Nitrogenase activity in a transfilter culture of rhizobia with a non-leguminous plant callus culture: transfer of fixed $^{15}N_2$ from bacteria to *Portulaca* callus

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Summary. A transfilter culture of the non-leguminous plant *Portulaca grandiflora* var. JR and *Rhizobium* sp. 'cowpea' 32H1 was established. Using $^{15}N_2$-analysis we demonstrated that ^{15}N-containing substances produced by the bacteria passed through the membrane and ^{15}N was enriched in the plant cells.

The activation of nitrogenase activity in rhizobia by non-leguminous plant cell cultures could be demonstrated in several cases using the acetylene reduction assay[2-6]. In order to demonstrate that acetylene reduction was due to nitrogenase activity ^{15}N analysis was used. Scowcroft and Gibson[4] observed ^{15}N enrichment in bacteria after exposure of a 32H1-tobacco cell association to an atmosphere containing about 64 atoms percent ^{15}N. Due to the fact that bacteria and plant cells were not separated in their system, they were not able to demonstrate the fate of the fixed nitrogen, but from the point of view of possible practical use, the partitioning of fixed nitrogen between the bacteria and the plant cells is a major question. Therefore, we investigated the ^{15}N enrichment and partition in a transfilter culture of *Rhizobium* 'cowpea' 32H1 and *Portulaca grandiflora* var. JR.

Callus from internodes of *P. grandiflora* var. JR was obtained by cultivation on MS-Medium[7-9]. *Rhizobium* sp. 'cowpea' 32H1 was cultivated on yeast-mannitol agar[10]. Tests for contamination of the rhizobium strain were routinely carried out[6]. These tests confirmed the absence of microbial infection. In vitro associations were established on 5 ml LNB-5 medium with 0.8 g callus and 0.4 ml samples of bacteria (2×10^{10} cells/ml), at 26 °C in light (10,000 lx) for 14 h per day. Bacteria and callus were separated by a dialysis membrane with a pore size of 150–200 nm in a transfilter apparatus developed for these experiments[11]. The cultures were incubated in gas-tight glass vessels (volume 180 ml). Nitrogenase activity was determined by the acetylene reduction assay[12]. After 10 days half of the gas atmosphere was replaced by $^{15}N_2$-gas (97.5 atom%). After 96 h samples of the bacteria and the callus culture were taken and lyophilized until we observed no further loss of weight. ^{15}N enrichment of the samples was determined by the Dumas method[13] followed by the optic emission method[14].

To establish a basis for our experiments with ^{15}N in associations between plant cells and rhizobia, we first studied, whether ^{15}N is enriched in cultures showing no nitrogenase activity (figure 2; 2, 3). The ^{15}N enrichment of the samples incubated in air correlates with the natural $^{15}N/^{14}N$-ratio (figure 1; 3a, 3b). The cultures incubated in the synthetic atmosphere showed an increase in ^{15}N compared with identical material incubated in air (figure 1; 2a, 2b). This may be the result of an unspecific absorption of molecular ^{15}N to cellular structures, because later experiments dealing with the ^{15}N content of proteins did not show this enrichment. It is possible, for instance, that despite prolonged lyophilization a small amount of water, containing ^{15}N, remains in the sample. The results indicate that a relatively high amount of ^{15}N can be enriched in plant callus cultures and bacteria without nitrogenase activity. It is therefore not sufficient to compare the experimental results only with control experiments incubated in air.

In the experiment concerning the in-vitro association in a transfilter culture, we detected an enrichment of ^{15}N in the plant callus compared with the values obtained in identical plant material without bacteria (figure1; 1a, 2a). The bacteria of the in-vitro association showed almost the same ^{15}N incorporation as bacteria in absence of the plant host (figure 1; 1b, 2b). The corresponding acetylene-dependent ethylene production could only be detected in the in-vitro association (figure 2; 1).

The enrichment of ^{15}N in the plant callus culture of the in-vitro association, combined with the fact that the ^{15}N-content of the bacteria in the same culture remained almost constant compared with the control experiment, immediately suggests a transfer of ^{15}N-containing compounds from bacteria to plant cells. This explanation is consistent with the results of O'Gara and Shanmugan[15]. They found that free-living bacteria excrete a major part of their fixed nitrogen. For example, as much as 65–80% of the ^{15}N fixed

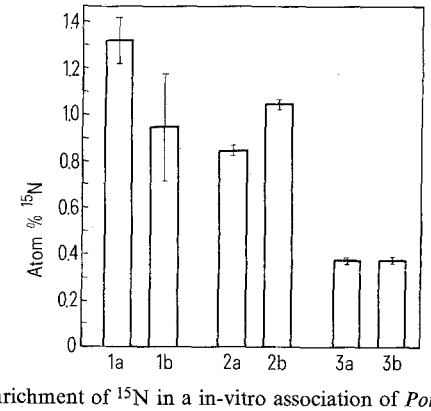

Fig. 1. Enrichment of ^{15}N in a in-vitro association of *Portulaca* and *Rhizobium* 'cowpea' 32H1. After an incubation period of 96 h in an atmosphere of about 10% O_2 and 90% N_2, containing 54 atom% ^{15}N, the ^{15}N incorporation was measured in *1a* plant callus of the in-vitro association, *1b* bacteria of the in-vitro association, *2a* plant callus not associated with bacteria, *2b* bacteria in the absence of plant host. Parallel to these experiments, the ^{15}N content of the following samples incubated in air was determined: *3a* plant callus not associated with bacteria, *3b* bacteria in the absence of plant host.

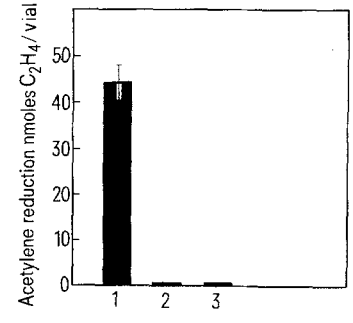

Fig. 2. Acetylene-dependent ethylene production of *1* an in-vitro association between *Portulaca* callus and rhizobia, *2* plant callus not associated with rhizobia, *3* bacteria in absence of the plant host.

by 32H1 was recovered as NH_4^+ from the cell supernatant[15]. Preliminary results indicate that, in our system also, NH_4^+ was excreted into the medium, transported to the plant callus and used for the synthesis of nitrogen-containing compounds.

One of the prerequisites for a quasi-symbiotic situation, the transfer of fixed N from rhizobia to plant cells in an in-vitro association, is suggested by our results. Further investigations concerning the physical nature of the transferred fixed N and the utilization of this compound by the plant cells can now be made. We consider that these approaches can provide information about the possible practical use and economic importance of in-vitro associations between non-leguminous plants and rhizobia.

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Insecticidal effect of trans-2-nonenal, a constituent of carrot root

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Summary. Vapour of trans-2-nonenal killed the carrot fly larva, *Psila rosae*, with an LC_{50} of 2.17 mg/24 h. The aldehyde was identified in the essential oil of carrot in concentrations of up to 40 µg/root.

Plants use chemicals in their defence against insects with effects that may be non-lethal such as repelling insects or inhibiting their growth[3] or lethal as caused, for example, by the pyrethrins[4]. A systematic investigation of the effects of compounds associated with the carrot root (*Daucus carota* L.) on the larva of its parasite, the carrot fly (*Psila rosae* (F.)) (Diptera: Psilidae), has identified a naturally-occurring toxin, trans-2-nonenal $CH_3 \cdot (CH_2)_5CH{:}CH \cdot CHO$.

The following amounts of trans-2-nonenal (purity 99% GC; Bush Boake and Allen Ltd, London) dissolved in chloroform (Merck analytical grade) were each pipetted onto filter paper discs (Whatman No. 4, 5 cm diameter): 0.05, 0.1, 0.5, 1.0, 2.5, 4.0, 5.0, 7.5, 10.0 and 15.0 mg (4 replicates of each); control discs received only solvent. After a 3-min interval to evaporate solvent, each disc was placed in a 400 cm^3 Kilner jar which was then sealed by a paraffin-coated glass plate. After 5 min a batch of 5 3rd-instar larvae, still active after dissection from carrot roots and subsequent starvation for 24 h at 19 °C, was introduced into each jar in a stainless steel tray containing moist vermiculite (70 ml distilled water/60 g vermiculite); each tray was placed on glass vials to provide a clearance of 1.5 cm above the paper disc. These conditions formed a rather stringent test of the compound as direct contact with the insects was not allowed. Tests lasted 24 h and the time taken to immobilize 50% of larvae per treatment was noted; dead larvae were recorded following a 4-h exposure to fresh air. These were shrivelled and had a browned cuticle.

2-nonenal has been previously identified in both steam-distilled[5] and cold-solvent[6] extracts of carrot root and to supplement these observations, steam-distilled extracts were made from 4 carrot cultivars, Chantenay-Red-Cored Elite, Regulus Imperial, Danro and Regol, for analysis by GLC.

Vapour from 15 mg of trans-2-nonenal induced 100% larval mortality in 24 h, 0.05 mg induced none and the LC_{50} was 2.17 (figure 1). 50% of larvae were immobilized in 15 min by 15 mg, in 30 min by 2.5 and in more than 4 h by 1 mg; larvae were neither immobilized nor killed by controls. Trans-2-nonenal was identified in the oils of the 4 carrot cultivars, representing 0.2 ppm Chantenay-Red-Cored Elite, 0.2 ppm Regulus Imperial, 0.5 ppm Danro and 0.6 ppm Regol (figure 2): the latter value is equivalent to 40 µg of trans-2-nonenal per average carrot root. Such GC identification is not definitive but there was insufficient of

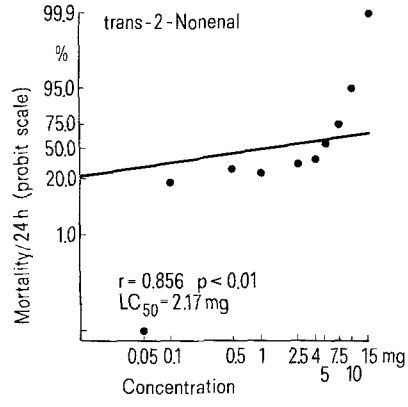

Fig. 1. Dosage/mortality relationship for 24 h exposure of the 3rd-instar carrot fly larva to trans-2-nonenal. As cis-2-nonenal is not commercially available the stereospecificity of this effect could not be ascertained.