

## Nitrogen-fixing endophyte *Frankia* in Polish *Alnus glutinosa* (L.) Gartn.

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### Abstract

The first successful isolation of *Frankia* from the nodules of Polish *Alnus glutinosa* have been described. The fine structures of endophyte (thick-walled spherical vesicles of 6 µm diameter, numerous sporangia, various in shape, 60–80 µm length, aseptate and highly branched filaments 1–3 µm) have been studied by light and scanning microscopy. The pure culture in BAP medium without combined nitrogen showed nitrogenase activity (94.51 nmoles of ethylene was formed from acetylene per 1.0 ml of packed cells per hr). The isolate was capable of forming N<sub>2</sub>-fixing symbiotic root nodules on *Alnus glutinosa* seedlings.

**Key words:** *Alnus glutinosa* – *Frankia* – symbiosis – nitrogen fixation – nodulation

### Introduction

*Frankia* species are sporulating filamentous actinomycetes capable of forming symbiotic nitrogen-fixing root nodules on several dicotyledonous plants, including alders. Alders are important to the long-term ecosystem productivity in unfertilized forest ecosystems because of their capacity of fixing atmospheric nitrogen and leading to accumulations of nitrogen, ranging from 500 to 750 kg ha<sup>-1</sup> in 50 years, in forest floor and mineral soil (Bormann *et al.* 1994). Alders have been used in crop rotation with millet (Silvester 1977) and planted in admixtures with pines, cedar, cypress and other trees for

land stabilization and soil improvement (Uemera 1964). *Frankia* is commonly prepared as aqueous suspensions and used for inoculation to improve seedling size and quality, by either mixing with soil or potting medium prior to planting (Benoit and Berry 1994), or by spraying over the surface of soil mix on large scale (Perinet *et al.*, 1985).

Pommer (1959) isolated a *Frankia* strain from *Alnus glutinosa* and reinfected the host with formation of nodules. But his work was never recognized and the culture was lost. Danilewicz (1965) claimed the first isolation of noduleforming *Streptomyces alni* from *A. glutinosa* in Poland. The results obtained with the methods used are doubtful (the strain grew on nitrogenless medium after 4–5 days at 27°C). In addition, the pure culture was not tested for nitrogen fixation. Since 1978 when Callaham *et al.*, (1978) accomplished first successful isolation of *Frankia* sp. strain Cp11 from sweet fern (*Comptonia peregrina*), numerous strains have been isolated from various actinorhizal plants and cultured for physiological, biochemical, inoculation and genetic studies. However there are many reports of unsuccessful isolation, including strains obtained from the nodules of *A. glutinosa* in Poland.

### Materials and methods

*Isolation of Frankia.* Root nodules of *A. glutinosa* growing in a mucky soil (pH<sub>H<sub>2</sub>O</sub> = 4.9) developed from sands accumulated in Vistula River Valley in Toruń, Poland were collected and shipped immediately via air mail to the Forest Sciences Laboratory at Corvallis where they

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Forest Sciences Laboratory at Corvallis where they were processed directly. The nodules were washed in a stream of water to remove loose soil particles and those nodules that floated on water were discarded. Subsequently the nodule lobes were surface sterilized. For this purpose they were placed in a breaker with 2.5% sodium hypochlorite solution plus one drop of Tween 20 and continuously agitated with a magnetic stir for 20 min. The lobes were rinsed thoroughly in sterile distilled water to remove chemicals. Then they were cut into small fragments and homogenized in small amounts of sterile distilled water in a tissue grinder to release vesicle clusters. The homogenate was passed through sterile nylon screen with mesh openings of 50 and 20  $\mu\text{m}$ , as designed by Benson (1982). Vesicle clusters retained on the 20  $\mu\text{m}$  screen were thoroughly washed with large quantities of sterile distilled water in order to remove possible contaminating microorganisms in the nodules. The vesicle clusters were collected with a Pasteur pipet, placed in tubes of modified BAP medium without nitrogen (Murry *et al.*, 1984) and incubated at 30°C with occasional gentle shaking for two months. Subsequently *Frankia* filaments mats were transferred to a tube of sterile distilled water and serial transfers were made with shaking and washing to remove bacteria still associated with *Frankia*. The mats homogenated into numerous filamental fragments were transferred to tubes with BAP medium containing 5 mM  $\text{NH}_4\text{Cl}$  to initiate active growth.

*Inoculation of Frankia.* *Frankia* inoculation onto *A. glutinosa* was carried out in the greenhouse. The plastic leach tubes of 165 ml capacity were filled with equal volumes of pasteurized vermiculite and sphagnum peat moss. Several seeds of *A. glutinosa* which have been surface-sterilized with 30% hydrogen peroxide for 20 min were sown in each leach tube. The emerging seedlings were thinned to one per tube. 4 weeks old *Frankia* culture in BAP medium containing nitrogen were harvested by centrifugation. The pellet was washed with sterile distilled water and centrifuged again. This procedure was repeated three times in order to remove metabolites produced by *Frankia*. *Frankia* was homogenized in sterile distilled water in a tissue grinder and such suspension was applied for inoculation of each seedling. The seedlings were grown in a greenhouse at 25°C. The natural light with sodium-vapor lamps at 11,000 Lx for a 16 hr period was applied. The seedlings were fertilized monthly with 1/4 Hoagland solution minus nitrogen (Hoagland and Aron 1950).

*Nitrogenase activity of Frankia cultures.* *Frankia* cultures growing in the nitrogen containing BAP medium were transferred to the tubes of nitrogen deficient BAP and incubated for 4 weeks at 30°C. Acetylene was injected into tubes to constitute 10% of the total gas

volume. After 2 hrs gaseous samples were removed from the tubes and analyzed for ethylene with a Hewlett-Packard 5830A gas chromatograph fitted with 2 m  $\times$  2.1 mm 80–100 mesh, Porapak R column with oven temperature of 70°C. Injection and flame-ionization detector temperatures were adjusted to 100°C and the flow rate of nitrogen carrier gas was 40  $\text{cm}^3$  per min. Nitrogenase activity was expressed as nmoles ethylene formation per packed cells volume per hr.

## Results and discussion

The nylon screen with mesh openings of 20  $\mu\text{m}$ , as designed by Benson (1982) was effective in isolation of vesicle clusters released from crushed nodules of *A. glutinosa*. They were thick-walled, spherical, 4–10  $\mu\text{m}$  in diameter, with dense mass of filaments (Fig. 1). When cultured in BAP minus combined nitrogen, the vesicle clusters produced sporangia, vesicles and filaments (Fig. 2). The vesicles of 6  $\mu\text{m}$  diameter were thick-walled spherical structures borne on vegetative filaments (Figs. 2–3). Sporangia were numerous and various in shape, ranging from 60  $\mu\text{m}$  to 80  $\mu\text{m}$  in length. The filaments, 1  $\mu\text{m}$  to 3  $\mu\text{m}$ , were aseptate and highly branched (Figs 2 and 4). The vesicles disappeared when the isolate was growing in BAP plus combined nitrogen and produced no nitrogenase activity. In BAP minus combined nitrogen, the isolate produced vesicles and nitro-

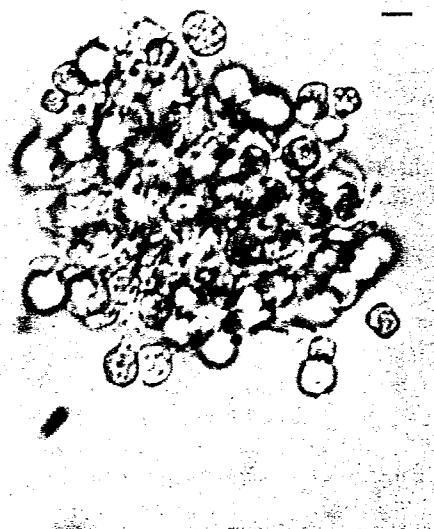


Fig. 1. Vesicle clusters isolated from nodules of *A. glutinosa*. Bar = 3  $\mu\text{m}$ .

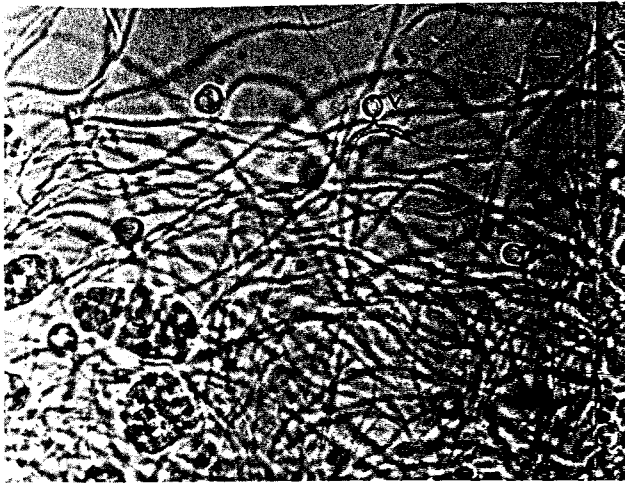


Fig. 2. *Frankia* showing sporangia and vesicles on branching filaments in BAP minus combined nitrogen. Bar = 3  $\mu$ m. Sporangium (s); vesicles (v).

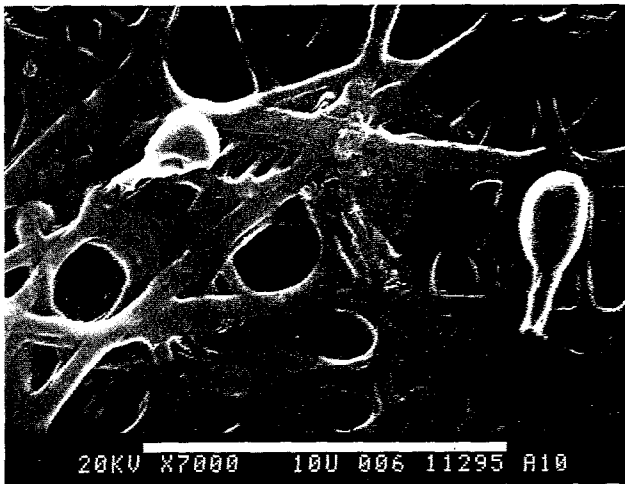


Fig. 3. SEM photographs of vesicles borne on filaments. SEM  $\times$  7000.

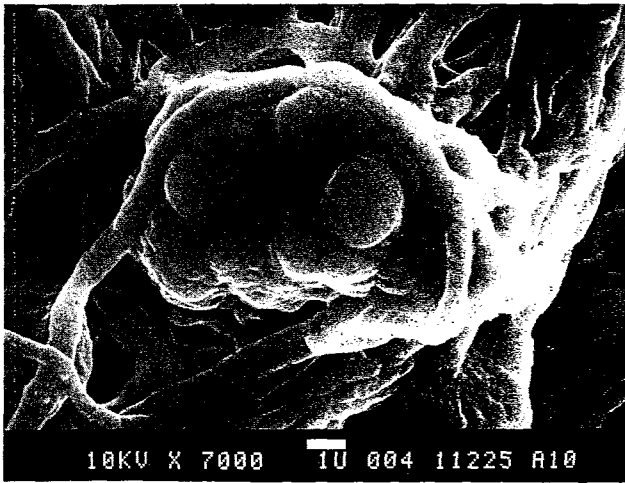


Fig. 4. SEM photographs of sporangium. Note immature spores within the sporangium. SEM  $\times$  7000.



Fig. 5. *A. glutinosa* seedlings inoculated with the *Frankia* strain NCP010301: the seedlings on the front row were not inoculated. Seedlings 4 months old.

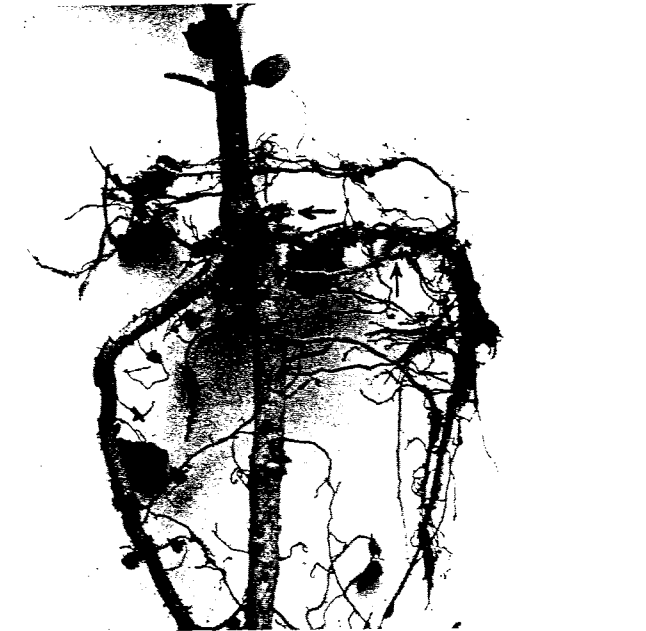


Fig. 6. Formation of root nodules on *A. glutinosa* upon inoculation of *Frankia* strain NCP010301. Arrows pointing to the nodules.

genase activity, which was equivalent to 94.51 nmoles ethylene ( $C_2H_4$ ) formed from acetylene ( $C_2H_2$ ) per 1.0 ml of packed cells per hr (Fig. 2, Fig. 7).

Upon inoculation with the culture of this actinomycete the growth of *A. glutinosa* was significantly stimulated and numerous root nodules were produced (Figs 5 and 6). The nodules possessed nitrogenase activity, as measured by acetylene reduction.

Based on the general morphological characteristics, growth in vitro, its infective and effective abilities, the

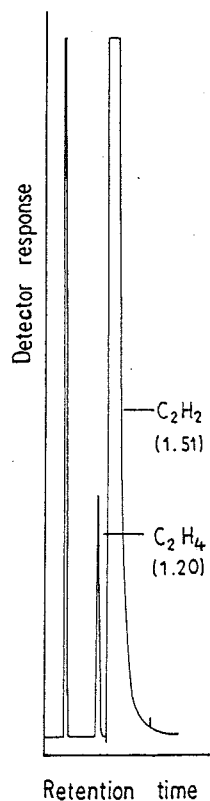


Fig. 7. Acetylene reduction of *Frankia* strain NCP010301 grown in BAP minus combined nitrogen.

isolate fulfilled the criteria for authentication of *Frankia* (Stowers, 1987). This is the first *Frankia* strain isolated from the Polish *A. glutinosa*. The authors designed the strain as NCP010301. NCP stands for Nicolaus Copernicus University of Poland.

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