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The Competitivity, Persistence and Dispersal of *Frankia* Strains in Mine Spoil Planted with Inoculated *Alnus rubra*

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Abstract

Polymorphisms in *Frankia* DNA sequences corresponding to part of the 16s rDNA were amplified by a PCR-based technique. The technique generated a sequence-specific "gene signature" that facilitated discrimination between two *Frankia* strains in nodules from *Alnus rubra*. Analysis of nodules from randomised plots of *A. rubra*, specifically inoculated 10 years earlier with the two strains or with a crushed nodule preparation, provided evidence for differential persistence of the two strains. *Frankia* UGL 013103 was detected in 28 nodules from trees inoculated originally with this strain whereas DDB010210 was not detected in any nodule from the specifically inoculated trees nor from other trees in the experimental plot. Strain UGL013103 was also found in 10% of samples from control plots (uninoculated prior to planting). Unidentified strains were detected in the remainder of nodule samples. Possible origins for these were from *Alnus glutinosa* trees, planted out with the experimental plot 6 years after establishment, from the crushed nodule preparations used to inoculate alders in some plots or from nursery soil carried over with outplants. Both strains were detected in nodules formed on plants grown in soil samples collected from an alder-free area

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immediately adjacent to the experimental plot. This suggests that the competitive pressures for survival of these strains, in the absence of alders as a host, were lower than within the experimental plot.

Keywords: *Alnus, Frankia*, competition, dispersal, nodules, PCR

1. Introduction

Frankia is a slow-growing, filamentous nitrogen-fixing actinomycete, which fixes nitrogen in symbiosis with the roots of a number of woody shrubs and trees from eight different families; collectively termed actinorhizal plants. In addition, at least *in vitro*, it is capable of fixing nitrogen in the free-living state. In Britain the only indigenous actinorhizal species are *Alnus glutinosa* (common alder), *Hippophae rhamnoides* (sea buckthorn) and *Myrica gale* (bog myrtle). These are important ecologically as pioneer species, providing input of fixed N, especially in riverine and other wetland areas, coastal dunes, and acid moorland respectively. Members of the genus *Alnus*, in particular *A. glutinosa, A. incana, A. viridis* and *A. rubra*, are used in Britain and elsewhere for a variety of purposes, including amenity planting, land restoration and stabilisation. In North America and Asia in particular, these trees are used for wood production or pulping, pole production and for special purposes such as veneers (Wheeler and Miller, 1990).

The ability to fix nitrogen in symbiosis with the actinomycete *Frankia* is central not only to the ecology of alders but also to their selection for use in forestry-related activities. The perennial root nodules ensure a high potential input of fixed N during the early years following inoculation while N difference measurements and ^{15}N natural isotope abundance data suggest that in some soils, symbiotic nitrogen fixation can satisfy much of the demand for N for growth of alders (Domenach et al., 1992).

The selection of elite *Frankia* strains and their use for inoculation of nursery plants can therefore be of immediate advantage for the growth of outplants (Wheeler et al., 1991; Prat, 1992). However, it is not clear what long term advantage for enhanced productivity and ecosystem N secretion is achieved when inoculated alders are planted into soils with an indigenous population of microsymbionts. While continued dominance of the introduced *Frankia* strains should continue until the first formed nodules senesce, new nodules on the developing root system may be initiated by highly infective and competitive strains that are less effective in N_2-fixation.

The ability to compete, to survive and to disperse in a particular soil are major determinants of the success of an introduced or naturally occurring strain in a particular environment. However, these factors have received little attention with respect to *Frankia*. Indeed, relatively little is known at present about most aspects of the soil ecology of this organism. *Frankia* grows slowly in culture, with a

doubling time usually much greater than 12–24 h, and sporulates prolifically. This latter ability may explain reports of the persistence of *Frankia* in soils for which there are no recent records of actinorhizal plants and possibly the low infectivity attributable to *Frankia* associated with other non-nodulating species or in soil *per se* (Rodriguez Barrueco, 1968; Arveby and Huss-Danell, 1988; Paschke and Dawson, 1992). *Frankia* is able to grow in the rhizosphere of some non-host plants, notably birch (Smolander, 1990; Ronkko et al., 1993).

This report describes an investigation of the competitivity, persistence and dispersal of two specific strains of *Frankia* (UGL 013103 – referred to as Ar125Q, and DDB 010210 – referred to as ArI4) used to inoculate a population of *Alnus rubra* planted out at a reclaimed open-cast mine site at Kyle Forest, Ben Bain, Dalmellington, Ayrshire ten years previously.

2. Materials and Methods

Field site, Frankia sources and planting details

The experimental site was located on newly reclaimed coal mine spoil at Dalmellington, Ayrshire. It was free of actinorhizal plants at the time of planting with specifically inoculated *Alnus rubra* that were grown at the Forestry Commission nursery, Penicuik (Wheeler et al., 1991). Prior to planting, nodules were not detected on plants of *Alnus rubra* grown from seed in soil from the site, showing an absence of *Alnus*-infective *Frankia* at this time. Saplings were infected in the nursery with either Ar125Q (UGL 013103), ArI4 (DDB 010210), crushed root nodules (CRN) or were not inoculated (NPK). The origins of *Frankia* isolates are described in Hooker and Wheeler (1987). ArI4 was isolated from nodules of *A.rubra* in the USA, Ar125Q from nodules from *A. rubra* in Scotland and the crushed nodule preparations originated from nodules of the same trees from which Ar125Q was isolated.

The four treatments were replicated four times in a randomised block design. Each plot consisted of 25 trees planted in rows of 5 trees and with 1.5 m spacing within and between rows. Each plot was separated from neighbours by a 3 m buffer which was planted with *Larix kaempferi* (Lamb.) Carr. A 3 m buffer zone was established around the entire block (Table 1).

About six years after the planting of *A. rubra* in 1985, other *Alnus* species, notably *A. glutinosa*, were introduced into areas adjacent to the experimental plot. The plants were nodulated but it is probable that the strains infecting these trees are different from those introduced in the original experimental plot since they were from different nurseries. At that time, nodulation of trees in control plots was compared with that of trees in the inoculated plots by digging up five trees from four plots per treatment for assessment of nodule number and dry weight.

Table 1. The distribution in plots within the experimental block at Kyle Forest of *Alnus rubra* trees inoculated with *Frankia* ArI4, Ar125Q, *A.rubra* crushed nodules from Lennox Forest (CRN) or control trees (NPK) that were not inoculated prior to planting. The identity of the *Frankia* strains determined by application of "single specific gene signature" technique to eight nodule samples from each plot is shown.

EAST

Top

ArI4 (plot 3) 8 unidentified	CRN (plot 1) 8 unidentified	NPK (plot 2) 8 unidentified	Ar125Q(plot1) 8 unidentified
CRN (plot 2) 8 unidentified	ArI4 (plot 4) 8 unidentified	Ar125Q (plot2) 8 unidentified	NPK (plot 1) 8 unidentified
CRN (plot 3) 3 Ar125Q 5 unidentified	NPK (plot 3) 8 unidentified	Ar125Q(plot 3) 8 Ar125Q	ArI4 (plot 2) 8 unidentified
NPK (plot 4) 8 unidentified	CRN (plot 4) 8 unidentified	Ar125Q (plot 4) 1 Ar125Q 7 unidentified	ArI4 (plot 1) 8 unidentified

Slope

WEST

Harvesting of samples

From within the experimental plot

A single nodule lobe cluster was collected from eight different trees from each of 16 plots of 25 trees. DNA was isolated as described below and analysed using the "gene signature" technique.

From outwith the plot

Soil samples were collected to a depth of 15 cm at 5 m intervals from points up to 30 m away from the plot:

- above the top right corner of the plot (South East)
- above the top left corner of the plot (North East)
- below the bottom right corner of the plot (South West)
- below the bottom left corner of the plot (North West).

Each soil sample was mixed with 50% perlite and placed in plastic "Transpots" (4 pots per sample). Each group of pots was isolated in a 15 cm deep bowl and

surface sterilised seeds of *A. rubra* were sown in each pot. Seedlings were thinned to three per pot after germination. Each pot received a supplement of mineral N-free Crone's solution (Hooker and Wheeler, 1987). Seedlings were grown for 8 months in a heated glasshouse lit by natural daylight supplemented with 16 h light from 400 W mercury vapour lamps.

All soil-grown *A. rubra* seedlings became nodulated but seedlings grown in perlite that were dispersed throughout the experiment remained non-nodulated.

Six nodule samples, selected at random, from the seedlings from each location were analysed by the sequence specific "gene signature" technique described below.

Isolation of DNA

DNA was isolated from a single lobe of each nodule by the method of Nalin et al. (1995). Nodules were removed from the plant and washed in water. The periderm from single nodule lobes was removed and the lobe placed in 300 µl extraction buffer which consisted of 100 mM Tris.HCl (pH 7.0), 20 mM EDTA, 1.4 mM NaCl, 2% (w/v) hexadecyltrimethyl-ammonium bromide (CTAB) and 1% (w/v) PVPP. Peeled lobes were homogenised using a piston pestle and incubated at 65°C for 2 hours. After incubation an equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added. The two phases were mixed by inversion. The phases were separated by centrifugation in a microfuge at 13,000 g for 20 minutes. A 200 µl sample of the aqueous (upper phase) was drawn off and transferred to a fresh tube. To this was added an equal volume of 100 mM Tris.HCl [pH 7.0], 20 mM EDTA, 0.7% (w/v) NaCl, 10% (w/v) CTAB, which had been pre-heated to 65°C. This was centrifuged further at 13,000 g for 20 min and the aqueous phase transferred to a fresh tube. To the aqueous phase, 0.1 volumes of 3 M sodium acetate buffer (pH 5.5) and 2.5 volumes of ethanol were added. DNA was allowed to precipitate by incubating at –20°C for 45–60 minutes. Precipitated DNA was collected by centrifugation, dried in air and resuspended in autoclaved ultrapure water.

Amplification of DNA

DNA sequences corresponding to part of the 16S *rDNA* were amplified by use of the ribosomal specific primers ATGGAGTTTGATCATGGCTCAGGAC and AAGGAGGGGATCCAGCCGCA. The PCR cocktail comprised the following: 50 mM KCl, 10 mM Tris.HCl [pH 8.3], 1.5 mM $MgCl_2$, 100 mg/ml gelatin, 0.25 mM of each primer, 200 mM of each dNTP, 2.5 units of Taq polymerase per 100 µl and approximately 5 ng/ml genomic DNA. Amplification was carried out using the following cycling protocol: (95°C for 5 minutes, 55°C for 1 minute, 72°C for 1

minute) for 1 cycle; (95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute) for 35 cycles and finally 72°C for 4 minutes.

After an initial PCR cycle for amplification of the 16S *rDNA*, around 10 ng of the resulting DNA was further amplified by a lower temperature PCR method, known as the sequence-specific "gene signature" technique, (Pena et al., 1994; Vanbelkum, 1995; Villa et al., 1995) using only one of the two specific primers described earlier; ATGGAGTTTGATCATGGCTCAGGAC, at double its previous concentration. Other components of the PCR cocktail were as described earlier. The cycling system was: (95°C for 5 minutes, 32°C for 1 minute, 72°C for 1 minute) for 1 cycle; (95°C for 1 minute, 32°C for 1 minute, 72°C for 1 minute) for 35 cycles; 72°C for 4 minutes. Amplified products were run on a 4% (w/v) TBE agarose gel.

3. Results

Nodulation of A.rubra in the experimental plot

All trees were nodulated at the time of the experiment. Comparison of the nodulation of the uninoculated Control trees with that of inoculated trees five years earlier showed no significant difference in either nodule number or dry weight per tree between treatments. The overall mean number of nodules per tree was as follows: Small (<0.5 cm diameter), 56.3; Medium (0.5–2.0 cm) 33.5; Large (>2.0 cm) 24.4.

Competition between Frankia strains for infection of inoculated A.rubra

Nodule samples were harvested from roots in the top 10 cm of soil, mid-way between trees in each treatment plot. In order to identify *Frankia* strains in nodule samples, gels of the amplified products of the specific sequences of 16S *rDNA* of *Frankia* strains ArI4 and Ar125Q, were obtained by the "single specific signature" technique. The amplified product obtained following initial PCR (using two primers) resulted in products of a single size. Fig. 1 demonstrates the reproducibility of the "single specific signature" technique by comparing the "signature" of DNA extracted from roots with DNA which had been extracted from pure cultures of the strain used originally to inoculate the seedlings. This demonstrates that the "signature" arising from the second round of PCR amplification at the lower temperature is characteristic of *Frankia* DNA, rather than of DNA from the plant.

Most of the nodule samples gave rise to patterns that did not correspond to either of the strains used originally for tree inoculation. Examples of the patterns obtained are shown in Fig. 2. Patterns identical to that of ArI4 were not detected

in samples from any trees, whether inoculated originally with this strain or not. However, patterns characteristic of Ar125Q were detected in all eight of the nodule samples from one plot and in one from eight of the samples from another plot with trees inoculated with this strain. In addition, this strain was detected in three samples of nodules from trees in one control plot.

Ar125Q was not detected in the remaining two plots trees inoculated originally with Ar125Q, nor in any of the plots which had initially been infected with crushed nodules obtained from the same site from which 125Q was originally isolated. Patterns characteristic of ArI4 were not found in nodule samples from any of the plots, including plots with trees inoculated originally with ArI4. All other patterns were of unidentified origin.

The distribution in the experimental block of the strains that were recognised is shown in Table 1.

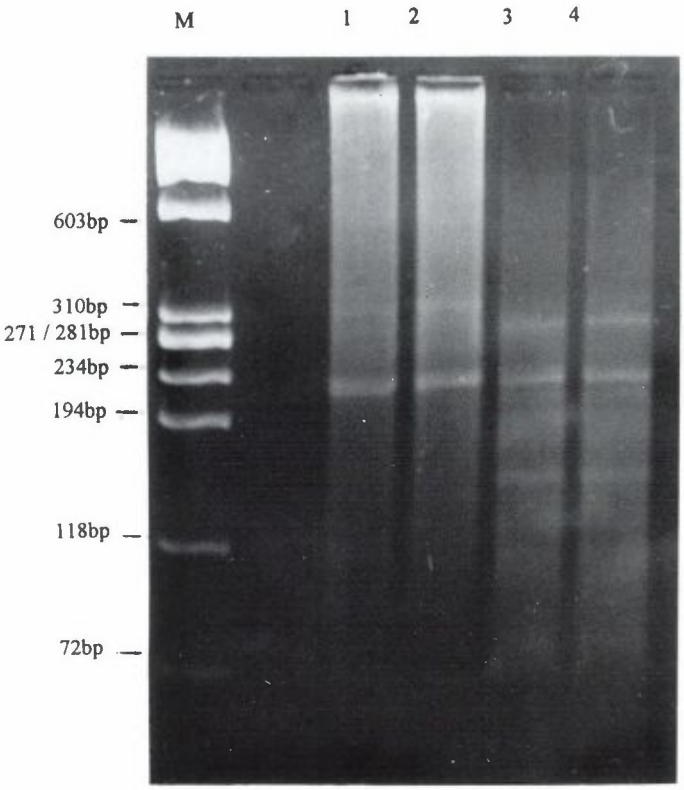

Figure 1. Sequence specific "gene signatures" for *Frankia* strains ArI4 and Ar125Q in nodules from specific strain inoculated *Alnus rubra*. Lane M: Molecular Weight Markers, Lanes 1: "Gene signature" for *Frankia* strain ArI4 grown in culture, Lanes 2: "Gene signature" for *Frankia* strain ArI4 from a nodule, Lanes 3: "Gene signature" for *Frankia* strain Ar125Q grown in culture, Lanes 4: "Gene signature" for *Frankia* strain Ar125Q from a nodule.

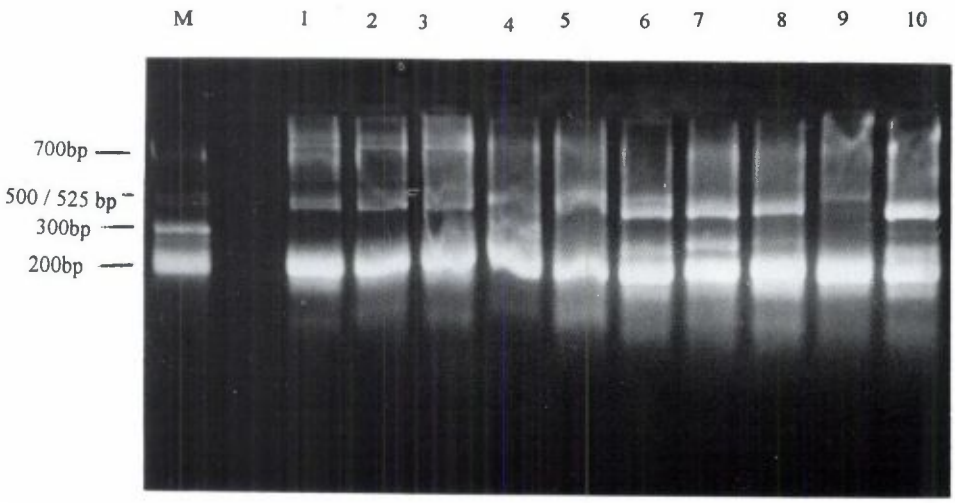

Figure 2. Sequence specific "gene signatures" for unidentified *Frankia* strains in nodule samples from the experimental plantation of *Alnus rubra* at Kyle Forest. Lane M: Molecular Weight Markers, Lanes 1–3: Nodules from Ar125Q plot 2 planted with *A. rubra* (inoculated originally with *Frankia* Ar125Q), Lanes 4–6: Nodules from CRN plot 1 planted with *A. rubra* (inoculated with crushed root nodules from *A.rubra* growing at Lennox Forest), Lanes 7–10: Nodules from ArI4 plot 2 planted with *A.rubra* (inoculated originally with *Frankia* ArI4).

The occurrence of introduced strains in soil out with the experimental plot

Nodulation of all seedlings was observed when seed was sown into soil collected at distances of up to 30 m from the experimental plot (Table 2). Using the "single specific signature" technique Ar125Q was recognised in 8.3% of nodule samples tested and ArI4 in 3.0% of nodules tested. There was no obvious relationship between distance from the experimental plot and occurrence of Ar125Q in soils but ArI4 was found only in samples collected up to 5m from the plot. Most (88.7%) samples contained *Frankia* of unidentified origin.

4. Discussion

The "single specific signature" technique developed in this work offers greater specificity for *Frankia* than techniques that have been applied previously to cultures of *Frankia* and which that have relied on RAPDs (Sellstedt et al., 1992; McEwan and Wheeler, 1995) or repetitive sequences (Murry et al., 1995). In addition, its potential for discrimination between individual strains is greater than techniques using RFLP-PCR analysis (McEwan et al., 1994) and it is more

Table 2. The identity of *Frankia* in nodules harvested from plants of *A.rubra* grown in soil dug to a depth of 15 cm at 5m intervals from four locations around the perimeter of the experimental plot. Six nodules from plants grown in each soil sample were analysed by the "single specific gene signature" technique.

Location of soil sample and identity of strain	Frequency of specific *Frankia* strain in nodules formed on *A. rubra* from soil samples at site
Ar125Q patterns	
20 m from the bottom North west of the plot	1 from 6 nodules
25 m from the top South west of the plot	2 from 6 nodules
0 m from the top South East of the plot	4 from 6 nodules
5 m from the top South East of the plot	3 from 6 nodules
25 m from the top South East of the plot	2 from 6 nodules
30 m from the top South East of the plot	2 from 6 nodules
ArI4 patterns	
0 m from the bottom South west of the plot	3 from 6 nodules
5 m from the bottom South west of the plot	2 from 6 nodules
Unidentified patterns	
All other samples (149 from a total of 168) gave patterns that differed from those of either ArI4 or Ar125Q	

practical than sequencing for application to large numbers of samples from the field (Nazaret et al., 1989). The specificity of the primer sequences used in the initial round of PCR ensures that the material available for amplification will not be from plant sources, while the low annealing temperature used in the second round of PCR increased the probability of detecting differences between strains. However, the technique is applicable only to strains in culture or in nodules, and is not suitable for identification of *Frankia* in soil. The experiments reported do not therefore separate the contributions to competitivity of survival in soil and effectivity of infection.

The use of ribosomal genes is preferable for this type of work, as *Frankia*'s high GC content of the third nucleotide in codons from translated sequences results in mutational pressure driving selection for specific nucleotides every third base (McEwan and Gatherer, 1998), which reduces the potential for sequence variation between strains.

The nodules sampled from the experimental plot were all relatively young (less than 1 cm diameter) and would have been initiated well after the trees were planted. The substrate on which the trees are growing presented particular difficulties for excavating old nodules but it is probable that degeneration of such

nodules would have been well advanced ten years after outplanting of *A. rubra*. Consequently, the experiments that were carried out determined the ability of *Frankia* strains released from the original nodules (i.e. those formed as a result of inoculation of nursery plants) to survive and to infect new roots in competition with other strains that were introduced into the environment. Possible origins for unidentified strains were the nodules of *Alnus glutinosa* trees, planted outwith the experimental plot six years after establishment; nursery soil carried over with outplants; the crushed nodule preparations from *A. rubra* at Lennox Forest, near Glasgow, which were used to inoculate alders in some plots and which was the location from which Ar125Q was isolated originally (Hooker and Wheeler, 1987).

The experiments confirmed the mobility of *Frankia* in field situations. Control plants were either not nodulated or bore few nodules when first planted from the nursery into the field (Wheeler et al., 1991). However, trees in all experimental plots were well nodulated prior to assessment for strain occupancy using the "single specific signature" technique. Ar125Q was of relatively low occurrence in the nodule samples analysed but was detected both in nodules from plots containing trees inoculated originally with this strain and in nodules from one of the control plots. By contrast, ArI4 was not detected in any of the experimental plots, including those with specifically inoculated trees. These data suggest that Ar125Q is a more persistent and more competitive strain than ArI4 although it is less competitive than some of the unknown strains in the environment (Table 1). Data supporting this conclusion have been obtained also using partially selective monoclonal antibodies to Ar125Q and ArI4 (Hahn, Kesavan and Hock, Technische Universität München, personal communications).

The origin of strains with patterns (Fig. 2) that do not correspond to either ArI4 or to Ar125Q (Fig. 1) is unknown. These could have been introduced by the spread of *Frankia* into the plantation from alders planted about four years earlier on surrounding areas, from nursery soil carried over with outplants or they could be strains other than Ar125Q in the *A.rubra* crushed nodules used to inoculate trees in a quarter of the plots. In this context, it is of note that, using the "single specific signature" technique, we were unable to detect Ar125Q in a number of nodule samples from Lennox Forest. Ar125Q is a spore(–) *Frankia* strain (i.e. although like most *Frankia* strains it sporulates in culture, it does not sporulate *in planta*) whereas spore(+) strains are common in nodules of *A. rubra* at Lennox Forest. It has already been documented that spore(+) and spore(–) strains may be discriminated at the genetic level (Simonet et al., 1994), and thus it would be reasonable to predict that spore(+) and spore(–) strains would have different signatures. It is possible, therefore, that Ar125Q is of low occurrence in nodules of the trees planted at Lennox Forest and may be poorly competitive for nodulation, even though it is a highly effective strain in symbiotic N_2-fixation

(Hooker and Wheeler, 1987). The strain can be isolated easily from nodules but this does not reflect its relative abundance in the natural environment.

The detection of patterns characteristic of both Ar125Q and ArI4 in nodules initiated on seedlings grown in soil from outwith the plot is of great interest. The area of ground assayed was devoid of alders and therefore the competitive pressures for survival of the latter strain in particular may have been less than within the experimental plot. This may explain the detection of ArI4 outwith the plot, even though it was not detected in nodule samples harvested inside the plot. Patterns typical of ArI4 were found only within 5 metres of the original test plot but Ar125Q patterns were found as much as 30 metres from the test plot which may reflect their relative ability to persist in the soil. The detection of specific *Frankia* strains outwith the plot confirms the considerable distance over which strains can move. Ar125Q was detected both uphill and downhill of the experimental site suggesting that movement in water alone is not the only means of movement of the organism. As well as possible movement in wind blown dust, dispersal of *Frankia* by worms and by birds has been described (Reddell and Spain, 1991; Burleigh and Dawson, 1995).

The data show that although Ar125Q is a highly effective strain for nodulation and N_2-fixation in *A.rubra* grown in controlled growth conditions or in pre-sterilised nursery beds (Hooker and Wheeler, 1987; Wheeler et al., 1991) it is not competitive for infection in field conditions against a broad background of other *Alnus*-infective strains. This observation underlines the importance of identifying strains combining both effectivity and infectivity in field conditions for use in inocula – a condition that may be met in practice only by preparing inocula with a wide mix of effective strains.

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