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EFFECTS OF NONPHOTOSYNTHETIC CARBON DIOXIDE FIXATION ON THE NITROGEN FIXATION IN ROOT NODULES OF THE SOYBEAN PLANT

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Abstract

The nonphotosynthetic CO_2 fixing activity of phosphoenol pyruvate carboxylase in nodules likewise the photosynthetic CO_2 fixation activity of ribulose-1, 5-diphosphate carboxylase in leaves was positively regulated by the CO_2 concentration supplied to root nodules. The activity of phosphoenol pyruvate carboxylase became more effective in promotion the nitrogen fixation of nitrogenase when plants were grown under dark condition or nodules were detached from the root system. However, the activity of nitrogen fixation reduced by the dark or by detachement of nodules from the plant could not be fully compensated by the phosphoenol pyruvate carboxylase activity to the level of nitrogen fixation that found in the nodules of plant grown under light condition. This suggests that photosynthetic CO_2 fixation catalyzed by ribulose-1, 5-diphosphate carboxylase in leaf can not be fully replaced by the nonphotosynthetic CO_2 fixation by phosphoenol pyruvate carboxylase in the nodules for the nitrogen fixation. The role of phosphoenol pyruvate carboxylase in nitrogen fixation may be to provide the carbon skeleton, oxaloacetate, for the ammonia assimilation and amino acid biosynthesis but may not have an anapleurotic function in replacement of tricarboxylic acid cycle that used in respiratory activities in the nodules for the nitrogen fixation.

Key words: Dark CO_2 fixation; phosphoenol pyruvate carboxylase; nitrogen fixation; carbon skeleton.

Introduction

It has been demonstrated that the availability of the sugars from the photosynthetic CO_2 fixation in leaves is the major factor limiting symbiotic nitrogen fixation in agronomically importany leguminous plants, such as soybeans (Hardy and Havelka, 1977; Huang *et al.*, 1975). Therefore, changes of CO_2 concentration around the aerial portion of soybean plants had a direct effect on the nitrogen fixing activity in nodules (Huang *et al.*, 1975). However, Mulder and Van Veen (1960) reported that growth and nitrogen fixation of red clovers, peas, and beans

were increased by increasing CO_2 concentration around the root environment. Lowe and Evans (1962) demonstrated that pure culture of *Rhizobium* required CO_2 for growth and that CO_2 was fixed into phosphoenol pyruvate and propionylCoA by carboxylase. Many other investigators also reported that the nonphotosynthetic CO_2 fixation catalyzed by phosphoenol pyruvate carboxylase was found in root nodules of broad beans (Lawrie and Wheeler, 1975) peas (Phillips *et al.*, 1976), cowpeas (Layzell *et al.*, 1979), garden beans (Cookson *et al.*, 1980), soybeans (Coker and Schubert, 1981; Laing *et al.*, 1979; Peterson and Evans, 1979). Mulder and Van Veen (1960) demonstrated that 4% CO_2 had no harmful effect on nodulation. Poel (1979) also showed that high concentration of CO_2 in root rhizosphere had little effect on respiration and acetylene reduction (nitrogen fixation) in attached nodules. The purpose of this study is to confirm the strong correlation between the rates of nitrogen fixation and CO_2 fixation in root nodules of soybean plants and to examine the extents and rates of CO_2 fixation in both roots and nodules of soybeans grown solely on symbiotically fixed nitrogen.

Materials and Methods

Growth of Plant

Soybean (*Glycine max.* var. Kaoshung No. 3) seeds were germinated for three days in vermiculite. Single healthy seedlings were inoculated with a commercial preparation of *Rhizobium japonicum* and were transplanted to a mixture of sand and vermiculite (1:1) in clay pots. The seedlings were grown in a constant environment chamber described previously (Huang *et al.*, 1975). During the first ten days after transplanting, the seedlings were watered with Hoagland solution containing small amount of nitrate every 3rd day and with water all other days. After ten days, plants were watered daily with nitrogen free nutrient solution or with water on alternate days. During the growing period, all branches were excised so that only the central stem remained. Plants were used for experiments when they were 4 to 5 weeks old.

Acetylene Reduction

Acetylene reduction was used to assay nitrogen fixing activity in root nodules of plant. Nodules were placed into flasks (125 ml). Each flask was sealed air-tighly with a serum bottle stopper, then 12.5 ml of air was withdrawn from the flask with a syringe before an equivalent volume of acetylene was injected into the flask. Gas samples (0.2 ml) were withdrawn every 10 min and chromatographed immediately. Acetylene and ethylene were separated with a gas chromatograph having a hydrogen ionization detector at an oven temperature of 65°C. A glass column 0.8 m long and 3 mm i.d. packed with Porapak R was used for the separation. The carrier gas was N_2 flowing at 30 ml per min.

Preparation of Crude Enzymes

Nodules were harvested from 4–5 week old plants, and rinsed thoroughly once with distilled water and twice with buffer, 50 mM Tris-HCl, pH 7.5, then ground with 1/3 (w/v) amounts of acid-washed polyvinylpolypyrrolidone and 5 volumes of 50 mM Tris-HCl containing 5 mM dithiothreitol with a mortar and pestle. The resulting slurry was sequeezed and filtered through three layers of cheesecloth. The filtrate was centrifuged at 15,000 g for 30 min. A half portion of supernatant fluid was left for organic acid analysis, and another half portion of supernatant was brought to 45, 50 and 55% saturated solution of ammonium sulfate. The precipitates from above three fractions were pooled together and resuspended in a small volume of 50 mM Tris-HCl buffer (pH 7.5), and dialyzed for 12 hours against three changes of the same buffer. All the procedures described above were conducted at 4°C. The dialyzed sample was used to determine the activity of enzymes including phosphoenol pyruvate carboxylase and isocitrate dehydrogenase.

Measurement of Phosphoenol Pyruvate Carboxylase Activity

A spectrophotometric and a coupled enzyme assay were used to determine the enzyme activity. The coupled enzyme reaction mixture was 50 mM Tris-HCl (pH 7.5), 1.0 mM $MgCl_2$, 10 mM $KHCO_3$, 1 mM NADH, 2 mM phosphoenol pyruvate, and 2 units of malate dehydrogenase. The reaction was initiated by the addition of 0.2 ml enzyme extract. The final volume of the reaction mixture was 1.5 ml. The reaction was linear for at least 10 min at 28°C. The rate of NADH oxidation was measured spectrophotometrically at 340 nm. One unit of enzyme activity was expressed as 1 μmol NADH oxidized per min.

Measurement of Isocitrate Dehydrogenase Activity

The enzyme reaction mixture contained 50 mM Tris-HCl (pH 8.0), 0.15 mM NAD^+, 3 mM $MgCl_2$, 0.15 mM isocitrate. The reaction mixture was incubated in a water bath at 28°C for 2 min before the reaction was started by the addition of 0.2 ml enzyme extract. The rate of NAD^+ reduction was also followed at 340 nm.

Extraction and Determination of Oxaloacetic Acid Concentration

The half portion of supernatant fluid obtained from centrifugation at 15,000 g during the preparation of enzymes was deproteinized by 1 M perchloric acid in a capped tube. The suspension was allowed to stand still in a cold room at 4°C for two hours. Then the suspension was centrifuged at 15,000 g for 30 min. The supernatant fluid was neutralized to pH 7.0 by 4 N KOH, and then was used as a sample for the measurement of oxaloacetic acid content. Oxaloacetic acid was measured spectrophotometrically in the presence of malate dehydrogenase and NADH as the method described in determination of phosphoenol pyruvate carboxylase activity,

except 10 mM $KHCO_3$ was substituted by 0.2 ml of supernatant fluid that had deproteinized by perchloric acid.

Carbon Dioxide Fixed by Nodules

The amounts of CO_2 uptake by detached nodules were measured according to the Warburg manometric method described by Umbreit *et al.* (1972). Nodules detached from the roots were placed in the manometric flasks with 3 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM dithiothreitol, 1.0 mM $MgCl_2$, and 2% sucrose. For the temperature equilibrium between inside and outside of flasks in a water bath at 28°C, the samples in flasks were incubated in a water bath for 10 min before $KHCO_3$ was added. Twenty mM $KHCO_3$ was enclosed in the side arm of flasks and CO_2 was released by the injection of 0.1 ml lactic acid (4 M) into the side arm. The nodules were incubated with $KHCO_3$ for one hour. The net amount of CO_2 fixed by nodules was estimated by the total amount of CO_2 uptake by nodules in the presence of $KHCO_3$ minus the amount of CO_2 fixed by nodules in the absence of $KHCO_3$.

Waterlogging Treatment

For the waterlogging, the plants were treated as the method used by Huang (1984).

Results

Table 1 shows the effect of CO_2 concentration around the rhizosphere influenced the initiation, growth, and development of nodules. Although the morphological appearances of both shoot and root systems of plant were slightly affected by CO_2

Table 1. *Effect of carbon dioxide concentrations on the nodulation and nitrogen content within nodules of soybean plants*

Plant grown in a plastic pot which containing a mixture of vermiculite and sand (1:1) was aerated with an air consisting of 300 ppm or 600 ppm of CO_2 at a flowing rate of 30 ml per min. A hole through which a serum stopper was fixed into the side wall of pot for pumping the CO_2 into root-soil system at 2 inches above the bottom of pot. Plants were harvested for experiments at 35 days after seed germination. Data represent average value of 5 plants.

	CO_2 Concentration (ppm)	
	300	600
Nodule number/plant	72	117
Nodule dry weight/plant (mg)	18.8	25.2
N-content in nodule/plant (mg)	13.8	20.6

enrichment, high CO_2 concentration, 600 ppm, tended to increase the number and dry weight of root nodules. Furthermore, the total nitrogen content in nodules was also increased by CO_2 enrichment (Table 1). This result indicates that dark CO_2 fixation may play an important role in regulating nodule development and nitrogen fixation. The amounts of oxaloacetic acid, a primary product of CO_2 fixation by phosphoenol pyruvate carboxylase, in nodules were increased with increasing the incubation time of nodules with CO_2 (Fig. 1). The data shown in Tables 2 and 3 indicate that the activity of phosphoenol pyruvate carboxylase plays an important role in fixing CO_2 and this nonphotosynthetic CO_2 fixation activity in nodules was positively regulated by CO_2 concentration supplied to root nodules, particularily, when plants were submitted to dark treatment (Table 2) or nodules were detached from root system (Table 3). To check whether the phosphoenol pyruvate carboxylase activity involves in an anapleurotic function in dark respiratory activity or in nitrogen fixation, other enzymes which are isocitrate dehydrogenase and nitrogenase in both nodules and roots of plants grown under the dark and light conditions were examined, respectively. As the data shown in Table 2, phosphoenol pyruvate carboxylase and isocitrate dehydrogenase had higher catalytic activity in the roots than in the nodules, and nitrogenase activity undoubtly restricted in the nodules. The activities of phosphoenol pyruvate carboxylase and nitrogenase in both nodules and roots were promoted by the

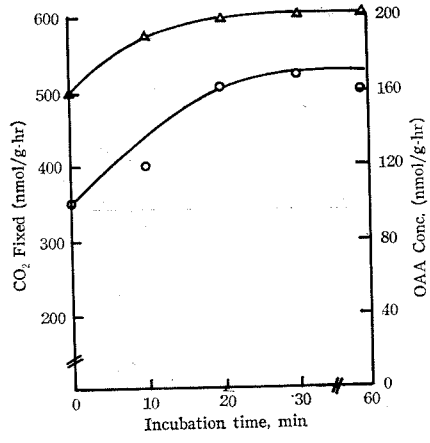

Fig. 1. The time courses of carbon dioxide fixion and the biosynthesis of oxaloacetic acid in the nodules of the soybean plant. The amounts of carbon dioxide fixed by nodules were measured by Warburg manometric method. The nodules which had been used to measure the activity of CO_2 fixation were then used to extract organic acids for the measurement of oxaloacetic acid. CO_2 fixed, △-△-△, OAA concentration, ○-○-○.

Table 2. *Comparison the activities of phosphoenol pyruvate carboxylase (PEPCase), isocitrate dehydrogenase (IDH), and nitrogenase (N_2ase) in the nodules of soybean plants treated with different carbon dioxide concentrations*

For the darkness treatment, plants were removed to a dark room for two days at 28°C. Nodules detached from roots were placed in a flask and aerated with an air mixture containing carbon dioxide of 300 ppm and 600 ppm, respectively. Two glass tubes were inserted through the serum stopper of flask, one tube is for pumping carbon dioxide into and another tube for outlet of the flask. The flowing rate of air mixture measured at the outlet of flask was 30 ml per min. The detached nodules in flask were incubated with 3 volumes of Tris-HCl buffer containing 1.0 mM $MgCl_2$, 2% sucrose, and 5 mM dithiothreitol. Two hours after the carbon dioxide treatment, the roots and nodules were used to measured enzymes activity and nitrogen fixing activity.

Carbon dioxide concentration (ppm)	PEPCase (nmoles NADH oxidized/ g fresh wt.•30 min)				IDH (nmoles NAD red./ g fresh wt.•30 min)				N_2ase (nmoles C_2H_4/ g fresh wt.•30 min)			
	Root		Nodule		Root		Nodule		Root		Nodule	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
300	5.32	9.67	1.32	2.27	0.30	0.31	0.03	0.06	—	—	118.7	77.47
600	5.89	11.80	1.85	4.95	0.34	0.31	0.05	0.06	—	—	152.7	117.4

Table 3. *Activities of enzymes in attached and detached nodules changed by carbon dioxide concentrations*

The activities of enzymes were measured at two hours after the nodules had aerated with carbon dioxide under dark condition. The carbon dioxide aeration method and the incubation media for nodules in flask were the same as that described in text of Table 2.

Nodules	CO_2 concentration (ppm)	N_2ase activity (nmoles C_2H_4/ g•30 min)	PEPCase (nmoles NADH oxidized/g•30 min)
Attached	300	117.10	5.19
	600	149.57	5.90
Detached	300	71.09	3.23
	600	109.84	7.67

increment of CO_2 concentration but CO_2 enrichment had little effect on the activity of isocitrate dehydrogenase. Light and nodules on intact root systems favor the nitrogenase activity because plants grew under the light condition and nodules on intact root had higher nitrogen fixing activity than those plants were grown under the dark condition (Table 2) or than those nodules were detached from the root systems (Table 3). On the contrary, lower activity of phosphoenol pyruvate carboxylase in both roots and nodules was found under the light growing condition. This indicates that dark treatment to plants or detachment of nodules from

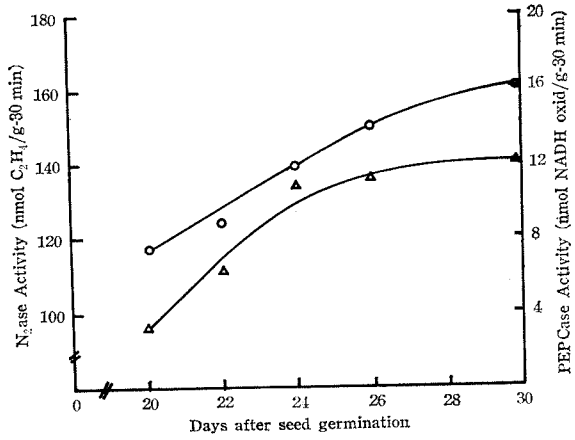

Fig. 2. Changes of nitrogenase and phosphoenol pyruvate carboxylase activity in the nodules with plant growth duration. The nitrogenase activity was measured ***in vivo***, ○-○-○, and the phosphoenol pyruvate carboxylase activity was measured ***in vitro***, △-△-△.

the root systems increases the phosphoenol pyruvate carboxylase activity in nodules. Although lower nitrogen fixing activities were found in the dark than in light condition and in detached nodules than in intact nodules, the nitrogen fixing activity of nitrogenase was compatible with the CO_2 fixing activity of phosphoenol pyruvate carboxylase in nodules (Tables 2 and 3). The positive correlation between the nitrogenase and phosphoenol pyruvate carboxylase activity is also shown in Fig. 2 and these two enzyme activities were all inhibited when plants were submitted to anaerobic (waterlogging) growth condition, and so the nitrogen fixation and CO_2 fixation in nodules were suppressed (Table 4).

Table 4. *Effect of waterlogging on activities of nitrogenase, and phosphoenol pyruvate carboxylase in nodules of soybean plants*

Plants were started to waterlogging treatment at 24 days after seed germination.

Treatment (day)	N_2ase (nmole C_2H_4/ g nodule·30 min)		PEPCase (nmole NADH oxid./ g nodule·30 min)	
	Control	Waterlogging	Control	Waterlogging
0	120	120	11.5	11.5
2	136	22	13.0	1.9
4	158	0.8	13.5	1.8
6	170	0.4	14.0	2.1

Discussion

It has been demonstrated that the biosynthesis of photosynthates in leaves regulates the rates of nitrogen fixation in root nodules of soybean plants (Huang *et al.*, 1975; Hardy and Havelka, 1977), because the photosynthates provide the sources of energy and reducing powers required for nitrogen fixation, and supply the carbon skeletons for ammonia assimilation into amino acids (Rawsthorne *et al.*, 1980). However, data in Fig. 2 indicate that dark CO_2 fixation in the nodules also plays an important role in regulating nitrogen fixation. The data shown in Tables 2, 3 and in Fig. 2 all provide evidences that nitrogenase activity may be influenced by the CO_2 fixation activity of phosphoenol pyruvate carboxylase. Consequently, nitrogen fixation of nodules was enhanced by CO_2 enrichment. Owing to the phosphoenol pyruvate carboxylase activity increased about double when plants were grown under dark condition (Table 2), and this enzyme activity became more sensitive to CO_2 enrichment when plants were under dark condition or when nodules were detached from the root systems (Tables 2 and 3), it seems that phosphoenol pyruvate carboxylase activity becomes more important to nitrogen fixation when plants supplies lesser amount of photosynthates to nodules for their nitrogen fixation. Although the phosphoenol pyruvate carboxylase activity in nodules was significantly promoted by the dark treatment to plants, this nonphotosynthetic CO_2 fixation could not completely restore the nitrogen fixation activity to that the activity found in nodules under light condition (Table 2). Besides, the nitrogenase had also much lower activity in detached than in attached nodules, no matter how the phosphoenol pyruvate carboxylase activity became more sensitive to CO_2 enrichment in detached nodules (Table 3).

One of the possible roles of nonphotosynthetic CO_2 fixation via phosphoenol pyruvate carboxylase in nitrogen fixation is to maintain the pools of tricarboxylic acid cycle intermmediates that are essential to support the respiratory activities within the nodules, because any consumption of acids of the TCA cycle for nitrogen fixation or for ammonia assimilation would result in a shortage of organic acids, such as oxaloacetic acid, and the shortage of acids would lead to a cease of TCA cycle. Therefore, an alternative source of organic acids is needed. However, the data shown in Table 2 indicate that isocitrate dehydrogenase activity in the nodules was not promoted by the enhancement of phosphoenol pyruvate carboxylase activity by CO_2 enrichment. This suggests that oxaloacetic acid produced by the phosphoenol pyruvate carboxylase has a minor function in anapleurotic replacement or complement TCA cycle acids used in nitrogen fixation or subsequent ammonia assimilation into amino acids but rather than directly involves in the ammonia assimilation by providing the carbon skeleton necessary for the biosynthesis of amino acids, and this function becomes more pronouncedly to nitrogen fixation when the amount of

photosynthates supplied to nodules is decreased because of the dark treatment to plants or of the detachment of nodules from the root system.

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非光合作用性二氧化碳固定作用對大豆根瘤固氮作用之影響

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根瘤中非光合作用性二氧化碳固定酶卽 phosphoenol pyruvate carboxylase，活性受到根瘤外界二氧化碳濃度所影響。此二氧化碳固定酶於植物正常光合作用低弱（如在黑暗中）或根瘤從植物根部摘下後，對根瘤固氮作用之影響比其在光合作用强或根瘤附着於根部時來得顯著。非光合作用性二氧化碳固定作用雖然對根瘤固氮作用有促進作用，但是在植物正常生長環境下，光合作用還是控制根瘤固氮作用之主要因素。此非光合作用性二氧化碳固定酶之功用可能是直接合成 oxaloacetic acid 以供氨同化作用合成氨基酸所需之碳架 (C-skeleton) 而不進入呼吸作用之 TCA cycle。