC h^{-1} pl^{-1}	Specific μgC h^{-1} $mgDW^{-1}$	%	
V3	29	43.31	0.78	9.33	0.41	82.28	10.67
V5	43	69.35	0.51	21.19	0.25	76.60	24.11
R2	49	158.63	0.81	87.55	1.04	64.44	40.88
R3	59	190.85	0.55	110.19	0.61	63.40	53.62
R5	71	263.51	0.58	191.74	0.61	57.88	73.50

Table 2. Soil culture: Nitrogenase activity and calculated values of root and nodule CO_2 fixation during the growth cycle of soybean. ($, stages of development, V: vegetative, R: reproductive, as defined by Fehr et al., 1971)

Growth stage $	Age of plants days	CO_2 Fixation					C_2H_2 Reduction
		Root		Nodule		Root/ Root + Nodule	μmol h^{-1} pl^{-1}
		Total μgC h^{-1} pl^{-1}	Specific μgC h^{-1} $mgDW^{-1}$	Total μgC h^{-1} pl^{-1}	Specific μgC h^{-1} $mgDW^{-1}$	%	
V3	38	234.73	1.13	2.46	0.07	98.96	12.98
V5	45	140.88	0.36	21.41	0.36	86.81	19.38
R2	52	322.54	0.70	39.03	0.29	89.21	31.58
R3	65	210.66	0.18	97.70	0.45	68.32	45.15
R5	72	198.68	0.17	185.98	0.84	51.65	74.98
R5	91	182.02	0.09	123.27	0.36	59.62	53.79
R6	100	150.23	0.08	128.46	0.35	53.91	55.54
R7	109	183.25	0.10	65.34	0.21	62.02	25.65
R8	119	106.69	0.08	—	—	—	—

Assuming that the correlation found between nitrogenase activity and nodule CO_2 fixation is the same for soybean grown in sand and in soil, the C_2H_2 reduction values measured in soil, made it possible to calculate the quantities of C fixed by nodules in soil (Table 2). Root CO_2 fixation was then obtained by difference between total and nodule fixation. As observed in sand, root CO_2 fixation accounted for more than 50% of the total activity. It was maximum during the early stage of development, reaching approximately 85% of the total. In alfalfa, Anderson et al. (1987) reported values ranging between 80 to 90% of the total for roots. Specific rates of root CO_2 fixation (expressed in $\mu gC\,h^{-1}\,mg^{-1}$ root DW) was higher than that of nodules at the beginning of the growth cycle (Table 1,2). It then decreased and became lower than that of nodules, which showed a maximum during the pod-filling stage in soil, and during the flowering stage in sand. Coker and Schubert (1981) reported, for soybean also, that the rate of nodules CO_2 fixation reached a maximum earlier, i.e. during the vegetative stage, before the decline of C_2H_2 reduction rate. The roots activity recorded in our study are markedly higher than those previously reported by these authors. This discrepancy may be explained by differences in experimental procedures i.e. plant culture, labelling conditions, time of sampling.

The molar ratio of CO_2 fixed per C_2H_2 reduced by nodules, as estimated from the correlation, was 0.25. Assuming that 4 moles of C_2H_2 were reduced for each one of N_2 fixed (Coker and Schubert 1981), this corresponds to 1 mole of CO_2 fixed per mole of N_2 fixed. This value is low compared with the molar ratio of 2.1 reported for lupin (Christeller et al., 1977) and the maximum of 3.4 estimated for soybean (Coker and Schubert 1981). An underestimation can be the consequence of our method, which does not take into account the respiratory losses of the ^{14}C fixed. This process in ureide-transporting plants could represent 60 to 80% of the $^{14}CO_2$ fixed during a 5-minute labelling period (Coker and Schubert 1981; King et al., 1986; Warembourg and Roumet 1989). These authors assumed that the carbon released as CO_2 is used to supply metabolic energy for the N_2 fixation process. Nevertheless, in our study, a significant correlation was found between N_2-fixation and the amount of fixed carbon remaining in the whole plant. This fraction could have at first produced carbon skeletons for amino acid synthesis in nodules, compounds which were then transported to other plant parts (Vance et al., 1985; Anderson et al., 1987). The C fixed by roots is known to be integrated into organic acids, to act as counter ions in the xylem sap (Davies 1979).

In conclusion, this study has shown that *in situ*, roots as well as nodules possess an active system for non-photosynthetic assimilation of CO_2. In natural conditions, the overall carboxylase activity, not including respiration losses, ranged from 350 to 400 $\mu gC\,h^{-1}\,pl^{-1}$. This is equivalent to approximately 5% of the concomitant plant C increment. Considering that in legumes, roots and nodules depend primarily upon translocated carbohydrates for their energy in biosynthesis and other activities, efficient conservation of carbon is of central importance. It's improvement through plant or *Rhizobium* strain selection should lead to a better efficiency of N_2 reduction, and therefore a better carbon economy for the whole plant.

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