Role of Plant Cell Conditioned Medium in the Phenotypic Expression of Nitrogenase Activity of *Rhizobium trifolii* Strain T1

**Minocher Reporter, Mary L. Skotnicki**\(^A\) and **Barry G. Rolfe**\(^B\)

Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387, U.S.A.
\(^A\) Present address: School of Biological Technology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033.
\(^B\) Present address: Department of Genetics, Research School of Biological Sciences, The Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

**Abstract**

The influence of substances from a conditioned medium of cultured plant cells on nitrogenase activity, respiration and ATP synthesis was investigated in *R. trifolii* strain T1. Nitrogenase activity in strain T1 was dependent on the addition of the plant cell conditioned medium. Studies showed that the initial effects of the plant substances on rhizobial cells was to increase their respiration rate and ATP production. Mutants of strain T1 which were uncoupled in their oxidative phosphorylation, were also tested. However, the plant factors had no effect on respiration and ATP synthesis and also failed to elicit *in vitro* nitrogenase activity in these mutants. It is proposed that these plant factors act by increasing the efficiency of oxidative phosphorylation, making more ATP available, and thus stimulating nitrogenase activity of *R. trifolii* cells.

**Introduction**

When rhizobia enter legume roots, a complex set of interactions between the two organisms can occur, leading to the development of a root nodule containing rhizobia in the nitrogen-fixing bacteroid form. As host specificity between *Rhizobium* spp. and legumes appears to be strict, an effective (nitrogen-fixing) nodule will only result if the correct pattern of recognition and interaction occurs between the host and bacterium. As an approach to the analysis of this interaction between plant and rhizobial cells, an *in vitro* system was developed which involved soybean plant cells, rhizobia and a double-membrane filter apparatus (Reporter and Hermina 1975). In a series of experiments, it was shown that the rhizobia could cause the soybean cells to release substances into their growth medium (plant cell conditioned medium, PCM) which in turn could induce the nitrogen-fixing phenotype in different *Rhizobium* strains (Reporter 1976, 1978; Bednarski and Reporter 1978).

*In vitro* nitrogenase activity in cultures of fast-growing rhizobia can be demonstrated by incubation of the bacterial cells under a low level of O\(_2\) together with appropriate dilutions of PCM (Bednarski and Reporter 1978) which may be produced by an initial incubation of rhizobia, separated by membrane filters or doubled dialysis bags, with cell suspensions from leguminous plants (Bednarski and Reporter 1978). The components of PCM which are active in aiding this expression of rhizobial nitrogenase have been partially characterized and described as plant peptidoglycans (Storey et al. 1979). Thus, PCM made with *R. japonicum* and soybean cells induced nitrogenase activity in other rhizobial species as well as *R. japonicum*. However, the effect was not simple and concentration optima for PCM or the active isolated compounds from PCM preparations varied for each *Rhizobium* strain tested (Storey et al. 1979).
Whether PCM acts as an effector molecule, or as an essential component, or as a substrate for some reaction, is unknown. To analyse this problem, the effect of these active plant substances on respiration, ATP synthesis and nitrogenase activity was studied in _Rhizobium trifolii_ T1 and mutants of this strain which were defective in their energy metabolism.

**Materials and Methods**

**Bacterial Studies**

Strains used are described in Table 1.

**Media**

All buffers, growth media and procedures have been described elsewhere (Reporter 1976, 1978; Skotnicki _et al._ 1979; Rolfe _et al._ 1980). A modified B5 medium which lacked nitrate and contained 1 mM NH₄Cl (Bednarski and Reporter 1978) was used to suspend the soybean cells for preparation of PCM. A nitrogen-free B5 medium (NNB5) was used to dilute bacteria for nitrogenase assays.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>R. trifolii</em></td>
<td></td>
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<tr>
<td>T1</td>
<td>Prototrophic parental strain effective (Eff⁺) (nitrogen-fixing) nodulation of white, red and subterranean clover</td>
<td>Schwinghamer and Dudman (1973)</td>
</tr>
<tr>
<td>T1Sp⁴</td>
<td>Spectinomycin-resistant, Eff⁺ derivative of strain T1</td>
<td>Schwinghamer and Dudman (1973)</td>
</tr>
<tr>
<td>ZT2</td>
<td>Neomycin-resistant (Nm⁴) mutant uncoupled in oxidative phosphorylation, unable to use succinate as sole carbon source (Suc⁻), defective Mg²⁺ + Ca²⁺-stimulated ATPase activity (ATPase⁻), Eff⁺ derivative of strain T1</td>
<td>Skotnicki and Rolfe (1979)</td>
</tr>
<tr>
<td>RT18</td>
<td>Nm⁴Suc⁻ATPase⁻ Eff⁺ uncoupled mutant of strain T1Sp⁴</td>
<td>Skotnicki and Rolfe (1979)</td>
</tr>
<tr>
<td><em>R. japonicum</em></td>
<td></td>
<td></td>
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<tr>
<td>31-1b-138</td>
<td>Prototrophic strain Eff⁺ nodulation of soybeans</td>
<td>Bednarski and Reporter (1978)</td>
</tr>
</tbody>
</table>

**Other Materials**

Gases were purchased from Matheson Gas Products as prepared mixtures of 95% Ar:1% O₂; 4% C₂H₂. Standards for gas chromatography were obtained for H₂, O₂, C₂H₂ and C₂H₄ from Scott Research Laboratories and Applied Research Laboratories, State College, Pennsylvania. S ephadex was obtained from Pharmacia, New Jersey. Dissicated firefly tails for ATP assays and lysozyme were purchased from Sigma Chemical Co., St Louis.

**Preparation of PCM**

The PCM used in these experiments was made from a suspension culture of Harosoy Soybean variety (obtained from Dewine Seed Company, Yellow Springs, Ohio), together with _R. japonicum_ strain 31-1b-138 (obtained from Dr S. Smith, Agriculture Laboratory Inc., Columbus, Ohio). PCM was prepared by the double dialysis bag method used previously (Bednarski and Reporter 1978), and was partially purified by passing the crude filtrate through a Sephadex G25 column (Reporter 1978; Storey _et al._ 1979). Seven samples of constant volume were collected from the column, and these fractions were stored under an argon atmosphere at −70°C.

**Estimation of Nitrogenase Activity**

The reduction of C₂H₂ to C₂H₄ as well as production of H₂ were used to estimate nitrogenase activity. These gases were determined by gas chromatography; C₂H₂ and C₂H₄ levels were measured...
using a Hewlett-Packard model 5710A gas chromatograph fitted with a Poropak column at 80°C with a flame ionization detector at 145°C. To determine \( \text{H}_2 \) and monitor \( \text{O}_2 \) levels, a Hewlett-Packard model 5750 gas chromatograph with a molecular sieve column 5A was used at 65°C with a thermal conductivity detector at 100°C.

Cultures to be tested for nitrogenase activity were inoculated into 19-ml assay vials. Each vial contained 1 ml cells, 0.5 ml NNBS medium and 10 mM succinate (pH 6.5), with or without PCM. The vials were closed with serum stoppers and were evacuated and flushed four times with the 95% Ar:1% \( \text{O}_2:4\% \text{C}_2\text{H}_4 \) mixture. Vials were incubated at 30°C, and regularly tested for the presence of \( \text{C}_2\text{H}_4 \) and \( \text{H}_2 \) over a period of up to 100 h. During this time, the rhizobial cultures went through the exponential phase of growth, and after 100 h were well into the stationary phase.

**Determinant of ATP Levels**

The synthesis of ATP from ADP was measured by the firefly luciferin–luciferase assay (Thore 1979). Rhizobial cells were first treated with a lysozyme solution containing 10 mg/l lysozyme, 0.3 mM sucrose and 0.5 mM EDTA in 0.1 M Tris-HCl buffer (pH 6.5) for 15 min to make spheroplasts. Microscopic examination of this suspension revealed that most cells were still rod-shaped, with some spherical cells and very few broken cells. ADP was then added to the spheroplasts, and the samples of the suspension were removed to test for ATP. The spheroplasts were broken open by forcing through a syringe needle of very small gauge (23 gauge) as fast as possible by hand, and were then mixed (0.1 ml cells) with 0.2 ml buffer containing 0.2 M sodium arsenate and 2 mM MgSO₄ (pH 7.5) and 0.1 ml firefly extract which was added just before measuring the level of ATP. The firefly extract was prepared by grinding 0.1 g firefly tails (Sigma Chemical Co.) with 0.3 g fine sand and 10 ml arsenate buffer (Reporter and Raveed 1977) followed by a 10-min centrifugation at 20,000 g and the supernatant was used as the firefly extract. All solutions were kept on ice and used in plastic containers to prevent adsorption of the enzyme on to glass. Light emission by the firefly enzyme in the presence of ATP was measured in a Kettering photometer at 560 nm, and cell samples were compared with a standard curve obtained with \( 10^{-10} - 10^{-8} \) moles of ATP. To ensure the reliability of this method, internal standards with duplicate samples were included and measurements were taken immediately after the addition of a sample.

**Measurement of Respiration Rates**

Respiration by *Rhizobium trifolii* cells was measured by following the rate of \( \text{O}_2 \) consumption at 28°C using an \( \text{O}_2 \)-electrode (Yellow Springs Instrument Co., Ohio). These measurements were made in 2 ml of air-saturated saline–phosphate buffer (Cannon et al. 1974). Respiration by spheroplasts was followed in saline–phosphate buffer containing 10% (w/v) sucrose to prevent cell lysis.

**Protein Determination**

The protein content of cells was determined using the Lowry method (Lowry et al. 1951).

**Results**

**Plant Cell Conditioned Medium and Nitrogenase Activity**

The ability of PCM to induce nitrogenase activity was tested in *Rhizobium trifolii* strain T1 (Fig. 1) and was found to give a level of acetylene reduction comparable with other fast-growing rhizobia (Bednarski and Reporter 1978). Nitrogenase activity was detected after about 40 h of incubation in the assay vials.

As found with other rhizobial strains, sequentially collected samples of fractionated PCM from a Sephadex G25 column gave different rates of acetylene reduction and hydrogen production (Fig. 2). Thus PCM affects nitrogenase activity in strain T1 in a manner similar to other rhizobia. For subsequent experiments, PCM fraction 5 from the column, which induced high rates of acetylene reduction in strain T1, was used. It was found that increasing concentrations of this active PCM fraction 5 first increased, and then decreased, the level of nitrogenase activity in strain T1 (Fig. 3). This concentration effect of PCM has been also observed with other rhizobia (Bednarski and Reporter 1978).
PCM and the P/O Ratio of Strain T1

The rate of O₂ consumption by strain T1 after PCM addition was tested to see whether PCM acts by altering respiration. It was found that addition of PCM fraction 5 (which induced nitrogenase activity) to a respiring suspension of strain T1, increased the rate of O₂ consumption by up to 32%, depending on the amount added. However, this increase in the rate of respiration was not simply due to PCM uncoupling O₂ consumption from oxidative phosphorylation since a further addition of CCCP (carbonyl cyanide m-chlorophenylhydrazone, an uncoupler) still had the
expected uncoupling effect leading to increased O$_2$ consumption (Fig. 4). If PCM had acted only as an uncoupler, it is unlikely that the addition of CCCP would have given such a significant increase in respiration above the high rate already attained.

The increase in respiration observed could have been due to the PCM simply providing extra carbon sources for strain T1. This possibility was eliminated by examining the rate of O$_2$ consumption with (a) extra glucose or succinate (known to be present in crude PCM preparations, but which came off the column in later, less-active fractions), (b) different fractions of PCM which induced either very good rates of acetylene reduction as in PCM fraction 5 from the experiment of Fig. 3, or poor rates, as in PCM fraction 7 of the same experiment, and (c) a sample of one active fraction of PCM (fraction 5) which was inactivated by exposure to air for 30 h at room temperature. Extra carbon sources above those already supplied did not significantly increase the rate of respiration (Fig. 4). The least-active fraction of PCM (fraction 7) collected off the column which induced 12% of the nitrogenase activity shown by fraction 5 in strain T1 also increased respiration much less than the active fraction 5 (4% increase for fraction 7, compared with 32% for fraction 5). The most direct evidence, however, was from that sample of active fraction 5 which was exposed to air at room temperature and gave very little increase in the rate of respiration (5%), whereas the rest of this fraction (stored at -70°C under argon) increased respiration by up to 32%.

Since PCM apparently increased the rate of O$_2$ consumption without uncoupling oxidative phosphorylation, ATP synthesis in strain T1 was tested to see whether PCM fraction 5 also increased oxidative phosphorylation. It was found that while the active PCM fraction 5 increased respiration by about 17%, ATP synthesis was increased by 44% (Table 2). The P/O ratio thus increased after the addition of PCM.
by nearly 30% (Table 2). In contrast, inactivated PCM did not stimulate ATP synthesis. It appeared, therefore, that PCM acted on strain T1 by increasing the efficiency of oxidative phosphorylation.

**Table 2. Effect of PCM on the P/O ratio of spheroplasts of R. trifolii strain T1**

<table>
<thead>
<tr>
<th>Additions</th>
<th>O₂ consumption [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>ATP synthesis [nmol min⁻¹ (mg protein)⁻¹]</th>
<th>P/O ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM succinate</td>
<td>187 (100%)</td>
<td>269 (100%)</td>
<td>0.72</td>
</tr>
<tr>
<td>20 mM succinate + 20 µl PCM</td>
<td>219 (117%)</td>
<td>402 (144%)</td>
<td>0.92</td>
</tr>
<tr>
<td>20 mM succinate + 25 µM ADP</td>
<td>204 (128%)</td>
<td>607 (128%)</td>
<td>1.49</td>
</tr>
<tr>
<td>20 mM succinate + 25 µM ADP + 20 µl PCM</td>
<td>239 (128%)</td>
<td>916 (128%)</td>
<td>1.29</td>
</tr>
</tbody>
</table>

* Both respiration and ATP synthesis were measured on the same spheroplast preparation at the same time. Without the addition of succinate, no O₂ consumption or ATP synthesis was detectable.

**Effect of CCCP on PCM-induced Nitrogenase**

The addition of the uncoupler CCCP abolished nitrogenase activity by up to 100% when added to various nitrogen-fixing E. coli hybrids containing the K. pneumoniae nitrogen-fixing genes (Skotnicki and Rolfe 1978). Similarly, if the uncoupler CCCP was added to a PCM-induced, acetylene-reducing culture, nitrogenase activity was also abolished (Fig. 5). In both organisms, it appears that a

![Fig. 5. Effect of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) on nitrogenase activity induced by PCM in R. trifolii strain T1. (a) Control suspension: strain T1 cells were incubated with 200 µl PCM under an atmosphere of 95% Ar: 1% O₂: 4% C₂H₂, and gas samples were periodically assayed for C₂H₄ (●) and H₂ (O). (b) T1 cells were incubated in the same way as the control, but at 91 h CCCP was added at a final concentration of 15 µM to vials to test the effect of this uncoupler on nitrogenase activity.

'high-energy membrane state' (Harold 1972) is necessary for nitrogenase activity and CCCP abolishes nitrogen fixation by dissipating this state. Thus, although PCM affects oxidative phosphorylation, its role cannot be that of an uncoupler since the uncoupler CCCP has a completely different effect from PCM on nitrogenase activity of *Rhizobium* cells.
Mutants of R. trifolii Uncoupled in Oxidative Phosphorylation

A set of mutants of strain T1, which had growth properties similar to E. coli strains defective in phosphorylation associated with electron transport, have been isolated and characterized (Skotnicki and Rolfe 1979). These mutants could respire a range of carbon and energy sources, but ATPase activity was very low and no rapid ATP synthesis could be detected with the addition of succinate or glucose (Table 3; Skotnicki and Rolfe 1979). Table 3 shows the physiological properties of two of these mutants, RT2 and RT18, as well as their symbiotic characteristics on either white, red or subterranean clovers.

Table 3. Physiological properties of R. trifolii strain T1 and T1Nm8Suc−ATPase− mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Respiration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ATP synthesis&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>Coupled</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RT2</td>
<td>Uncoupled</td>
<td>132</td>
<td>109</td>
<td>11</td>
</tr>
<tr>
<td>RT18</td>
<td>Uncoupled</td>
<td>128</td>
<td>109</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Respiration rates of glucose and succinate for strains RT2 and RT18 expressed as percentage of those for parent strain T1 which respired glucose at 227 and succinate at 228 nmol O<sub>2</sub> consumed min<sup>−1</sup> (mg protein)<sup>−1</sup>, respectively.

<sup>b</sup> Activities for strains RT2 and RT18 expressed as percentage of that for strain T1 which was 2.55±0.12 μmol Pi min<sup>−1</sup> (mg protein)<sup>−1</sup>.

<sup>c</sup> ATP synthesis for strains RT2 and RT18 expressed as percentage of that for strain T1 which was 269 nmol min<sup>−1</sup> (mg protein)<sup>−1</sup>.

These mutants were used to further examine the effect of PCM on rhizobial cells and test the hypothesis that its main action is on the efficiency of oxidative phosphorylation. However, it was not possible to induce nitrogenase activity in the uncoupled mutants of strain T1 with PCM addition, even when added at three times the optimal concentration for strain T1. Furthermore, PCM addition had very little or no effect on respiration by the uncoupled mutants, and no ATP synthesis could be detected with or without PCM addition.

Discussion

The experiments reported above have demonstrated that R. trifolii strain T1 was also influenced by these plant-derived compounds. Moreover, strain T1 was shown to behave like other rhizobia, since it exhibited similar patterns of induced nitrogenase activity with different fractions and concentrations of added PCM. When PCM was added to cultures of strain T1, it was about 40 h before nitrogenase activity could be detected, and then only after the O<sub>2</sub> concentration in the culture vials had fallen to less than 0.2%. By this time, the cells were entering the stationary phase of growth. However, when low concentrations of O<sub>2</sub> (0, 0.1, 0.2, 0.5, 1 and 2% initial concentration) in an argon-acetylene atmosphere were tested with strain T1 in similar conditions except for the absence of PCM, no induction of nitrogenase activity occurred. Thus some additional factor, supplied by PCM, is required for nitrogenase activity.
When PCM was added to strain T1 cells, a significant increase occurred in the O₂ consumption and ATP synthesis, which led to an increase of about 30% in the P/O ratio measured for the cells. Thus it seems that these plant factors may act on *R. trifolii* by increasing the efficiency of oxidative phosphorylation, causing an increased consumption of O₂ and making more ATP available for the rhizobial cell. Presumably, this combination of events provides the environmental factors necessary for a functional nitrogenase complex in rhizobial cells.

This explanation of the mechanism of action of PCM was supported by the experiments with mutants of strain T1 defective in electron transport-mediated phosphorylation and therefore unable to synthesize ATP by the ATPase complex. When PCM was added to the uncoupled mutants of strain T1, nitrogenase activity could not be detected even if three times the optimal concentration of PCM for strain T1 was used. Moreover, PCM had no effect on the rate of respiration by the mutants, and did not cause detectable levels of ATP to be synthesized. In *R. trifolii* the oxidative phosphorylation system is necessary to supply the large amounts of ATP needed for nitrogen fixation to occur in the nodule (Appleby *et al.* 1975; Bergersen and Turner 1975; Laane *et al.* 1978). It should be noted, however, that the uncoupled T1 Suc⁻ ATPase⁻ mutants form effective nodules, and do have a functional ATPase complex when they differentiate into the bacteroid form within a nitrogen-fixing nodule (Skotnicki and Rolfe 1979). Therefore, the addition of PCM to T1 Suc⁻ ATPase⁻ mutants either does not completely mimic all the changes that occur during the differentiation into the bacteroid form, or else the PCM from soybean cells cannot induce these changes to occur in *R. trifolii* cells.

Recently, we have reported on the isolation of spectinomycin-resistant mutants of *R. trifolii* which no longer require PCM for the induction of their nitrogenase activity in cultures (Skotnicki *et al.* 1979). An investigation with PCM, spectinomycin and these T1Sp⁸ mutants indicated that spectinomycin may also affect oxidative phosphorylation in strain T1 (M. L. Skotnicki, B. G. Rolfe, and M. Reporter, unpublished data). This finding may help to explain why nitrogenase activity is more readily expressed in these T1Sp⁸ mutants. Further studies, using a combination of these mutants and PCM, should give a greater insight into the molecular signalling between the bacteria and plant cells within the nodule, as well as the regulation of nitrogen fixation in *Rhizobium* spp.

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