FACTORS EFFECT ON THE NITROGEN FIXATION OF RHIZOBIA IN THE SYMBIOTIC RHIZOBIUM-CALLUS TISSUES

1. Carbon sources

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Abstract: Activity and span of nitrogen fixation (acetylene reduction) of rhizobia in the symbiotic rhizobium-callus tissues changed by the carbon sources and concentrations. Hexoses were better than pentoses, and sugars were better than those organic acids, except the fumaric acid, for the rhizobial acetylene reduction in the symbiotic tissues. High concentration of sugar promoted the symbiotic tissues to senescence.

No leghemoglobin was found in the symbiotic tissues. The lignification of callus tissues might have a regulatory function to prevent the nitrogenase in rhizobia of callus tissues from oxygen-denaturation.

Negligible amount of N_2O realeased by symbiotic system. The nitrate respiration might not provide energy to support the rhizobial acetylene reduction in symbiotic callus tissues.

INTRODUCTION

It had been reported that the nitrogen-fixing activity of rhizobia in root-nodules of leguminous plants is regulated by photosynthesis (Huang, Boyer and Vanderhoef, 1975), because the photosynthate provides both energy and reductant via the respiratory pathway for the rhizobial nitrogen fixation (Bergersen, 1971 and Yates, 1980). In previous studies had been shown that rhizobia in callus tissues could be induced to have the nitrogen fixing activity if carbohydrate was added to the culture medium (Huang, 1980). It was assumed that sugars involved in the initiation of nitrogenase synthesis or in activation of enzyme activity. However, the activity of rhizobial nitrogenase in callus tissues was much lower than that of rhizobia (bacteriods) in root-nodules (Huang, Boyer, Vanderhoef, 1975 and Huang, 1980). This differences in nitrogen fixing activity of rhizobia between in nodules and symbiotic rhizobium-callus system might be due to differences of carbon source supplied to the rhizobia. In this study, it is attemped to figure-out what kind of carbon sources including the sugars and organic acids will be the effective carbon sources for rhizobial nitrogen fixation in the symbiotic rhizobium-callus system.

MATERIALS AND METHODS

Culture of Callus Tissue

Seeds of Shih-Shih, a cultivar of soybean, were selected and sterilized with a 0.12% sodium hypochloride. The seeds after had been rinsed several times with deionized water were germinated in petri-dish in the dark room with a constant temperature of 26°C. Three days later, small sections of stems cut from seedlings were incubated on the B_5 medium

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(Gamborg, Miller and Ojima, 1968) to induce formation of callus tissues. Those callus tissues were subcultured once at a ten-day interval.

Rhizobial Suspension

The purified rhizobia isolated from the nodules of Shih-Shih soybean were transferred from the stock culture (Huang, 1980) to freshly prepared liquid medium (Gamborg, Miller and Ojima, 1968) and shaked on a shaker for 72 hours. This rhizobial suspension was then used as an inoculant of callus tissues.

Modification of B_5 medium

Essentially the B_5 medium was used as the culture medium except the carbon source was been modified. The disaccharide, sucrose, was replaced by monosaccharid, fructose, galactose, glucose, xylose, or arabinose. Besides, sucrose was also substituted by organic acid, fumaric acid, malic acid, succinic acid, α-ketoglutaric acid, or isocitric acid.

Development of symbiotic rhizobium-callus system

One and half grams of actively growing callus tissues were transferred from B_5 medium into 250 ml flask containing 50 ml of modified B_5 medium. Each flask was capped with aluminum foil and shaked on a shaker in the dark room at 25°C for 12 days. The vigorus callus tissues were then inoculated with rhizobia by adding 0.1 ml of rhizobial suspension, whose $O.D._{540nm}$ was 0.9, to culture flasks to establish the symbiosis between callus tissues and rhizobia. After three days of inoculation, the liquid culture containing the symbiotic rhizobium-callus tissues were filtered through four layers of cheesecloth. The rhizobium-callus tissues retaining onto the cheesecloth were rinsed several times by fresh modified B_5 medium to remove those extracellular rhizobia adhering to the surface of callus tissues. Those washed tissues were then used to determine the rhizobial nitrogen fixing (acetylene reduction) activity.

Measurement of symbiotic nitrogen fixation

The nitrogen fixing activity of rhizobia in the symbiotic rhizobium-callus system was measured by convenient acetylene reduction as method described (Huang, 1980). At a 30 minute interval, 0.2 ml of gas sample was withdrawn from each culture flask and the concentrations of ethylene produced by the nitrogenase was assayed by gas chromatography (Huang, 1980).

Dry weight determination

The rhizobium-callus tissues after had been finished the measurement of nitrogen fixing activity were dried in an oven at 70°C for 48 hours, then the dry-weight was measured.

Determination of nitrate respiration

The symbiotic rhizobium-callus tissues were incubated in flasks containing freshly prepared B_5 medium and 25 mM KNO_3. The flasks were then flushed thoroughly with Argon gas to replace the air in flasks, and then capped tightly with rubber stopper. The gas samples were withdrawn from the flasks every three-day interval and assayed by Varian 3700 gas chromatography (thermal conductivity detector, steel column packed with Porapak Q, Helium was the carrier gas) to determine the amount of N_2O released by nitrate respiration.

Determination of total N

Total nitrogen content in dried symbiotic rhizobium-callus tissues was dertermined by the Kjeldahl method (Umbreit, Burris and Stauffer, 1964).

Determination of leghemoglobin

The leghemoglobin content in symbiotic rhizobium-callus tissues was determined as the method described by LaRue (LaRue and Child, 1979).

RESULTS

Carbon source plays an important role in regulating the nitrogen fixing activity of rhizobia in the symbiotic rhizobium-callus tissues because the rhizobia in the symbiotic tissues showed different levels of nitrogen fixing activity while different monosaccharides supplied individually to the modified medium as a solely carbon source for the rhizobia in symbiotic tissues (Fig. 3, Table I). Based on the levels of acetylene reduction (nitrogen fixation) activity of rhizobia, the fructose was the best carbon source for the rhizobial activity and the xylose was the worst one, and hexoses were better than those pentoses for rhizobial acetylene reduction. However, the acetylene reduction activity of rhizobia was decreased as the concentrations of sugar increased from 20 g/l to 40 g/l or to 60 g/l, and higher the concentration of sugars added to medium, higher the inhibitory effect on acetylene reduction of rhizobia was found (Fig. 1, 2, 3, Table I). The similar results were obtained if the organic acids supplied as a carbon source for the rhizobia in replacement of sugars. Among the organic acids, the fumaric acid was the best carbon source, and the isocitric acid was the worst one for the rhizobial activity (Table II). In general, sugars were better than organic acids for rhizobial acetylene reduction activity (Table I, II).

For the time course studies, the acetylene reduction activity of rhizobia in the symbiotic callus tissues were started to measure at every 3-day interval after the callus tissues had been inoculated with rhizobia. The time took to reach the maximal acetylene reduction activity of rhizobia were varied with kinds of sugar added to the incubation medium. It

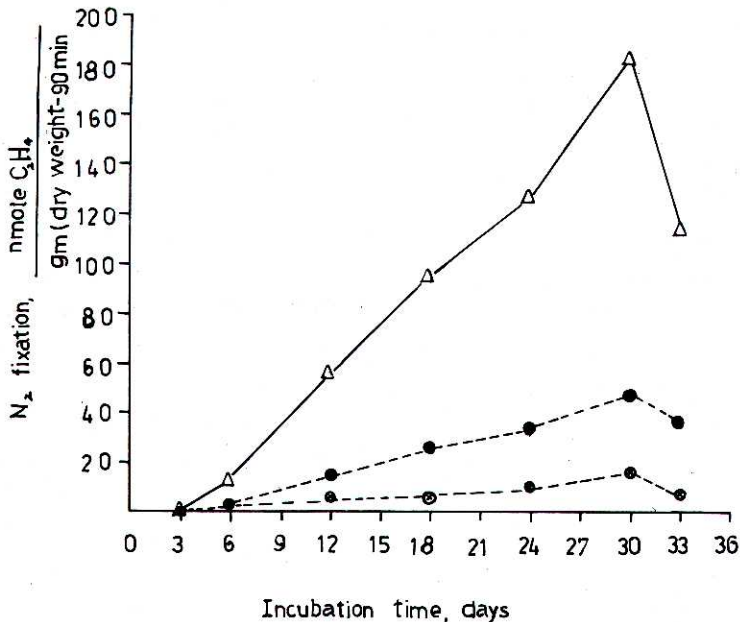

Fig. 1. Effect of fructose on the nitrogen fixing activity of rhizobia in symbiotic rhizobium-callus tissues.
20 g/l, ●—●—●; 40 g/l, ⊗—⊗—⊗; 60 g/l, △—△—△.

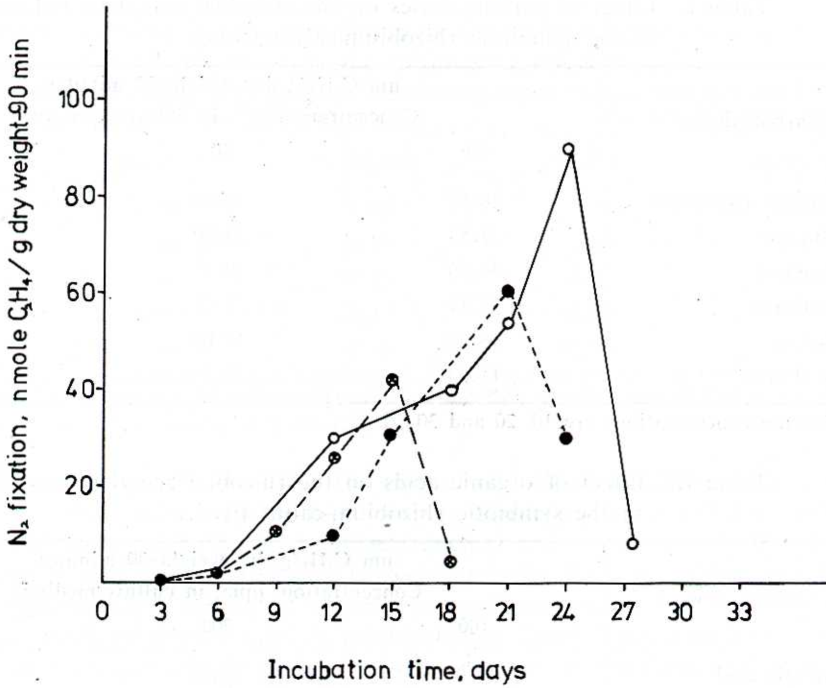

Fig. 2. Effect of glucose on the nitrogen fixing activity of rhizobia in symbiotic rhizobium-callus tissues.
20 g/l, ●—●—●; 40 g/l, ⊗—⊗—⊗; 60 g/l, ○—○—○.

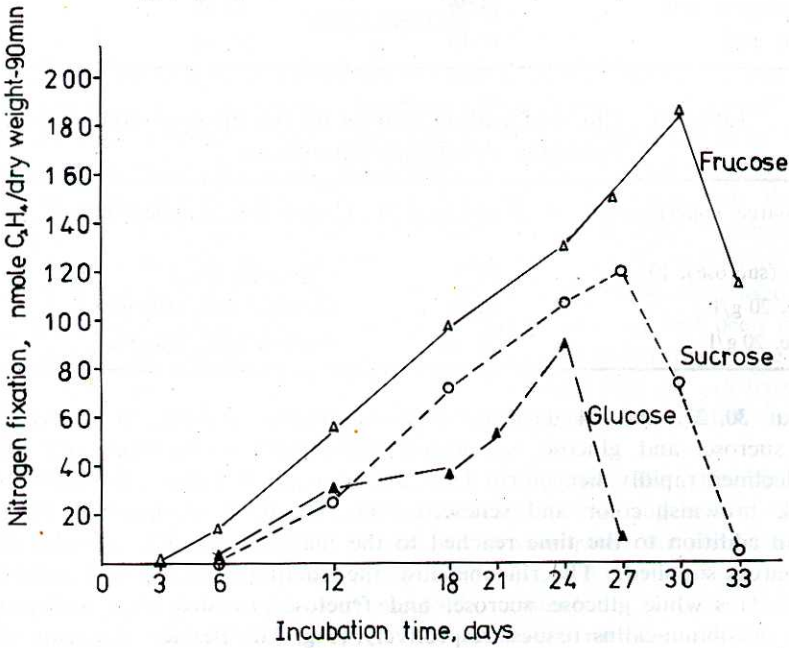

Fig. 3. Comparing the effects of C sources on the nitrogen fixing activity of rhizobia in symbiotic rhizobium-callus tissues. Fructose (20 g/l), △—△—△; glucose (20 g/l), ▲—▲—▲; sucrose (10 g/l); ○—○—○.

Table I. Effect of carbohydrates on the rhizobial acetylene reduction in the symbiotic rhizobium-callus tissues

Carbohydrate	nm C_2H_4/g dry weight-30 minutes Concentration, g/l, in culture medium		
	20	40	60
Control (Sunose)*	28.55	27.09	23.61
Glucose	27.63	24.80	23.56
Fructose	30.70	29.53	23.98
Galactose	25.14	24.41	23.44
Xylose	16.54	17.00	13.79
Arabinose	17.23	14.53	11.00

* Sucrose concentrations are 10, 20 and 30 g/l.

Table II. Effect of organic acids on the rhizobial acetylene reduction in the symbiotic rhizobium-callus tissues

Organic acid	nm C_2H_4/g dry weight-30 minutes Concentration, ppm, in culture-medium		
	100	200	300
Fumaric acid	30.04	21.30	16.00
Malic acid	20.25	22.10	20.96
Succinic acid	17.42	15.41	13.95
α-ketoglutaric acid	18.36	17.48	17.00
Isocitric acid	17.10	15.46	14.15

Table III. Effect of carbon sources on the nitrogen content in the symbiotic rhizobium-callus tissues

Carbon source, concentration	% of the total N	Carbon source, concentration	% of the total N
Control (sucrose), 10 g/l	18.50	Galactose, 20 g/l	15.87
Glucose, 20 g/l	18.30	Fumaric acid, 100 ppm	20.22
Fructose, 20 g/l	20.49	Isocitric acid, 100 ppm	15.17

took about 30, 27, and 24 days to reach the highest activity of acetylene reduction if fructose, sucrose, and glucose was added individually to medium, respectively, and the activity declined rapidly henceforth (Fig. 3). Around this time, the callus tissues had become dark brownish color and senesced. The duration of rhizobial acetylene reduction activity, in addition to the time reached to the maximal activity, was also changed by the carbon sources supplied. The rhizobia lost their acitivity to an undetectable levels in 27, 33, and 37 days while glucose, sucrose, and fructose provided as a carbon source for the symbiotic rhizobium-callus tissues. respectively, (Fig. 3). Besides, the span of rhizobial acetylene reduction was also be changed by the concentrations of sugars. For instance, the length of acetylene reduction activity in time was shortened from around 27 days to 24 days or to 18 days while the glucose concentration in incubation medium was increased from 20 g/l to

Table IV. Effects of nitrate respiration on the nitrogen fixing activity of rhizobia in symbiotic rhizobium-callus tissues

Treatment (Day)	Nitrate Respiration		Nitrogen Fixation	
	aerobic	anaerobic	aerobic	anaerobic
0	0.00	0.00	0.11	0.00
3	0.00	0.19	2.32	0.10
6	0.00	0.26	35.33	2.90
9	trace	0.47	50.06	3.72
12	trace	0.46	62.95	3.08

* N_2O released by rhizobia = N_2O released by rhizobium-callus symbiotic system minus N_2O released by callus tissues without inoculating the rhizobia.

40 g/l or to 60 g/l, respectively (Fig. 2). The amount of total nitrogen content in symbiotic rhizobium-callus tissues was matched with the activity of nitrogen fixing activity (Table III). The rhizobia had higher activity of acetylene reduction and percentage of total N while fructose or fumaric acid was supplied as a carbon source for them in culture medium.

The acetylene reduction activity of rhizobia in symbiotic callus tissues was drastically inhibited by deficiency of oxygen (air) in the incubation flasks. They remained an insignificant amount of acetylene reduction activity while the air in the flasks was replaced by Argon. Although the nitrate respiration occurred under this anaerobic condition, the activity was very low (Table IV).

DISCUSSION

Kinds and concentarations of carbohydrate or organic acid affected the acetylene reduction activity of rhizobia in the symbiotic rhizobium-callus tissues. The optimal concentration of sugars for the rhizobial activity was 20 g/l. If the concentration of sugar was higher than this concentration, the level of acetylene reduction and life-span of symbtiotic callus tissues were adversely affected. The harmful functions of sugar on the rhizobial activity and senescence of symbiotic callus tissues might be due to water potential of sugar was so low that caused the rhizobia and callus tissues to be dehydrated. This effect was similar to the rhizobial acetylene reduction activity in root-nodules decreased by water stress (Huang, Boyer and Vanderhoef, 1975). In 1975, Huand *et al* had been shown that the rate of acetylene reduction of soybean plants was reduced drastically while their leaf water potenial or nodule water potential lowered to −12 bars bars or −6 bars. Besides, it had been reported that water stress caused plant to senescence (Salisbury and Ross, 1978).

The intermediates of TCA cycle were also able to support the rhizobial acetylene reduction but they were not so efficient carbon sources as the sugars for the rhizobial activity, except the fumaric acid. In 1982, Huang had demonstrated the combined nitrogens such as nitrate and ammonium salts reduced the levels of acetylene reduction because those combined nitrogen salts decreased the leghemoglobin contents in nodules (Huang, 1980). However, there was no such inhibitory effects of inorganic nitrogen on the nitrogenase activity in symbiotic callus tissues. The rhizobia remained their activity in the presence of nitrogen salts in the culture medium until the symbiotic tissues became senescent.

It had been well documented that Nif genes expression can be inhibited by the presence of those inorganic nitrogenous compounds in culture medium or in soil (Brill, 1977,

Roberts and Brill, 1981). Consequently, there are no nitrogenase synthesis found in rhizobia or other nitrogen fixing micoorganisms. However, there was no such adverse effect of nitrate on the enzyme synthesis in rhizobia of symbiotic callus tissues. Although it was found that rhizobial acetylene reduction in callus tissues was not so high as the rhizobia in nodules, this might not be the problem of nitrogenase synthesis but the enzyme activity. Nitrogenase is an oxygen-labile enzyme (Bergersen, 1971; Brill, 1977; and Yates 1980). In the nodules of legumes, the leghemoglobin plays an important role in regulating the oxygen diffusion rate into the rhizobia to prevent the nitrogenase from oxygen-denaturation (Bergersen, 1971; Brill, 1977; and Yates 1980), but there was no leghemoglobin found in this symbiotic callus tissues. Therefore, there must have an alternative protectory mechanism for rhizobia in the callus tissues. It had been reported that the tannin or anthocyanin might have an oxygen-protectory funtion for nitrogenase in nonleguminous plants (Sprent, 1979). However, the lignification of cell walls of callus tissues might have an important role in limitation the oxygen diffusion rate from the exterior to interior of cells. The symbiotic callus tissues lignified and became dark brownish color after they were inoculated with rhizobia.

Theorectically, there will release 545 Kcal of energy while one mole of glucose is catabolized via the nitrate respiration ($C_6H_{12}O_6 + 6\,NO_3^- \rightarrow 6\,CO_2 + 3\,H_2O + 3\,N_2O + 6\,OH^-$) (Delwiche and Bryan, 1976) but this kind of carbohydrate break-down process did not have significant beneficial effect on rhizobial acetylene reduction because negligible nitrate respiration was found in this symbiotic callus system. It seems that the energy required for the rhizobial activity is likely the rhizobia in nodules derived from the aerobic respiration (Bergersen, 1971 and Yates).

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影響大豆癒合組織與根瘤菌共生現象及其固氮活性因子之研究

I. 碳　　源

黃　啓　穎

摘　要

1. 碳源之不同可影響癒合組織中根瘤菌之固氮活性及固氮壽命。其中以 fructose 爲碳源者效果最好，xylose 者最差。若以有機酸爲碳源者 fumaric acid 最佳而 isocitric acid 較差，在同一濃度下，若以 fructose，sucrose 或 glucose 作爲碳源時癒合組織中根瘤菌固氮活性於第 30 天、27 天或 24 天達到最高，並於第 37 天、 33 天或 27 天失去活性，此時癒合組織已老化。

2. 碳源濃度以 20 g/l 最佳，若是給予碳濃度高於此濃度時根瘤菌固氮作用反而減低並縮短根瘤菌之固氮活性壽命。

3. 癒合組織中沒有血紅素存在，癒合組織之木質化作用可能有助益於癒合組織中氧氣濃度之控制，使固氮酶活性不受氧氣所抑制。

4. 癒合組織之 nitrate respiration 活性很低，對根瘤菌固氮作用所需之能量之供給沒有多大貢獻。