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Effect of a Clay Mineral (Montmorillonite) on the Nodulation of *Alnus* and on the Nitrogenase Activity of *Frankia* in Pure Culture

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Abstract

The addition of a clay mineral, montmorillonite, to *Alnus* seedlings inoculated with *Frankia* strain Ai1, growing in gravel and in Leca gravel, led to a two-fold increase in the nodule number. A liquid culture experiment in which the culture solutions, collected from the gravel jars, were used as the 'inoculation media', indicated that the stimulatory effect of the clay could be partly attributed to the adsorption by the clay of inhibitory compounds secreted by alder.

Montmorillonite affected the nitrogenase activity (acetylene reduction) of *Frankia* Ai1 in pure culture by shortening the lag phase. Both light and electron microscopy indicated interactions between the *Frankia* strain and the clay.

Keywords: acetylene reduction, actinorhizal plants, *Alnus*, clay, electron microscopy, *Frankia*, montmorillonite, nitrogen fixation, nodulation

1. Introduction

Clay minerals affect the activity, ecology and population dynamics of microorganisms in soil (reviewed by Stotzky and Burns, 1982). Their primary influence on microbes apparently takes place through the modification of the physical and chemical characteristics of the microbial habitats, and this either enhances or attenuates the growth and metabolic activities of individual microbial populations. In addition to these indirect effects, direct surface interactions between clays and microbes can also occur (Stotzky, 1985).

Of the various clay minerals so far studied, montmorillonite has the strongest influence, primarily because of its high cation exchange capacity, although its large surface area and ability to swell also appears to be involved in some microbial responses (Stotzky and Burns, 1982). Montmorillonite has been shown to both stimulate and inhibit the growth and metabolic activity of fungi (Campbell and Ephgrave, 1983; Stotzky, 1972) as well as actinomycetes (Martin et al., 1976) and other bacteria (Marshman and Marshall, 1981), depending on the concentration of clay and on the microbe species.

Actinorhizal plants, due to their root nodule symbiosis with the nitrogen fixing actinomycete *Frankia*, considerably increase soil nitrogen status (Mikola, 1966; Wheeler et al., 1986). In field studies, some indirect indications have been obtained concerning the importance of clay in the formation of the symbiosis (Righetti et al., 1986; Vogel and Dawson, 1985). However, the mineral composition of the clays is unknown. Apart from these few observations, nothing is known about the effect of clays on *Frankia* or on its symbiosis with actinorhizal plants.

The present study describes on the one hand the effect of montmorillonite on the nitrogenase activity (acetylene reduction) of *Frankia in vitro*, and, on the other hand, on the nodulation of alder, the most common and important actinorhizal plant in Finland.

2. Materials and Methods

Clay mineral

Commercial HCl-treated montmorillonite (K10, Fluka), with a surface area of 220–270 m^2g^{-1}, was shaken in 0.1 M KOH or 0.05 M $Ca(OH)_2$ for 18 hr, and washed twice with distilled water. The KOH-treated montmorillonite was, in addition, washed twice with half-strength nitrogen-free nutrient solution (Huss-Danell, 1978). After drying, the montmorillonite was sterilized by autoclaving.

Cultivation of the plants

The progeny and sterilization of seeds of an *Alnus* hybrid (*A. incana* × *A. glutinosa*) have been described earlier by Smolander and Sundman (1987), the growing conditions by Weber et al. (1987), and the nutrient solution by Huss-Danell (1978).

The plants were cultivated in gravel (diam. 2–4 mm) and Leca gravel (diam. < 3 mm) which had been sieved (mesh size 2 mm), washed with distilled water and autoclaved. Lecagravel is expanded clay (Lohja Ltd, Finland),

made from field clay (mainly swelling layer silicates) which, when heated in a rolling oven (1150°C) forms light, porous particles, called Leca gravel.

Frankia strain

Frankia strain Ail (catalog no. UHF 01112; Lechevalier, 1985), isolated from an *A. incana* nodule, was precultivated at 28°C in TPC medium (Weber et al., 1988), modified to contain 0.5 ml Tween 80, 0.8 g sodium propionate, and 0.5 g casaminoacids per litre. For inoculation purposes the cells were harvested by centrifugation, washed 3 times and resuspended in sterile water. The suspension was subsequently homogenized by repeated flushing through an injection needle.

Light and electron microscopy

Light and electron microscopy were used to reveal possible interactions between *Frankia* Ail and montmorillonite both in young cultures in nitrogen-free TP-N medium and in old cultures in nitrogen containing TPC medium.

For light microscopy, the samples were mounted in water and observed with phase contrast optics with a Jenaval microscope (Carl Zeiss, Jena).

For transmission electron microscopy, the samples were gently prefixed for 2 hr by adding glutaraldehyde (Leiras, Finland) to the growth medium to give a final concentration of 3% (v/v), and washed 3 times in 0.1 M sodium phosphate buffer (pH 7.2). The specimens were postfixed and examined as described earlier (Weber et al., 1988).

For scanning electron microscopy, the samples were prefixed as described above, dehydrated in a graded series of ethanol, and dried in a Balzers CPD 020 critical point drying apparatus. The specimens were mounted on specimen stubs and coated with gold in a Jeol Fine Coat ion-sputter JFC-1100. A Jeol JMS-820 scanning electron microscope operating at 10 kV was used for examining the samples.

Statistical analyses

Statistical significance of the differences between the means was tested using analysis of variance, followed by Tukey's test.

Nodulation experiments

The effect of montmorillonite on the nodulation of *Alnus* seedlings was studied in gravel and in Leca gravel. Sterilized alder seeds were transferred, after germination on water agar, to 1 l glass jars containing 250 ml gravel or

Leca gravel, with or without supplementation of 25 ml of nutrient-solution-treated montmorillonite, and moistened with 80 ml of nutrient solution (either nitrogen-free or supplemented with KNO_3, 1 mM final concentration). The jars were covered with petri dishes, the holes in the dishes being plugged with cotton to provide ventilation. Water lost by evapotranspiration was replaced by adding sterile distilled water. After 3 weeks, part of the jars were inoculated with *Frankia* Ail (the inoculum had been harvested from 0.8 ml of a 3-week-old culture grown in TPC medium). The number of nodules and the dry weight (80°C for 18 hr) of the inoculated plants were determined 2 months after inoculation. The uninoculated plants, grown in parallel, did not form any nodules during the experiment.

In order to determine whether the effect of montmorillonite on nodulation is to counteract the effect of antagonistic secretions from alder, culture solutions were collected from jars containing uninoculated 4-week-old plants by sieving and centrifuging, and from corresponding jars without plants. The supernatants were diluted with distilled water (3:1), the pH adjusted to 5.9 when necessary, and then filter sterilized (mesh size 0.45 μm). Five-week-old alder seedlings, grown in liquid culture as described by Smolander and Sundman (1987), were transferred to the supernatant solutions in 120 ml glass bottles and inoculated with *Frankia* Ail (the inoculum had been harvested from 1 ml of a 3-week-old culture grown in TPC medium). The number of nodules was counted after 3 weeks.

Assay of nitrogenase activity

Nitrogenase activity was estimated as acetylene reduction (Hardy et al., 1968) in 25 ml serum bottles containing 10 ml TP-N medium (pH 6.6), i.e. TPC medium without supplementation of NH_4Cl and casaminoacids. Montmorillonite, treated with nutrient solution or with Ca^{2+} and autoclaved separately, had been added to part of the medium (concentration 0.25% w/v, according to Martin et al., 1976). After being inoculated with *Frankia* Ail (the inoculum had been harvested from 0.5 ml of a 2-week-old culture grown in TPC medium) and plugged with cotton, the bottles were incubated at 28°C and gently shaken daily by hand. For the acetylene reduction measurements, triplicate bottles were taken, in the beginning at daily and later with 2 day intervals, stoppered, and 10% acetylene added. Ethylene was measured by gas chromatography immediately, and after incubation for 24 hr. Parallel bottles, not containing the *Frankia* inoculum, were incubated in the same way; no ethylene production was detected in these control bottles.

Table 1. Effect of montmorillonite (mo) on the nodulation and growth of alder, inoculated with *Frankia* Ail, in a 3-month experiment. The results are means of 6 parallel jars (each containing 5 seedlings), SD in parenthesis. Values in each column with a common letter as superscript do not differ at a statistical significance of $p < 0.05$.

Growth substrate (pH in parenthesis*)	Nodule number	Dry weight, mg		
		Nodules	Roots	Shoots
Gravel (6.0–5.6)	21 (2.1)^a	2.8 (0.35)^a	6.5 (0.81)^a	94.5 (11.20)^a
Gravel + mo (5.9–5.7)	43 (2.8)^b	4.4 (2.28)^a	9.3 (1.19)^b	129.1 (9.88)^b
Leca gravel (6.1–5.8)	23 (4.2)^a	3.7 (1.16)^a	9.0 (1.26)^b	98.9 (8.52)^a
Leca gravel + mo (6.2–6.3)	45 (7.8)^b	4.5 (0.97)^a	9.8 (1.12)^b	98.6 (12.37)^a
Gravel + N** (6.0–6.1)	24 (6.2)^a	3.9 (0.69)^a	9.2 (2.04)^b	133.2 (23.38)^b

* pH at the time of inoculation and at the end of the experiment.

** Nutrient solution was supplemented with 1 mM KNO_3.

3. Results and Discussion

Effect of montmorillonite on the nodulation of alder

The effect of montmorillonite on the nodulation and growth of alder is shown in Table 1. Montmorillonite doubled the number of nodules both in gravel and in Leca gravel. The root biomass was higher in the gravel but not in the Leca gravel when montmorillonite was present. Addition of the nitrogen-containing nutrient solution increased the root biomass in the gravel to the same extent as montmorillonite, but did not affect nodulation. The pH values of the gravel or the Leca gravel containing montmorillonite do not appear to have differed sufficiently from those without montmorillonite to have affected nodulation (Table 1).

The results of the liquid culture experiment in which the presence of montmorillonite was eliminated in the actual nodulation situation are shown in Table 2. The number of nodules was similar in culture solutions from jars without alder seedlings, irrespective of whether montmorillonite had been present or not, and the same as in fresh nutrient solution. However, when the culture solution had been exposed to alder, there were differences in nodule number. Fewer nodules were formed in the solution from alder jars without montmorillonite than in the solution from alder jars containing montmorillonite. The apparent stimulatory effect of montmorillonite can therefore be at least partially attributed to the adsorption by montmorillonite of inhibitory compound(s) secreted by alder. The release of stimulatory compound(s) from montmorillonite (compounds which the original nutrient solution contained but had been used by the 4-week-old alder seedlings) seems a less probable explanation although can not be excluded.

Both seeds and roots are known to excrete a number of compounds, such as organic acids, amino acids, proteins, sugars and phenols (Hale et al., 1978). Allelopathic roles have been proposed for certain root exudate components (Smith, 1976). The seeds and roots of some leguminous plants can secrete compounds toxic to rhizobia (Lowendorf, 1977). It is well established that the surface of montmorillonite provides adsorption sites for both ions and a wide range of macromolecules (Stotzky, 1985). The marked stimulatory effect of montmorillonite on the nodulation of alder (Tables 1 and 2) could be partly explained on the basis of the adsorption of nodulation inhibitors. This hypothesis is in accordance with the work of Turner (1955) with clover. Adding activated charcoal to clover plants inoculated with *Rhizobium* strains led to a stimulation in nodule production. The stimulation appeared to be due to the adsorption by the charcoal of the inhibitory compounds secreted

Table 2. Nodulation of alders in culture solutions originating from jars (see Table 1) where uninoculated alder seedlings had been grown for 4 weeks, and from similar jars but without seedlings. Alder seedlings, pregrown in liquid culture, were transferred to the culture solutions and inoculated with *Frankia* Ail. The results are means of 4 parallel bottles (each containing 2 seedlings), SD in parenthesis. The values with a common letter as superscript do not differ at a statistical significance of $p < 0.05$.

Origin of the culture solution	Nodule number
Jars without seedlings	
Gravel	52 (7.9)[a]
Gravel + mo	55 (8.6)[a]
Jars with seedlings	
Gravel	39 (5.7)[b]
Gravel + mo	54 (6.7)[a]
Fresh nutrient solution	56 (5.3)[a]

by the clover roots. These compounds were eluted from the charcoal and shown to affect nodule production. "Clover sickness" has been observed in the field after repeated cultivation of clover crops in the same soil. It has been suggested that this is due to the accumulation of toxic compounds secreted by the clover plants in the soil, and can be alleviated by the addition of charcoal to the soil.

Whether or not a similar situation exists in *Alnus-Frankia* symbiosis remains to be solved, but unknown factors in soil under aging alder stands have been given as the explanation for the relatively low nodulation capacities of *Frankia* of the Sp^- type in soil under the host plant, especially compared to the nodulation capacities found in soil under stands of *Betula*, a non-host plant (Smolander and Sundman, 1987; Smolander, manuscript in preparation; Van Dijk, 1984).

Effect of montmorillonite on the nitrogenase activity (acetylene reduction) of Frankia Ail in vitro

The effect of montmorillonite, saturated either with the nutrient solution or with Ca^{2+} ions, on the nitrogenase activity (acetylene reduction) of *Frankia* Ail in pure culture was also studied. In cultures containing montmorillonite, especially that treated with the nutrient solution, the lag phase was shorter than that in cultures which had not received montmorillonite (Fig. 1). In another experiment (data not shown), using a smaller size of inoculum (1.7 μg

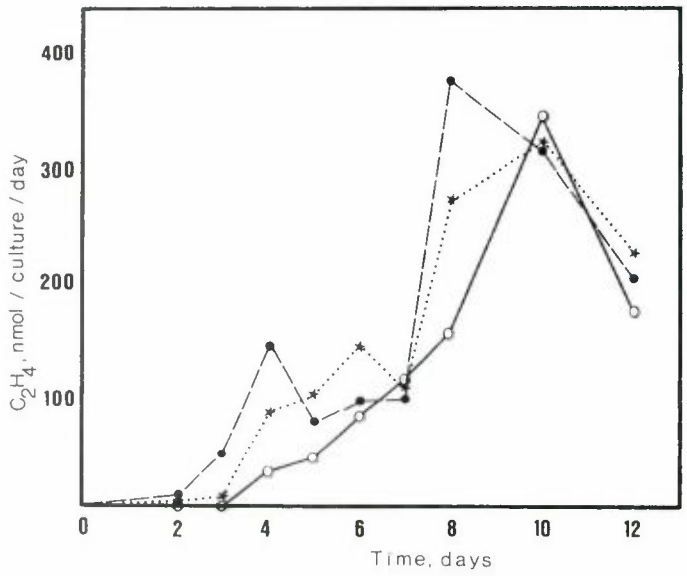

Figure 1. The effect of montmorillonite on the nitrogenase activity (acetylene reduction) *in vitro* of *Frankia* Ai1 in TP-N medium (——o——), and in the same medium supplemented with nutrient-solution-treated (——•——) and Ca-treated (- - - ⋆ - - -) montmorillonite. Inoculum contained 13.6 μm cell protein. Plotted values show the mean (n=3). The standard errors in most cases were below 15% of the mean value.

cell protein), the effect of montmorillonite on the length of the lag phase was even more pronounced; in cultures containing montmorillonite, acetylene reduction started 4 days after inoculation, whereas in cultures without montmorillonite acetylene reduction was not measurable until 10 days after inoculation.

The nitrogenase activities were of the same order of magnitude as the activities earlier reported for *Frankia* strains (Burggraaf and Shipton, 1983; Burggraaf and Valstar, 1984; Zhongze et al., 1986).

Martin et al. (1976) described the accelerated growth, carbon dioxide evolution and glucose consumption of various *Streptomyces, Micromonospora* and *Nocardia* species in the presence of Ca-montmorillonite. Another adsorbent, activated charcoal, promoted the formation of *Frankia* colonies (Diem and Dommergues, 1985). The responses of bacterial cultures to these or other compounds with a large surface area have been attributed to the adsorption of inhibitory metabolites. Adsorption of carbon sources in the medium was observed in this study; we tried to measure the growth of *Frankia* as total

organic carbon (Salonen, 1979), but this was not successful because montmorillonite adsorbed, in addition to *Frankia*, also the carbon sources of the medium. We also tried to assess the growth of *Frankia* by measuring soluble protein, using Coomassie Brilliant Blue G250 (see Smolander et al., 1988), but protein was adsorbed by the montmorillonite, as has been well reported in the literature (Stotzky and Burns, 1982).

The positive effect of montmorillonite was most evident when the culture was young, and observed as a shortening of the lag period (Fig. 1). The same phenomenon has been noticed with the growth of *Escherichia coli* (Novakova, 1977), as well as with the nitrogenase activity of *Azotobacter* sp. and two *Anabaena* species (Sharma, 1984). This has been attributed to the high cation exchange capacity and strong buffering effect of montmorillonite. In the present study, the pH did not change by more than 0.2 pH-units during the incubation, and the change was similar irrespective of whether montmorillonite was present or not. In some so far undetermined way, montmorillonite provided a chemically and/or physically favourable environment for *Frankia*.

Interaction between Frankia and montmorillonite

In order to reveal any possible interactions between *Frankia* and montmorillonite, light microscopy and both scanning and transmission electron microscopy were used. Only the figures for the nutrient-solution-treated montmorillonite (Figs. 2–5) are shown but those of the calcium-treated montmorillonite were similar.

Although the montmorillonite particles were attached to *Frankia* mycelia (Fig. 2b) there was no clear flocculation or other changes in the structure of the clay (Fig. 3b and c). Flocculation of clays by microbial metabolites has been reported by Stotzky (1973). Incubation with *Rhizobium* resulted in aggregation of silt particles (Fehrmann and Weaver, 1978). Furthermore, soil particles were aggregated by the mycelium of a mycorrhizal fungus, *Glomus* (Clough and Sutton, 1978).

Frankia, examined by light microscopy (Fig. 2a and b) and scanning electron microscopy (Fig. 3a and c), looked similar irrespective of whether montmorillonite was present or not. Transmission electron microscopy revealed extra electron-dense material distributed unevenly over the surface of the hyphae in cultures containing montmorillonite (Fig. 4a). Campbell (1983) observed such material on the hyphae of a fungus, *Gaeumannomyces graminis* and on some bacteria, and considered that it consisted of small particles of clay.

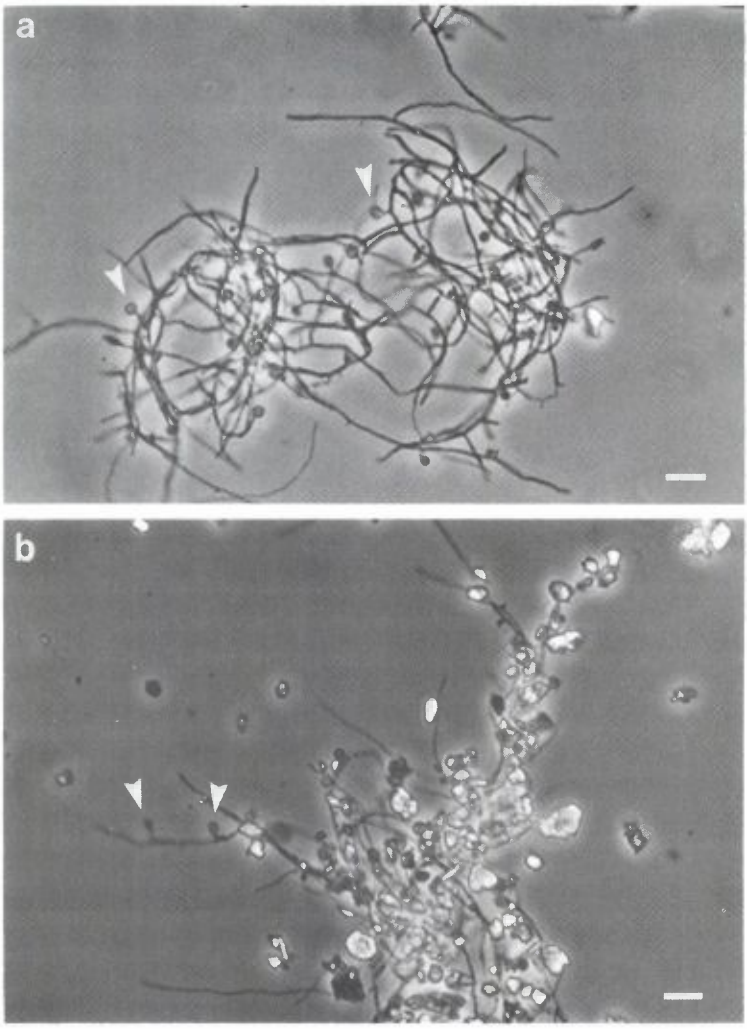

Figure 2. Light micrograph of *Frankia* Ai1 grown for 5 days in (a) TP-N medium, (b) the same medium supplemented with montmorillonite. Vesicles indicated by arrows. Phase contrast optics. Bars represent 10 μm.

Numerous vesicles, the sites for nitrogenase activity (Meesters, 1987), were formed in all nitrogen-free media (Figs. 2 and 3). The outer layer of the vesicle (Fig. 4b), when grown in media containing montmorillonite, was thicker and more electron-dense than that which has usually been observed (e.g. Weber et al., 1988). Microscopical observations did not reveal whether the shorter lag phase in the nitrogenase activity of cultures containing montmorillonite compared to those without montmorillonite was due to a differ-

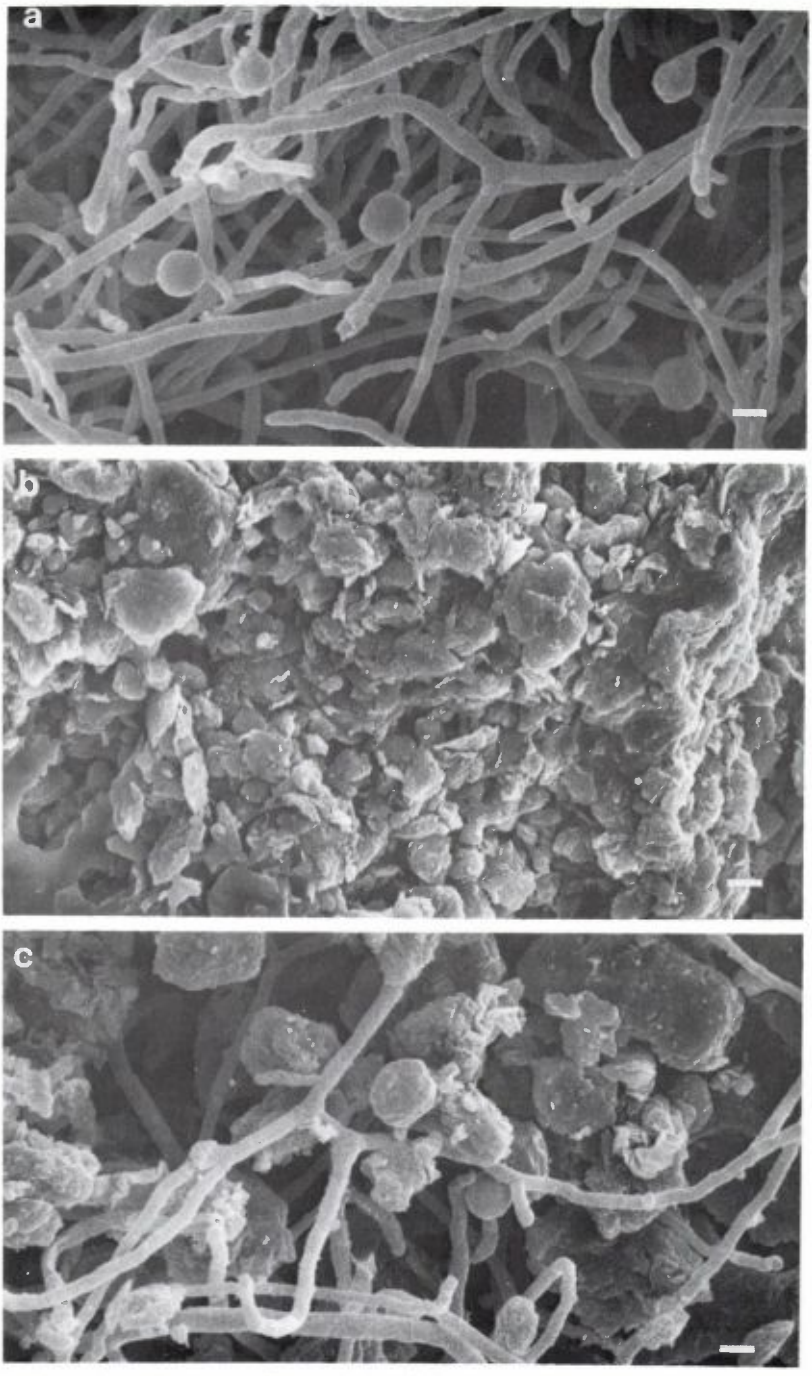

Figure 3. Scanning electron micrograph of (a) *Frankia* Ail grown for 6 days in TP-N medium, (b) montmorillonite, and (c) as (a) but supplemented with montmorillonite. Bars represent 1 μm.

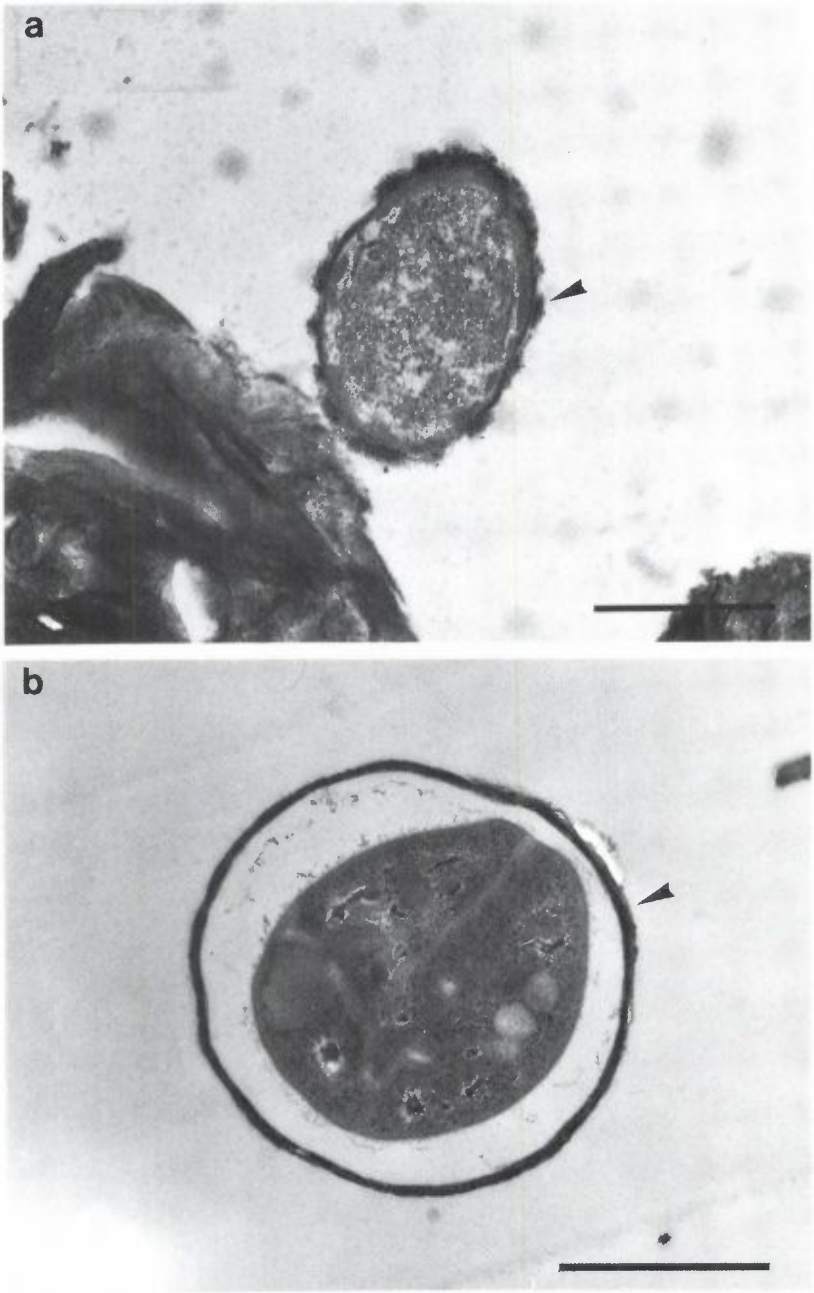

Figure 4. Transmission electron micrograph of *Frankia* Ail grown for 6 days in TP-N medium supplemented with montmorillonite. (a) Electron-dense material (arrow) on the surface of the cross section of a hyphae closely adjacent to montmorillonite. Bar represents 0.5 μm. (b) Electron-dense and thick outer layer (arrow) of the vesicle. Bar represents 1 μm.

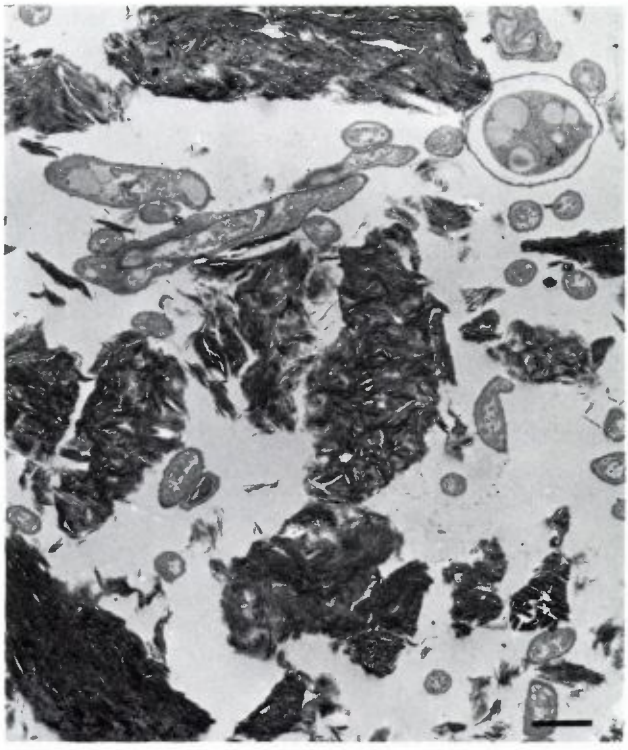

Figure 5. Transmission electron micrograph of *Frankia* Ail grown for 41 days in TPC medium supplemented with montmorillonite. In the upper corner montmorillonite appears to have affected the shape of the outer layer of a vesicle. Contact sites between hyphae and montmorillonite are frequent. Bar represents 1 μm.

ence in the frequency of vesicles, or to enhanced nitrogenase activity per vesicle.

In an old culture growing in nitrogen-containing medium, there was a closer interaction between *Frankia* and montmorillonite (Fig. 5). Although the culture had been thoroughly mixed, *Frankia* appeared to have close contact with montmorillonite, which even affected the shape of the outer layer of the vesicle. Further studies are needed to evaluate further the interaction between *Frankia* and the clay.

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