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Review article

Host Specificity and Efficiency of Nitrogenase Activity of *Frankia* Strains from *Alnus incana* and *Alnus glutinosa*

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Abstract

The nitrogen-fixing actinomycete *Frankia* nodulates dicotyledonous plants belonging to 21 different genera, among them the alder, which is the most important actinorhizal plant in temperate forests. Two types of actinorhizal nodules can be formed: spore-containing nodules (Sp+) and nodules without spores (Sp–).

Two alder species are native in Finland, *Alnus incana* (L.) Moench (and *Alnus glutinosa* (L.) Gaertn. A field survey of the nodule distribution indicated an association of nodule type with alder species; on *A. incana*, Sp+ nodules predominated, whereas on *A. glutinosa* the majority of the nodules were of the Sp– type. Inoculation experiments showed that this association was connected with host specificity. Whether originating from *A. incana* or *A. glutinosa*, Sp– *Frankia* strains were infective and effective (capable of fixing nitrogen) on both alder species. In contrast to the general opinion, the Sp+ type *Frankia* populations differed in their host specificity. Since pure cultures of Sp+ type *Frankia* were not available, only nodule homogenate could be used for this *Frankia* type. Field-collected Sp+ nodules from *A. glutinosa* were effective on both native alder species and also on the non-native species *Alnus nitida* Endl. Sp+ nodules from *A. incana* were able to induce effective nodules only on the original host; on *A. nitida* no nodules at all were formed and on *A. glutinosa* only a restricted number of prenodule-like structures were found. As *A. glutinosa* plants with these structures died on nitrogen-free media, the nodules were apparently ineffective.

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Although all the native *Frankia* strains produced effective nodules on *A. incana*, a great difference was evident in their efficiency. Apart from one exception, Sp-*Frankia* isolates produced almost three times as much biomass on *A. incana* as did nodule homogenates of the Sp+ strain, which is the dominant endophyte on *A. incana* in nature.

This work thus revealed remarkable variation in efficiency between the different *Frankia-Alnus* combinations. Through strain selection, it should hence be possible to exert considerable influence on the productivity of *Alnus* species.

Keywords: actinorhizal plants, *Alnus glutinosa*, *Alnus incana*, *Frankia*, host specificity, nitrogen fixation, nodule types

1. Introduction

The actinomycete *Frankia* possesses the enzyme complex nitrogenase and is hence capable of biological nitrogen fixation. It is also able to infect a wide range of dicotyledonous plants, giving rise to nitrogen-fixing symbioses, which have been referred to as actinorhizal. At present more than 200 different species of angiosperms, representing eight plant families, are known to bear actinomycetous nodules (Lechevalier, 1986).

Actinorhizal plants have a world-wide distribution, but with the exception of genera in the family Casuarinaceae, they mostly occur in temperate regions or at high altitudes in the tropics. They inhabit a wide variety of ecosystems, from swamps to deserts, often being found on nitrogen-poor sites as pioneers in early stages of plant succession, after disturbances such as fires, vulcanic eruption and flooding. Here, they improve their environment thanks to their ability to fix nitrogen, but also by stabilizing the soil and reducing erosion.

The utilization of actinorhizal plants includes soil melioriation (Tarrant and Trappe, 1971; Mikola et al., 1983; National Research Council, 1984; Dawson, 1986), land reclamation (Perinet et al., 1985; Wheeler et al., 1986), afforestation (Mikola, 1975), and biomass production (Leikola, 1976; Gordon and Dawson, 1979; National Research Council, 1984; Pregent and Camire, 1985). The amount of nitrogen fixed by the actinorhizal plants rivals that of the legumes, not only on a global basis, but also in the amounts fixed per hectare and year. For the alder, this has been estimated to lie between 20 and 300 kg (Tarrant and Trappe, 1971). Investigations carried in Finland by Virtanen (1957) and Mikola (1966) showed that even at these latitudes significant amounts of nitrogen can be fixed. The nitrogen input through litter fall was shown by Mikola (1966) to reach levels of 100 kg per ha each autumn.

Alders are potential energy trees in short rotation forestry, where an optimal combination of host tree and the nitrogen-fixing endophyte is desired. In this review *Frankia* strains from the two native alders in Finland (*Alnus incana* and *Alnus glutinosa*) are evaluated as symbionts on both native and non-native alder species. For this purpose *Frankia* strains were isolated. Since not all *Frankia* strains were available in pure culture, nodule homogenates were also included.

2. The endophyte *Frankia*

As symbiont

The actinorhizal nodule is a modified lateral root. *Frankia* grows in the cortex cells of the nodule in hyphal form. In some nodules sporangia are formed. Two types of nodules can thus be distinguished; those with sporangia and those without. The terms spore-positive (Sp+) and spore-negative (Sp-) were introduced by van Dijk (1978) for these two nodule types. The sporulation capacity of the nodules is considered to be a genetically stable character of the endophyte, which is not influenced by the host plant (van Dijk, 1978; VandenBosch and Torrey, 1985).

At the tips of some of the hyphae special structures called vesicles are formed (Fig. 1). The vesicles are considered to be the site of nitrogen fixation. The special role of the vesicle in nitrogen fixation is suggested by its ultrastructure; the vesicle is surrounded by a multilaminate envelope (Torrey, 1985). The vesicle has been compared to the heterocysts produced by blue-green algae, whose specialized structure is thought to protect the oxygen-labile nitrogenase; both vesicles and heterocysts have laminate cell wall layers, which may function as physical barriers to O_2 diffusion. Haemoglobins have been found in some of the actinorhizal symbioses and a correlation appeared to exist between the degree of tissue aeration and the haemoglobin concentration (Tjepkema et al., 1988).

Hydrogenase has, with one exception (Sellstedt et al., 1986), been found in all tested actinorhizal nodules (Benson et al., 1980; Sellstedt, 1989). The relative efficiency of nitrogen-fixation of the actinorhizal nodules is high (Schubert and Evans, 1976). Several studies have shown, however, that nitrogenase activity between different host-endophyte combinations varies (Dawson and Sun, 1981; Dillon and Baker, 1982); Hooker and Wheeler, 1987; Normand and Lalonde, 1982; Sellstedt et al., 1986; Weber et al., 1987; Weber et al., 1989), giving scope for improving the symbiosis.

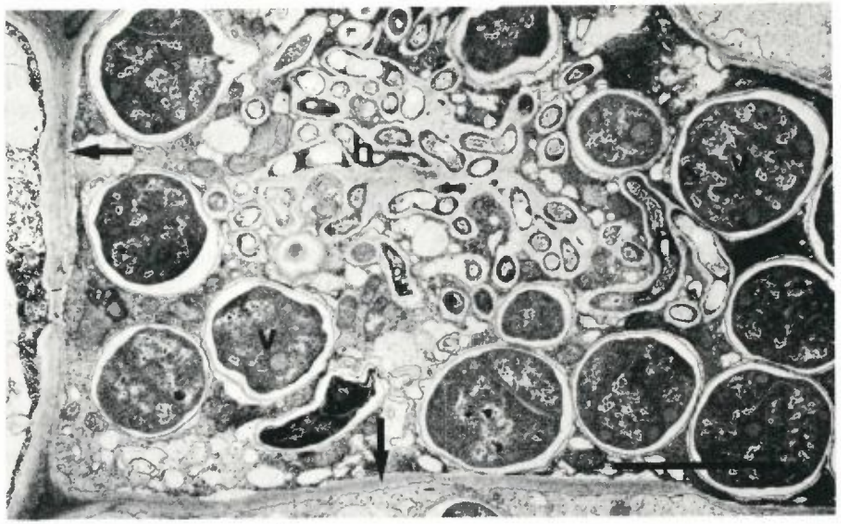

Figure 1. Electron micrograph of a thin section from a nodule from *Alnus incana*. The plant cell is filled with vesicles (v) and hyphae (h). The plant cell wall is indicated by arrows. Bar = 5 μm.

The free-living Frankia

Frankia was considered an obligate symbiont until 1978, when the research group at Harvard University (Callaham et al., 1978) finally succeeded in obtaining *Frankia* in pure culture, and by now several hundred *Frankia* strains are available in pure culture (Lechevalier, 1986). Most of them originate from Sp- nodules and it is still uncertain whether any Sp+ *Frankia* is available (Torrey, 1987). The isolation attempts in our laboratory were successful when the nodules were of the Sp- type. In contrast, numerous attempts with many different media failed to yield any *Frankia* isolates from Sp+ nodules (Weber et al., 1988).

The criteria used in classifying an actinomycete as a member of the genus *Frankia* include morphology, chemistry, infectivity and effectivity for a host plant, but he species definition is at the moment not clear (Lechevalier and Lechevalier, 1989).

The free-living *Frankia* has the same morphology as the endophytic one; hyphae, vesicles and sporangia also occur in pure cultures (Fig. 2). A surprising difference between the symbiotic and the pure cultured *Frankia* lies in the production of sporangia. In pure culture all strains produce sporangia although they originate from Sp- nodules.

The carbon sources used by *Frankia* in symbiosis have not yet been identified. In pure culture the best carbon sources are short-chain fatty acids, such

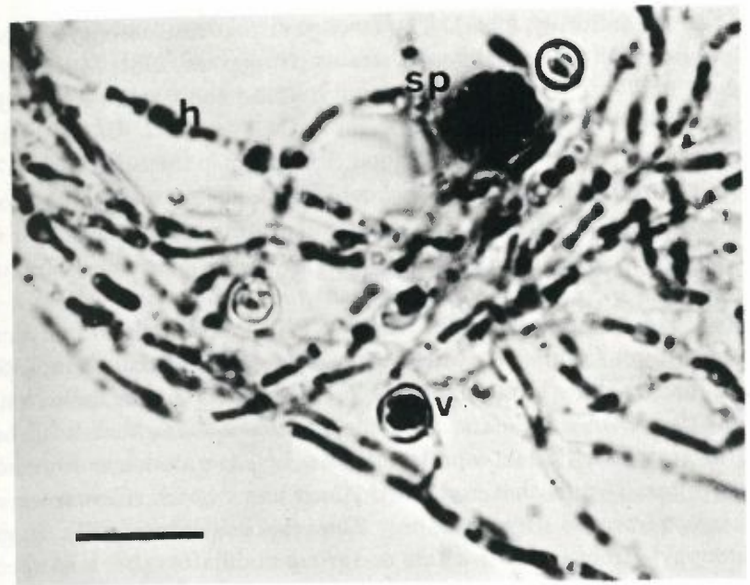

Figure 2. Light microscopic picture of a living *Frankia* culture. Hyphae (h) vesicles (v) and sporangia (sp) are seen. Bar = 10 μm.

as propionate and acetate, and fatty acid derivatives, such as Tween 80. Some frankias use tricarboxylic acid cycle intermediates, and organic acids, but sugars are used by some strains only as reviewed by Tjepkema et al. (1986). The isolates obtained in our laboratory were, in spite of their diverse origin, physiologically very similar; only propionate, acetate or Tween 80 supported good growth (Weber et al., 1988).

The occurrence of *Frankia* in soils without actinorhizal plants has been documented in several studies (van Dijk, 1984; Huss-Danell and Frej, 1986; Weber, 1986; Smolander and Sundman, 1987), which suggests that *Frankia* is a common soil organism, and may even be capable of saprophytic growth.

Most frankias fix nitrogen in pure culture under normal laboratory conditions. The observation that nitrogenase activity (acetylene reduction) correlates with vesicle production (Fontaine et al., 1984) has been taken as indirect evidence that the vesicles are the site for fixation. Further support for this hypothesis is provided by the findings that isolated vesicles reduced acetylene, whereas no nitrogenase activity was found in the hyphae (Tisa and Ensign, 1987), and that the enzyme nitrogenase was located only in the vesicles (Meesters, 1987). In pure culture also, the vesicles possess the multilaminate envelope that has been postulated to be involved in oxygen protection

(Murry et al., 1984; Torrey, 1985). The *in vitro* nitrogenase activity has been shown to vary greatly between different strains (Burggraaf, 1984; Murry et al., 1984; Weber, 1989). The nitrogenase activity is also the feature it would be most desirable to develop, in order to optimize the symbiosis. Unfortunately, inoculation experiments showed that good fixation in pure culture does not guarantee high symbiotic efficiency (Weber, 1989).

3. Host Specificity of the *Frankia-Alnus* Symbiosis

Our knowledge of the host-endophyte specificity is incomplete, due to the lack of pure cultures from some of the actinorhizal genera (Lechevalier, 1986). In addition, many isolates have completely lost their infectivity or are no longer infective on their own host. The available *Frankia* isolates that have been evaluated in cross-inoculation experiments can be separated into four host-specificity groups: strains that nodulated *Alnus* and *Myrica, Casuarina* and *Myrica, Elaeagnacea* and *Myrica*, or only *Elaeagnacea* (Baker, 1987). In general the endophyte from any host within one group nodulates other host species within the group.

The distribution pattern of Sp+ and Sp− nodules on the two native alders in Finland, *A. incana* and *A. glutinosa* (Table 1), indicates an association of nodule type with alder species (Weber, 1986). On *A. incana*, Sp+ nodules predominated. In pure stands of *A. glutinosa* only Sp− nodules were found; Sp+ nodules were recorded on *A. glutinosa* only in sites where this species grew together with *A. incana*. These observations prompt speculation on the factors responsible for the difference in nodule type. Can it be attributed to selection by the host plant (recognition and specificity) or by the soil (nutrient status, water level, etc.)? Differences exist in the habitat of the two alders; *A. glutinosa* prefers wet sites whereas *A. incana* thrives on drier soils.

Table 1. Nodule types in natural stands of the two native alder species in Finland

Alnus species	Number of nodules investigated and distribution of nodule types		
	Total	Sp+	Sp−
A. incana	360	301 (84%)	59 (16%)
A. glutinosa			
— pure stands	140		140 (100%)
— with *A. incana*	80	22 (28%)	58 (72%)

Indications were found for a connection between Sp− nodules and high soil water potential (Weber, 1986) as has been reported earlier (van Dijk, 1984), but no evidence was obtained of a relationship between nodule type and soil pH, in contrast to the findings of Holman and Schwintzer (1987), nor was the distribution of nodule types explained by any of the soil nutrients studied.

To examine the possibility of host plant selection, inoculation experiments were performed (Weber et al., 1987; van Dijk et al., 1988) and these results are summarized in Table 2. All the *Frankia* strains produced nodules in combination with the native alders, but the type of symbiosis varied. The Sp− *Frankia* type, whether originating from *A. incana* or *A. glutinosa*, was effective (capable of fixing nitrogen) on both host species, but Sp+ nodules from *A. incana* induced effective nodules only on the original host; on *A. glutinosa* only small prenodule-like structures were found. Such *A. glutinosa* died on nitrogen-free medium, thus showing that these nodules were ineffective. Recently the same intrageneric specificity was found in a French investigation (Kurdali et al., 1988). A non-native alder species, *A. nitida*, originating from Pakistan, failed

Table 2. Results from cross-inoculations between alders (Ai = *A. incana*, Ag = *A. glutinosa*, An = *A. nitida*) and nodule homogenates of *Frankia*. E = effective (nitrogen-fixing) symbiosis, I = ineffective (not nitrogen-fixing) symbiosis, No = no nodulation. Nodulation capacity is given as minimum numbers of infective *Frankia* particles (mg dry weight nodule)@−1, nd = not determined.

Inoculum original host (nodule type)	Inoculated plant species	Type of symbiosis	Nodulation capacity
A. incana	Ai	E	86, 000
(Sp+)	Ag	I	1, 000
	An	No	0
A. glutinosa	Ai	E	52, 000
(Sp+)	Ag	E	41, 000
	An	E	160, 000
A. glutinosa	Ai	E	nd
(Sp−)	Ag	E	nd
	An	E	145
A. incana	Ai	E	nd
(Sp−)	Ag	E	nd

to develop nodules when AiSp+ was used as inoculum (Table 2), whereas both AgSp+ and AgSp– formed effective symbioses with this host (Table 2).

In compatible crosses a much higher nodulation capacity (several hundred-fold) was found for the Sp+ nodule type than for the Sp– nodules (Table 2), which agrees with earlier observations (van Dijk, 1984). The incompatible combination between AiSp+ and *A. glutinosa* showed a greatly reduced nodulation capacity (Table 2). Since nodules were still formed, although these were ineffective, the recognition mechanism was functioning. The reasons for the ineffective symbiosis are unknown, but metabolic disturbance was indicated by the occurrence of amyloplasts in infected plant cells. Surprisingly, vesicles were also found (Weber et al., 1987), although their absence is considered to be a salient feature of the ineffective symbiosis (Berry, 1984). Since the plants died on nitrogen-free medium, the vesicles were evidently not providing them with enough nitrogen — due to their scarcity, lack of nitrogenase or lack of a suitable energy source.

The few Sp+ nodules that were observed on *A. glutinosa* (Table 1) resembled the AgSp+ strain type frequently found in Holland in having an equally high nodulation capacity on their own host and on *A. incana* (Table 2). AgSp+ had a high nodulation capacity on *A. nitida* as well, unlike AiSp+, which completely failed to induce nodulation in this host. These differences in host range between AgSp+ and AiSp+ and the differences in productivity on *A. incana* (Fig. 3) showed that the two strain types represent different genotypes. The reason why AgSp+ is found only in mixed stands and yet is different from AiSp+ remains unclear.

These results show that the pure cultures of Sp– type and the Sp+ nodule homogenate from *A. glutinosa* fit into the *Alnus* host-specificity group, but the Sp+ nodule homogenate from *A. incana* does not. The observation that crushed nodules are non-infective or produce ineffective nodules within their own host-specificity group is not an isolated one. VandenBosch and Torrey (1983), who compiled data on cross-inoculation experiments for the *Alnus-Myrica* group, reported similar results and Reddell and Bowen (1985) made the same observation with *Casuarina*. To obtain a clearer picture of the host-specificity groups, we need isolates from all actinorhizal species, and of both Sp– and Sp+ type.

4. Efficiency of the *Frankia-Alnus* Symbiosis

The successful interaction between *Frankia* and a host plant gives rise to nodules, which are the site of nitrogen-fixation. Symbiotic nitrogen fixation

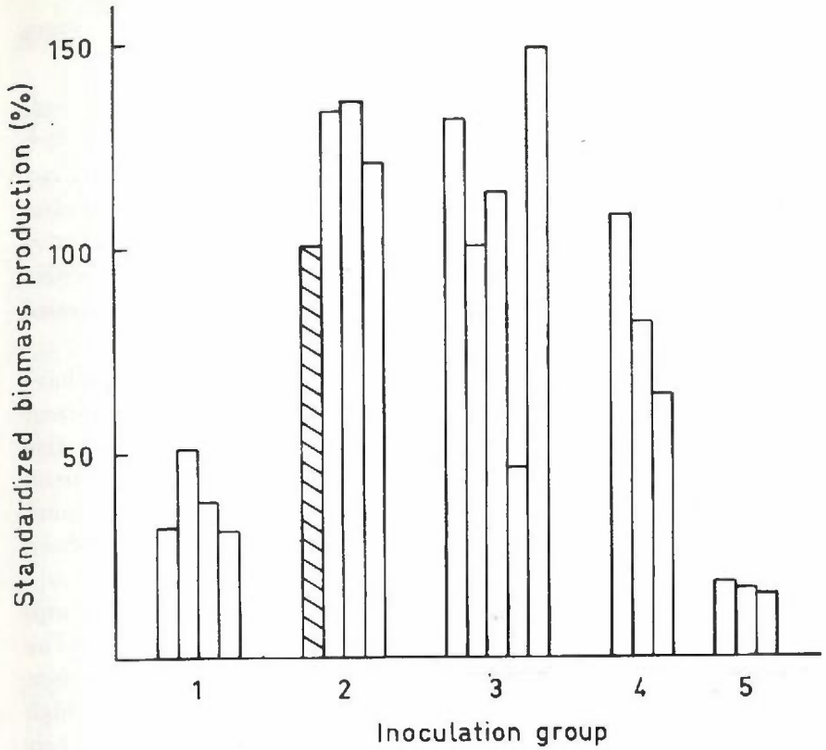

Figure 3. Biomass production of *Alnus incana* seedlings inoculated with 16 different *Frankia* strains. The data have been standardized by transformation to percentages of the values for a comperator strain (hatched column). The strains have been grouped according to nodule type and origin: Group 1, Sp+ nodule homogenates from *A. incana*; Group 2, Sp– strains isolated from *A. incana*; Group 3, Sp– strains isolated from *A. glutinosa* and *A. viridis crispa*; Group 4, Sp+ nodule homogenates from *A. glutinosa*; Group 5, uninoculated controls.

depends on both the host plant genotype (Gordon and Wheeler, 1978; Huss-Danell, 1980; Palmgren et al., 1985; Hahn et al., 1988) and the *Frankia* strain. Efficiency of the nitrogenase activity has been shown to vary between different endophyte-host combinations (Dawson and Sun, 1981; Dillon and Baker, 1982; Normande and Lalonde, 1982; Sellstedt et al., 1986; Hooker and Wheeler, 1987; Weber et al., 1987). The optimal use of actinorhizal plants includes improvement of the symbiosis through selection of superior genotypes of both partners. In order to evaluate the *Frankia-Alnus* symbiosis, local *Frankia* strains were isolated from the two native alder species (Weber et al., 1988), and compared with alders growing on nitrogen-free substrate (Weber et al., 1989). Since all

isolates obtained in pure culture were of the Sp– type, but the dominant nodule type of *A. incana* in nature is Sp+ (Weber, 1986), crushed nodules of Sp+ type were also included.

A remarkable variation in efficiency was found between the different *Frankia-Alnus* combinations. Some of the local *Frankia* strains induced ineffective nodules on *A. glutinosa* (Table 2). On *A. incana* all strains produced effective nodules, though the efficiency, measured as biomass production, varied considerably among the different *Frankia* strains (Fig. 3).

In Fig. 3, data from three inoculation experiments (Weber et al., 1989) have been summarized. The strains have been grouped according to nodule type and origin. Apart from a few exceptions, the results can be said to agree with this arrangement. The first group consists of AiSp+ strains, which predominate on *A. incana* in nature. Surprisingly enough, the lowest productivity was found in this group. In the second group, consisting of Sp– strains isolated from *A. incana*, the biomass production was three times as high. The third group, composed of Sp– pure cultures isolated from *A. glutinosa* or *A. viridis* ssp. *crispa*, also supported high rates of plant growth, except in one case. The highest productivity was obtained with the American reference strain from *A. viridis* ssp. *crispa*. Although the pure cultures generally supported high rates of plant growth, statistically significant differences were found in their symbiotic efficiency (Weber et al., 1989). The fourth group, three Sp+ strains from *A. glutinosa*, shows overlapping with all other groups.

Through strain selection, it is hence possible to exert a considerable influence on the productivity of both *Alnus incana* and *Alnus glutinosa*. The intrageneric specificity and the variation in efficiency found in the *Frankia-Alnus* symbiosis emphasizes the importance of choosing the right endophyte, if actinorhizal plants are used for afforestation, soil melioration, or biomass production.

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