Nitrogen Fixing Activity in *Rhizobium japonicum* Separated from Plant Cell Cultures

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Induced by soy bean tissue cultures in socalled "tissue chambers", *Rhizobium japonicum* str. 61-A-96 developed nitrogenase activity separated from the plant cells. The activity proceded for 48 h with a rate of 1×10^{-8} nmol C_2H_4 h^{-1} $cell^{-1}$, which is about 6% of the activity measured for bacteroids from *Rhizobium japonicum* in nodules of *Glycine max*.

A major step in the study of the developmental aspects of symbiotic nitrogen fixation was the first sucessful *in vitro* symbiosis between soybean tissue cultures and rhizobia by Holsten *et al.* [1], Child and LaRue [2], and Philipps [3]. A main feature of the method used by the last two authors was the transfer of liquid grown plant cells to a solid medium and a development of the *in vitro* association lasting 3 – 4 weeks. With similar experiments, in pea [4] and in lupine with *Rhizobium lupini* [5], an active *in vitro* symbiosis has also been established. Our knowledge however, is scarce of how far the *in vitro* symbiosis resembles the *in vivo* development. With the cowpea strain 32-H-1 of *Rhizobium* recently, the establishment of *in vitro* symbioses with non-legume tissue cultures of rapeseed, brome grass [6] and tobacco [7] were described. This strain also infects *in vivo* non-legume plants [8]. Also with this possibly unique *Rhizobium* strain, the first nitrogenase activity of *Rhizobium*, separated from plant cells was recorded, where the free bacteria contributed between 5 and 25% [6] or 16% of the activity of the association [7].

The present study was conducted with a modified method [9], using tissue chambers, in which the plant cells are separated from the agar by a 10 μm Perlon gauze. The advantage of this method is the ability to transfer the plant cell – *Rhizobium* association from one medium and container to another and, apparently very important, the facility for the bacteria to grow through the cell pad and develop on the agar around the plant tissue.

In the experiments for Fig. 1 the tissue chambers with 15 d old soybean cells var. Mandarin were infected with 0.3 ml of *Rhizobium japonicum* str. 61-A-96 (about 10^9 cell/ml). The rhizobia were cultivated in the newly developed low phosphate and low nitrate medium 20 E with the following composition: in 1000 ml medium 68 mg KH_2PO_4, 87 mg K_2HPO_4, 370 mg $MgSO_4 \times 7\,H_2O$, 73.5 mg $CaCl_2 \times 2\,H_2O$, 560 mg KNO_3, 4.84 mg $Na_2MoO_4 \times 2\,H_2O$, 6.95 mg $FeSO_4 \times 7\,H_2O$, 9.3 mg EDTA, 4.6 g glycerol, 1.82 g mannitol, 2.0 g yeast extract, the pH was made up to 6.8. After another 14 days the tissue chambers were transferred to other gas tight test jars. The remaining bacteria, developed around the site of the tissue chambers on the agar and the separated tissue chambers themselves were tested for acetylene reduction. The data show that more than 95% of the total activity is produced by the rhizobia separated from the plant tissue. The activity continues for at least 48 h. Because the activity was almost totally present in the separated rhizobia, the specific activity on a per rhizobial cell basis could be calculated. It is compared with some data for aerobic, free-living, diazotrophic bacteria and with bacteroids from nodules, measured in the intact nodule and calculated by evaluating the number of bacteria and bacteroids in the nodules.

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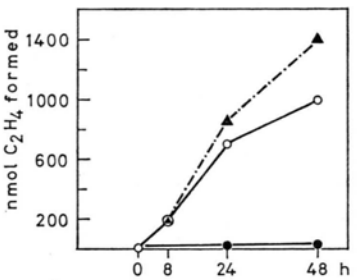

Fig. 1. Acetylene reduction assay [9], ●, with *Glycine max* var. Mandarin-*Rhizobium japonicum* str. 61-A-96 associations in tissue chambers. The activity given is the total of four tissue chambers contained in two gas tight jars. The total dry weight of the biological material was 0.50 g. Activity ▲, with 3.2×10^9 and ○, 5.2×10^9 free living, separated rhizobia after removal of the tissue chamber. The plant cells are cultivated as described previously [2] and the rhizobia in the low phosphate-low nitrate medium 20 E. The bacterial counts (colony plating) were made after 48 h.

Table I. Specific activity of induced, free living *Rhizobium japonicum* and of other aerobic nitrogen fixing bacteria in the acetylene reduction assay.

Bacteria	nmol C_2H_4 h^{-1} $cell^{-1}$
Rhizobium japonicum str. 61-A-96 induced by tissue culture, free living	1×10^{-8}
Rhizobium japonicum-bacteria and bacteroids in nodules of *Glycine max*.	1.5×10^{-7}
Mycobacterium flavum calculated from [10]	5×10^{-6}
Azotobacter vinelandii	5×10^{-5}
Azotobacter chroococcum calculated from [11]	1×10^{-4}

From these experiments we conclude, that the most appropriate index for the effectiveness of the *in vitro* association is the activity of the induced rhizobia on the basis of per rhizobial cell, rather than on the basis of per g dry weight of plant tissue. In this type of experiment also plant tissue strains, which had, in previously published work [2, 6], only low activities, like *Glycine max* var. Mandarine, gave a similar overall activity as other varieties like *Glycine max* var. Acme [8].

The following tests were made to ensure axenic cultures. The plant cell cultures were rountinely tested for aerobic and anaerobic bacterial contaminants on yeast peptone agar and in thioglycollate medium. Single colonies from the induced bacteria in experiments for Fig. 1 were reisolated and compared with the original strain microscopically and by using antibiotic diffusion tests with 13 different antibiotics (Oxoid U1 – U4). 32 plates of single colony isolates were screened with antibiotic test discs and were indentical with the original strain. Subcultures on new medium from the induced rhizobia in the experiments for Fig. 1 and cultures of the original strain were tested for acetylene reduction. The activity was zero in all cases. The ethylene production by the plant cells themselves in the tissue chambers is less than 10% of the very low activity of the association including the plant cells in Fig. 1 and therefore negligible.

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