INDUCTION OF NITROGEN FIXING (ACETYLENE REDUCTION) ACTIVITY OF RHIZOBIA GROWING SYMBIOTICALLY AND ASYMBIOTICALLY WITH SOYBEAN CALLUS TISSUES

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Abstract: The rhizobium-soybean callus association in both symbiotic or asymbiotic system caused the *R. japonicum* to establish an acetylene reduction (nitrogen fixation) system. The symbiosis between rhizobium and callus was not an obligative requirement for the induction of nitrogenase synthesis in rhizobia. However, some unknown critical substance(s) produced by the callus is required to initiate the nitrogenase synthesis in the rhizobium. The rhizobia in the symbiotic rhizobium-callus system had 10^3 times higher nitrogen fixing activity than those in the asymbiotic system. The development of nitrogenase in rhizobium in both systems, unlike in intact plant. was not inhibited by the presence of combined nitrogen in medium. The nitrogen fixation might not be interferred by the nitrate assimilatory processes.

The callus could regulate the nitrogen fixing activity of rhizobia but the level of rhizobial nitrogen fixation could not delay the senescence of callus tissues. The rhizobial nitrogen fixing activity declined at the time of callus tissues senesced.

INTRODUCTION

The genus Rhizobium is able to form nodules and fix dinitrogen in the roots of leguminous plants, but the symbiotic interaction between soybean root cells and the rhizobia to initiate the synthesis of nitrogenase is poorly understood. This might be due to the diversity and complexity of the experimental systems using intact plants. However, more recently the tissue culture symbiotic system between plant tissues and rhizobia has been developed[1,2,3,4,8,9]. The present study was carried out in order to interprete the regualtory functions of callus tissues in initiating and regulating the rhizobial nitrogen fixing acitvity while the rhizobia grow symbiotically or asymbiotically with them, and also to provide evidences that rhizobia in the asymbiotic rhizobium-callus system had lower nitrogen fixing activity than those in the symbiotic system. Besides, both quantity and quality of nitrogen assismilatory products released by the rhizobia into the liquid medium were examined.

MATERIALS AND METHODS

Induction of callus tissues

Seeds of the cultivar of soybean (Glycine max.), Shih-Shih, and Kaoshiung #3, were selected and surface sterilized with a 1.2% sodium hypochloride solution for 10 minutes, then washed and rinsed three times with sterile water. The seed coats were removed before seeds were germinated on the surface of Difco agar solidified medium composed of a 2% of sucrose in

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the dark culture room with a constant temperature of 26°C. The seedlings 5 cm tall were dissected into stem and root sections. Those sections were then incubated on the agar solidified B_5 medium[2,3], to induce the formation of callus tissues. Those callus tissues produced from either root sections or stem sections were subcultured once at ten day intervals to maintain vigorous stock.

Preparation of the rhizobial suspension

The *Rhizobium japonicum* used for inoculation the soybean root- or shoot- callus tissues was isolated from the mature root—nodule of the cultivar of Shih-Shih, and purified by the conventional procedures. The purified *R. japonicum* was cultured on agar slants at 30°C. Before the rhizobia were used for inoculation, they were transferred from the agar slants to freshly prepared liquid medium and put on a shaker for 48 hours[2,3]. This rhizobial suspension was then utilized to inoculate the callus tissues.

Establishment of symbiotic rhizobium-callus system

Three tenth grams of actively growing callus tissues produced from stem or root explants were transferred from the agar solidified B_5 medium into the 250 ml Erlenneyer flask containing 50 ml of liquid B_5 medium. Each flask was tight with sterilized cotton and then capped with an aluminum foil. The flasks were shaked rotatinly with a speed of 120 r. p. m. in the dark room. Having cultured in the liquid B_5 medium for 12 days, the callus tissues were then inoculated with rhizobia by adding 0.1 ml of rhizobial suspension to the liquid B_5 medium to establish a symbiosis between the rhizobium and callus[2,3]. After 2 to 4 days of inocualtion, the liquid culture medium contained symbiotic rhizobium-callus tissues and free living rhizobia were filtrated through three layers of miracloth. The filtrates were kept in refrigerator for the chemical components analysis, and the rhizobium-callus symbiotic tissues retained onto the miracloth were rinsed three times by a fresh liquid B_5 medium to remove those extracelluar rhizobia adhering to the callus surface. Those washed rhizobium-callus tissues were then incubated further on the agar solidified LNB_5 medium[2,3], and the rhizobial nitrogen fixing (acetylene reducion) activity in this symbiotic rhizobium-callus system were determined henceforth.

The controls were made by the rhizobia or callus tissues incubated in medium without the callus tissuses or the rhizobia.

Measurement of the symbiotic nitrogen fixation

Those symbiotic rhizobium-callus tissues were placed into flasks (125 ml). Each flask was sealed tightly with a serum bottle stopper, then one tenth of air was withdrawn from the flask with a syringe before an equivalent volume of acetylene was injected into the flask. At a 30 minutes intervals, 0.2 ml of gas sample was withdrawn from the flask and assayed by the gas chromatography[7]. Having finished the measurement of the nitrogen fixation of the rhizobium-callus tissues, the tissues were then dried in an oven at 65°C for 48 hours, then their dry-weight was determined.

Chemical analysis

Those filtrates obtained from the filtration of cultured liquid B_5 medium were centrifuged with a $10000 \times g$ for 15 minutes. The precipitates were discarded but the supernatant was diluted by 200 fold with distilled water for the following chemical determinations.

(a) Quantity of sugar: The total concentration of sugar were determined by the Anthrone method[10].

(b) Quality of sugars: The components of sugar were determined by the paper chromatographic method. The stationary phase was the Whatmenn filter paper (3 mm), and the mobile phase was a mixture of *n*-butanol, acetic acid, and water (4:1:1). The color development agent was the Anisidine-HCl solution[10].

(c) Quantity of amino acids: The amount of amino acids was determined by the Ninhydrin method[10], and also by the amino acid analyzer, Yanagimoto LC-5[3].

(d) Quality of amino acids: By the amino acid analyzer, Yanagimoto LC-5[3].

Establishment of asymbiotic rhizobium-callus system

At the center of fresh agar solidified LNB_5 medium in Petri-dish, a cavity was drilled into, then 0.1 ml of rhizobial suspension was poured into the cavity. The active growing un-inoculated callus tissues either from root- or stem- explants were then incubated surrounding the cavity. Thus, the rhizobia in the cavity were prevented from contacting with the callus tissues, so the asymbiotic interaction between the rhizobia and the calli could be established. The Petri-dishes were all sealed by the parafins. The culture conditions in this experiment were same as that in the establishment of symbiotic rhizobium-callus system. After a few days, those rhizobia growing in the cavity were removed out and diluted by 10 ml of 0.85% NaCl solutiou. This rhizobial suspension was then used to determine the nitrogen fixing (acetylene reduction) activity.

The controls were made by incubating the rhizobia in the center cavity but no callus tissues were incubated around them, or by incubating the callus tissues around the cavity but no rhizobia were in the cavity.

Measurement of asymbiotic nitrogen fixation

Ten ml of the rhizobial suspension obtained from the asymbiotic rhizobium-callus system was poured into a flask (100 ml). The procedures for the measurement of this nitrogen fixation were same as that described previously in the symbiotic rhizobium-callus system. The population of rhizobia in each flask was determined by the turbility of rhizobial suspension at an optical density of 660 nm ($O.D._{660\,nm}$) before their nitrogen fixing activity was measured.

RESULTS

The free living rhizobia or the un-inoculated callus tissues, which were induced either from the explants of stem or root origin, cultured in the B_5 or LNB_5 medium were unable to reduce acetylene to ethylene. However, the acetylene reduction (nitrogen fixing) activity of rhizobia could be detected after the rhizobia had interacted with the calli in the callus tissues (Fig. 1, 2, 3, 4). This symbiotic nitrogen fixation of rhizobia in the rhizobium-callus system had also been demonstrated by Holsten *et al.* (1971), Child and LaRue (1974), and Phillips (1974). The nitrogen fixing activity of the rhizobia in this symbiotic rhizobium-callus system could be found at the third day after inoculation. The activity increased linearily with increasing length of incubation on the agar solidified LNB_5 medium, and reached to a maximum level at the 12th or 26th day, depending upon the origin of explants, after they were transferred from B_5 medium to the LNB_5 medium (Fig. 1, 2, 3, 4). This maximum nitrogen fixing activity leveled off while the host callus tissues began to senesce, which occurred around the 26th day after incubation on the LNB_5 meium. The level of nitrogen fixing activty of rhzobia in symbiotic system depended upon the origin of soybean explant and the cultivar, The rhizobia is in symbiotic association with the callus originated from stem had higher activity than those in association with root-originated callus (Fig. 1, 3). This is unexpected result because in intact plant system only the roots instead of stem form nodules with rhizobia. The rhizobia interacted with the callus tissues from the cultivar of Shih-Shih had more vigorous activity than those from the cultivar Kaoshiung #3 (Fig. 3, 4). This might be the cultivar Shih-Shih was more effectively in association with this strain of *R. japonicum* than the cultivar Kaoshiung #3, because the rhizobia used as an inoculum in this study were isolated from the mature root-nodules of the cultivar shih-Shih. It had been reported that strains of rhizobium have different level of affinity toward the cultivar of legumes[1,8].

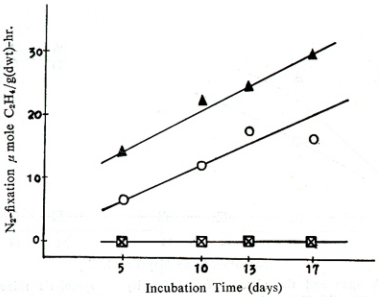

Fig. 1. The nitrogen fixation of rhizobia in the symbiotic rhizobium-callus system as a function of time. The soybean cultivar Shih-Shih callus tissues were inoculated with the rhizobia in a liquid B_5 medium for three days before they were transferred onto this agar solisified LNB_5 medium.
▲-▲: Stem callus tissues inoculated withR. japonicum.
○-○: Root callus tissues inoculated with R. japonicum.
×-×: Stem or root callus tissues without rhizobia.
□-□: Rhizobia without callus tissues. Above data are from ten replicates.

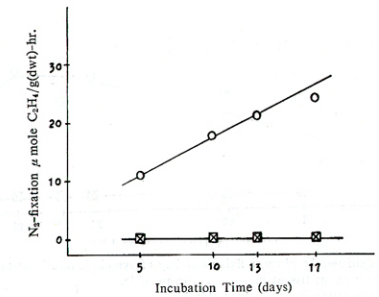

Fig. 2. The nitrogen fixation of rhizobia in the symbiotic rhizobium-callus system as a function of time. The callus tissues which produced from the explants of root of soybean cultivar Kaoshiung #3 had inoculated with rhizobia in a liquid B_5 medium for three days before they were transferred onto the agar solidified LNB_5 medium.
○-○: Root callus tissues inoculated with R. japonicum.
×-×: Root callus tissues without rhizobia.
□-□: Rhizobia without callus tissues. Above data are from seven replicates.

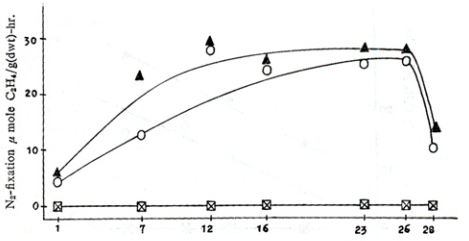

Fig. 3. The life span and the nitrogen fixing activity of symbiotic rhizobiium-callus system. The Shih-Shih callus tissues were inoculated with the rhizobia in a liquid B_5 medium for three days before they were transferred onto the agar solidified LNB_5 medium.
▲-▲: Stem callus tissues inoculated with rhizobia.
○-○: Root callus tissues inoculated with rhizobia.
×-×: Stem or root callus tissues without rhizobia.
□-□: Rhizobia without callus tissues. Above data are from seven replicates.

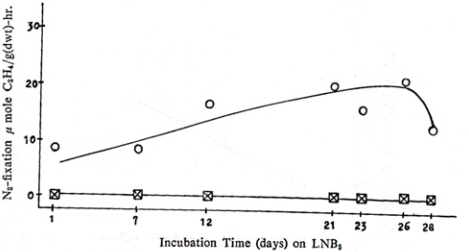

Fig. 4. The life span and the nitrogen fixing activi-ty of symbiotic rhizobium-callus system.
○-○: Root callus tissues inoculated with rhizobia.
×-×: Root callus tissues without rhizobia.
□-□: Rhizobia without callus tissues. Above data are from seven replicates.

The symbiosis between the rhizobia and the callus tissues was not the essential requirement to induce the rhizobia to fix nitrogen. As the Figs. 5 and 6 show that rhizobia which were incubated, but did not contact, with the callus tissues on the same agar solidified LNB_5 medium had the nitrogenase activity. The nitrogen fixation of rhizobia in this asymbiotic rhizobium-callus system could be detected no sooner than seven days after they were cultured on the

LNB_5 medium with callus. It took seven more days to exhibit the nitrogenase activity on the LNB_5 medium than those in the symbiotic system. The nitrogen fixing activity was also increased linearily with increasing incubation times until it reached to a maximum livil at 24th–28th day after incubation, and then the activity declined rapidly (Fig. 5, 6). Obviously, the kinetics of the rhizobia nitrogen fixation in this asymbiotic system was similar to that in the

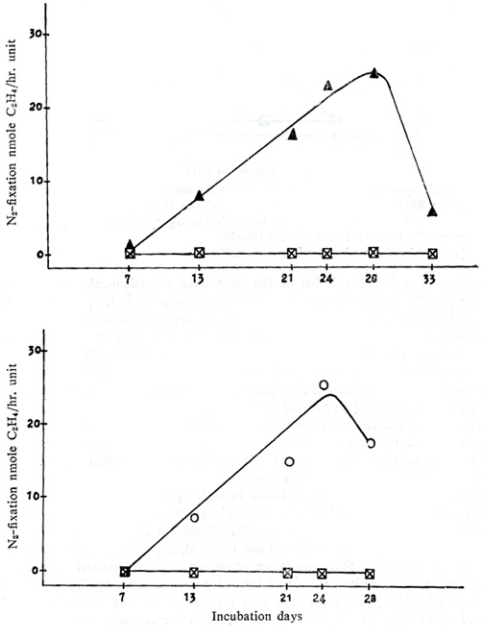

Fig. 5. The nitrogen fixing activity of rhizobia in the asymbiotic rhizobium-callus system. The rhizobia and Shih-Shih callus tissues were incubated adjacent to but not in contact with each other onto the agar solidified LNB_5 medium.
▲-▲: Rhizobia in the presence of stem callus tissues.
○-○: Rhizobia in the presence of root callus tissues.
×-×: Callus tissues (stem or root origin) without the rhizobia.
□-□: Rhizobia without the callus tissues. Unit=The optical density (O. D.) of the rhizobial suspension at 660 nm equal to one.

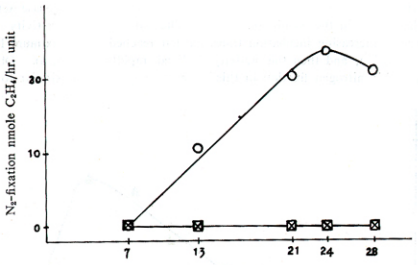

Fig. 6. The nitrogen fixing activity of rhizobia in the asymbiotic rhizobium-callus system.
○-○: Rhizobia and the root callus tissues of Kaoshiung #3 were incubated adjacent to but not in contact with each other on agar solidified LNB_5 medium.
×-×: Root callus tissues without rhizoba.
□-□: rhizobia incubated without the calli.

symbiotic system, even if the rhizobia in the asymbiotic rhizobium-callus system had lower nitrogen fixing activity than those ones in symbiotic system (Fig. 3, 4, 5, 6). The calli of the symbiotic rhizobium-callus system became brownish and appeared to be lignified, but the calli in the asymbiotic system remained yellowish brown. The life span of the callus tissues was about 26 to 28 days. The rhizobial nitrogen fixing activity in both symbiotic and asymbiotic system declined co-incidently at the time of senescence of callus tissues. This suggests that the plant callus tissues might regulate the nitrogen fixing activity of rhizobia, and the regalator(s) might be a chemical substance(s) produced by the calli.

The factors which induced the callus tissues to senesce need further study but it may confirm that ageing of the tissues was not the result of nutrient (either organic or inorganic) deficiency, or the accumulation of substance(s) released by the tissues into the culture medium, because they were subcultured at a 10-day intervals.

The nitrogen fixing activity of the rhizobia in both symbiotic and asymbiotic rhizobium-callus system, unlikely those of rhizobia in intact plant system, was not depressed by the presence of combined nitrogen, because those rhizobia cultured in B_5 or LNB_5 medium contained NO_3^- (25 mM) and NH_4^+ (2.0 mM) or 25 mM and 0.64 mM, respectively, retained active nitrogen fixation. It had been reported that ammonium or nitrate at a concentration as low as 0.1 mM had the inhibitory effects on nitrogenase activity in legumes[4]. Not much amino acid could be detected in the liquid B_5 medium in the first three days, but the concentration of amino acids increased about three- or six folds, depending upon the explants, after a three days' lag period of nitrogen fixation (Table 1). Although some of the amino acids might be assimilated from the activity of inducible nitrate reductase in the calli or rhizobia because of the presence of nitrate or ammonium in the medium, the rhizobia in the symbiotic rhizobium-callus system, undoubtly, could release some of their nitrogen fixing products, amino acids, into the liquid medium. As the Table 2 shows that the rhizobia in symbiotic association with the root-calli of cultivar Kaoshiung #3 had higher amino acid contents in the culture liquid medium than those of free living rhizobia. The compositions of amino acids differed in the symbiotic system.

Table 1. Changes of amino acid and sugar contents in the incubation medium (B_3) by the symbiotic rhizobium-callus system

Species	Amino acid (μg/ml)			Sugar (μg/ml)		
	Inoculation-days					
	0	3	4	0	3	4
S. S.	0.00	24.91	149.20	20.00	10.51	8.41
S. R.	0.00	40.49	114.80	20.00	10.34	10.35
K. R.	0.00	20.52	108.55	20.00	10.82	9.06

S. S.=Calli induced from shoot of Shih-shih seedling.
S. R.=Calli induced from root of Shih-shin seedling.
W. R.=Calli induced from root of Kaoshiung #3 seedling.

Table 2. The changes of the amino acid concentrations and compositions in the liquid B_5 medium by the inoculated and uninoculated callus tissue system

Amino acid \ Treatment	Amina acid concemtration (%)		
	K. R.+R (A)	K. R. (B)	A. A. synthesised by R. japonicum (A-B)
NH_3	46.15	83.54	−37.39
Lysine	7.64	1.66	+ 5.97
Histidiue	2.99	—	+ 2.99
Arginine	2.68	—	+ 2.68
Aepartic acid and asparaagine	6.70	1.91	+ 4.79
Threouine	6.74	1.35	+ 5.39
Serine	2.92	1.56	+ 1.36
Glntamic aoid and glntamine	1.91	1.42	+ 0.49
Glycine	2.44	1.81	+ 0.64
Alamine	3.88	6.76	− 2.88
Proline	0.35	—	+ 0.35
Valine	3.34	—	+ 3.34
Iso leucine	2.37	—	+ 2.37
Leucine	4.31	—	+ 4.31
Tyrosine	2.93	—	+ 2.93
Phenylalanine	2.66	—	+ 2.66

K. R.+R.=The calli induced from root of Kaoshiung #3 were inoculated with Rhizobium japonium.
K. R.=Un-inoculated callus tissues.
A. A.=amino acid.

The amino acids, such as histidine, arginine, proline, valine, isoleucine, leucine, tyrosine, and phenylalanine were found only in the rhizobium-callus culture. The aspartic acid (asparagine), threonine, and lysine were the major amino acids found in the culture liquid medium of symbiotic system. The changes of sugar concentration in the liquid medium of symbiotic rhizobium-callus culture are also shown on Table 1. It demonstrates that about 50% of sucrose was consumed during the lag phase of nitrogen fixation. According to the paper chromatographic analysis, the sucrose and its hydrolytic products, glucose and fructose, were the sugars found in

the medium. This suggests that glucose or fructose was the carbon source for the rhizobial nitrogen fixation.

DISCUSSION

The rhizobia interacting with the callus tissues either in the symbiotic or asymbiotic system might develop nitrogen fixing activity. However, the free living rhizobia cultured in the medium without the soybean callus, or the callus tissues cultured in the medium in the absence of rhizobia did not have the capacity for nitrogen fixation (Figs. 1, 2, 3, 4, 5, 6). This suggests that some unknown critical substance(s) produced by the calli and diffused into the rhizobia are the indispensable factor(s) to initiate nitrogenase synthesis in the rhizobia. Based upon this suggestion, it may interpiete that rhizobia in the asymbiotic system needed take a longer time to exhibit the nitrogen fixing activity than that in the symbiotic system. In the latter system, the rhizobia, which were penetrated into and embedded in the callus cells[5,6], could easily obtain the essential substance(s) produced by the host-callus to initiate the synthesis of nitrogen fixing enzyme, but in the former system, the rhizobia, which were kept away from in contact with the calli, could'nt sysnthesize the enzyme until the substance(s) released from the calli and diffuesed through the agar medium into the rhizobia. In the root-nodules of intact plant, the nitrogenase activity could be inhibited by the presence of combined nitrogen, such as nitrate or ammonium[4]. However, no such harmful effect of combined nitrogen on nitrogenase synthesis or activity was observed in this symbiotic rhizobium-callus system or asymbiotic rhizobium-callus system. It had been reported that one of the inhibitory effects of combined nitrogen on the nitrogen fixing activity of intact plant was attributed to the carbohydrate deprivation, because the sugar was utilized in the nitrate assimilatoty processes (ref. Gibson *et al.*). Besides, it had also been suggested that nitrite, the primary product of nitrate reduction, could form a complex with leghaemoglobin, so the leghemoglobin lost its role as a supplier of oxygen to the bacteriod, consequently, the nitrogen fixing activity of bacteriodal rhizobia was reduced or inhibited (ref. Gibson *et al.*). However, these two harmful effects of combined nitrogen on the nitrogen fixation might not occur in this symbiotic or asymbiotic rhizobium-callus system, because there was a high concentration of sugar, 20 g/l or 30 g/l, contained in the culture medium, B_5 or LNB_5, respectively. Therefore, the nitrogen fixation of rhizobia in both system might not be reduced by the nitrate assimilation because of the sugar deprivation. Besides, no leghaemoglobin has been found in the rhizobium-callus system[5,6], so the nitrogen fixation could not be reduced by the nitrite because of the denaturation of leghaemoglobin. From above interpretations, it can be seen that the rhizobial nitrogen fixing activity in the rhizobium-callus system is less sensitive to the combined nitrogen than that in intact plant system.

The rhizobia in the symbiotic rhizobium-callus system had 10^3 higher nitrogen fixing activity than those in the asymbiotic system[3,4,5,6]. Such a huge diversity in the activity of nitrogen fixation between in these two systems might not be induced by C-sources or any other nutrient factors, because both systems were cultured or incubated in the same kinds of medium. However, the oxygen-regulatory function of callus tissue might be the critical factor determining the level of nitrogen fixation. In the symbiotic system, rhizobia were invaded into the callus cells are likely in the micro-aerobic environment that favours the nitrogen fixation. On the contrary, in the asymbiotic system, the rhizobia are free exposed to the air, their nitrogen fixing activity can easily be inhibited by the oxygen because nitrogenase is an enzyme extremely sensitive to oxygen[4].

The senescence of the callus tissues was followed by a rapid reduction of nitrogen fixation of rhizobia. Thus, the nitrogen fixing activity of rhizobia was closely related with the active growth of callus in this rhizobium-callus system. However, the nitrogen fixation of rhizobia

did not delay the occurrence of senescent of callus. This may interprete that the senescence of callus tissues was not the result of nitrogen source deficiency, and neither was induced by the accumulation of nitrogen fixing products, or C_2H_4, because the infected and the non-infected callus tissues senesced at the same time, and no ethylene could be detected in either incubation systems.

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