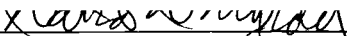


## AN ABSTRACT OF THE DISSERTATION OF

Kendall J. Martin for the degree of Doctor of Philosophy in Soil Science presented on June 5, 2001. Title: Inoculation Potential of Soil-Borne *Frankia* on Red Alder (*Alnus rubra* Bong.).

Abstract approved:

*Redacted for Privacy*

  
David D. Myrold

Inoculation trials were set up in fumigated nursery beds for red alder (*Alnus rubra* Bong.) seedling production. *Frankia* inoculum was applied in eight treatments: control, nodule suspension, and three levels of cell suspension (strain ArI5) applied with and without a peat carrier. Seedlings were evaluated at midseason and lifting. The peat inoculum treatment produced larger seedlings than the other treatments, both at mid-season and at lifting. The other treatments had little effect on growth. Size differences paralleled differences in degree of nodulation. Differences in percentage of seedlings nodulated were most pronounced at midseason, indicating that the inoculation conferred primarily an early-season advantage.

In a second nursery experiment, *Frankia* inoculum was applied in a peat mix carrier, in four treatments: uninoculated control, strain ArI5, strain AvcI1, and combined dual-inoculum. Seedlings were evaluated at midseason and at lifting. *Frankia* DNA from nodules was analyzed by DNA fingerprinting. The controls were poorly nodulated and grew slowly. The single strain inoculum for ArI5 produced

larger seedlings than for AvcI1, both at mid-season and at lifting. The dual-inoculum treatment produced larger seedlings than all other treatments. The AvcI1 single-inoculum nodules primarily produced fingerprints identical to AvcI1, but nodule fingerprints from all other treatments were dominated by ArI5 patterns.

In a field experiment, nodulation capacity was determined for soils from red alder stands in the Oregon coast range covering a wide span of ages. Total nitrogen, total carbon, nitrate, ammonium and pH were measured on the same soil samples. Soil pH was most highly correlated with nodulation potential, with nitrate content also significant. Cluster analysis of the sites using these two variables produced two groups with distinctly different nodulation capacities. The higher capacity cluster was lower in nitrate and higher in pH than the majority of sites in the lower capacity cluster. There was general overlap in the age ranges for the two groups but the lower capacity cluster contained the oldest sites.

Inoculation Potential of Soil-Borne *Frankia* on Red Alder (*Alnus rubra* Bong.)

by  
Kendall J. Martin

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of  
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degree of

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Doctor of Philosophy dissertation of Kendall J. Martin presented on June 5, 2001

Approved

*Redacted for Privacy*

  
Major Professor, representing Soil Science

*Redacted for Privacy*

Chair of Department of Crop and Soil Science

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Kendall J. Martin, Author

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## CONTRIBUTION OF AUTHORS

Yasu Tanaka provided nursery space at the Weyerhaeuser Mima Nursery in Washington for Chapters 2 and 3, as well as editorial comments on Chapter 2.

Nancy Ritchie provided laboratory assistance for Chapter 4 and Appendix.

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# Inoculation Potential of Soil-Borne *Frankia* on Red Alder (*Alnus rubra* Bong.)

## **Chapter 1**

### **Introduction**

*Frankia* are N<sub>2</sub>-fixing, sporulating, Gram-positive, filamentous bacteria (Actinomycetales) capable of forming root nodules on a variety of predominantly woody perennial plants, including *Alnus*. These actinorhizal associations contribute significant quantities of nitrogen (50 to 150 kg N ha<sup>-1</sup> yr<sup>-1</sup>) to temperate forest ecosystems (Torrey 1978, Dawson *et al.* 1983). In addition to enhancing forest productivity by serving as a major input of N, some actinorhizal plants are commercially valuable, for example, many alder species (*Alnus rubra*, in the Pacific Northwest of the U.S.A., and *Alnus incana*, in northern Europe) are important timber resources (Hibbs and Cromack 1990, Plank and Willits 1994).

Since the first successful isolation of *Frankia* from nodule tissue over two decades ago (Callaham *et al.* 1978), much has been learned about *Frankia* taxonomy and host specificity groupings, metabolism, biochemistry, and genetics (Huss-Danell 1997, Wall 2000, Hahn *et al.* 1999). But ecological studies of *Frankia* in soil are in their infancy, primarily because of the difficulty of isolating *Frankia* from soil and of having suitable means of differentiating *Frankia* strains from each other and from other soil microorganisms.

*Frankia* populations in soil are typically studied quantitatively using a plant bioassay-based most probable number (MPN) system (van Dijk 1984). The MPN method, which measures infective units (IU), has been useful in surveying *Frankia* populations in various forest soils (Smolander and Sundman 1987, Smolander *et al.* 1988, van Dijk *et al.* 1988) and in following the survival of *Frankia* introduced into soil under laboratory conditions (Smolander and Sarsa 1990). These studies have given similar results: (1) numbers of *Frankia* range from zero to a few thousand IU g<sup>-1</sup> soil, (2) *Frankia* populations are higher under certain tree species, notably more IUs often are found under *Betula* spp. than *Alnus* spp., and (3) native and introduced populations of *Frankia* are favored by higher soil pH or liming. Although the MPN method provides quantitative numbers, it is unclear whether these numbers reflect *Frankia* biomass in soil. Some studies indicate that spores appear to be much more infective than hyphae (van Dijk 1984, Burleigh and Torrey 1990). Calculations based on *Frankia* DNA extracted from soil (Myrold *et al.* 1990, Myrold and Huss-Danell 1994) suggest that numbers of *Frankia* genomes present in soil may be much higher than indicated by other methods.

*Frankia* may be considered as a nominal part of the microbial community. *Frankia* have been known to persist for extended periods in soils in the absence of actinorhizal hosts (Smolander and Sundman 1987, Weber 1986). In microcosm experiments, nodulation capacity of *Frankia*-free soils inoculated with *Frankia* dropped to stable levels just above 10 IU cm<sup>-3</sup> in year-long incubations at pH 6.3-6.8

and lower levels at lower pH (Smolander *et al.* 1988). It is likely that a variety of environmental factors affect *Frankia* populations.

*Frankia* strains are divided between spore-plus (sp(+)) and spore-minus (sp(-)) types. All *Frankia* strains that have been isolated from sporangia in pure culture, but actinorhizal nodules are typically either sp(+), having abundant numbers of large sporangia or sp(-), with very few if any sporangia within the nodule. This trait is considered to be controlled by the endophyte, but there are indications that the host plant species and the physiological state of the nodule may play a role in expression of this trait (Torrey 1987, Weber *et al.* 1986, van Dijk *et al.* 1988). The sp(-) nodule trait appears to be characteristic of alders growing as pioneer colonizers in locations previously without actinorhizal plants (Holman and Schwintzer 1987), whereas sp(+) nodules may be more common in sites where the host plant has persisted for long periods (Weber 1986). Akkermans and van Dijk (1975) postulated that sp(+) strains require the presence of the host to grow and proliferate, whereas sp(-) strains can grow independently of the host but are sensitive to factors like low soil pH. It is not clear whether spore production yields a greater store of inoculum in the soil or what effect this difference would have on MPN bioassays.

### **Alder stand development**

In younger alder trees, N<sub>2</sub> fixed by *Frankia* typically provides for the plants' N needs and other nutrients or conditions are more important in limiting growth. Alder can thrive in nutrient poor sandy soils by fixing large amounts of N<sub>2</sub> (Bormann *et al.*

1993). Van Miegrot and others (1990) found that red alder growth rates were higher when planted after Douglas fir than when planted in N-rich sites previously under red alder, and attributed this to lower cation availability in the alder impacted soils. The higher nitrate in alder soils provides a mobile anion that can accelerate leaching of cations. In younger soils this may be offset by cations derived from increased weathering under alder stands (Bormann *et al.* 1994).

As alder forests age, site characteristics change somewhat predictably over time. Most notably N content of the soil will tend to increase over time (Binkley 1986, Bormann *et al.* 1994). A decline in N<sub>2</sub>-fixation and N accretion rates with stand age has also been observed, while the energy input for nodulation and fixation per unit N fixed was calculated to increase with stand age (Sharma & Ambasht 1988). There is also typically an increase in soil organic matter and cation exchange capacity (Hibbs and Cromack 1990, Bormann *et al.* 1994), a decrease in pH (Binkley 1986), and a decrease in available phosphorus (Compton *et al.* 1997). Together, these factors could have a strong effect on soil *Frankia* populations and N<sub>2</sub> fixation.

Sharma and Ambasht (1988) found that the contribution of N<sub>2</sub> fixation to total plant N uptake decreased from 33% in a 7-year-old stand to 11% in a 56-year-old stand of Himalayan alder. Lundquist (1993) proposed a model for decline in *Frankia* populations in developed alder forests in which decreased carbon allocations may affect the oxygen protection for nitrogenase and accelerate *Frankia* cell turnover. The resulting loss of N<sub>2</sub> fixing capacity leads to further declines in carbon allocation further accelerating the decline of the *Frankia*. It would appear that the strong

selective pressure the symbiosis confers on the two participants early in alder stand development, is lost in older stands.

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## Chapter 2

### **Peat Carrier Increases Inoculation Success with *Frankia* on Red Alder (*Alnus rubra* Bong.) in Fumigated Nursery Beds**

K.J. Martin<sup>1</sup>, Y. Tanaka<sup>2</sup>, and D.D. Myrold<sup>1</sup>

<sup>1</sup> Department of Crop and Soil Sciences, Oregon State University, Corvallis, OR 97331-2213, USA, <sup>2</sup> Weyerhaeuser Co. Western Forestry Research Center, 505 North Pearl Street, Centralia, WA 98531, USA

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#### **Abstract**

Inoculation trials were set up in fumigated nursery beds for red alder (*Alnus rubra* Bong.) bare-root seedling production. *Frankia* inoculum was applied either as nodule homogenate or as pure culture (strain ArI5). The plots were laid out in 4 blocks of 8 treatments consisting of: control, nodule suspension, and three levels each of cell suspension and cells applied with a peat carrier. Numbers, height, and percentage nodulation on the seedlings were determined at mid-season. Numbers, dry weights, and degree of nodulation were determined at lifting. The peat inoculum treatment produced larger seedlings than the other treatments, both at mid-season and at lifting. The other treatments had little effect on growth. Size differences paralleled differences in degree of nodulation. Differences in percentage of seedlings nodulated were most pronounced at midseason, indicating that the inoculation conferred primarily an early-season advantage.

## Introduction

Red alder (*Alnus rubra* Bong.), the most economically valuable hardwood in the Pacific Northwest (Resch 1979), is a woody perennial that forms a nitrogen-fixing symbiosis with the actinomycete *Frankia*. As nitrogen-fixing trees, alder have several useful applications; they may be used in land reclamation, as nurse trees for silviculturally important plants, and as an economic crop. Alder is also capable of rapid juvenile growth. *Alnus glutinosa* has been shown to produce as much as 15.8 ton ha<sup>-1</sup> above-ground biomass over 4 years using year-old, nodulated, bare-root stock (Pregent and Camire 1985).

Commercial use of red alder requires large-scale production of seedlings that readily survive out-planting. Nodulated stock has been shown to survive better after transplanting (Dommergues 1982). Greenhouse production of such seedlings has been studied (Perinet *et al.* 1985, Berry and Torrey 1985), but field trials for nursery production are scarce (Hilger *et al.* 1991). McNeill *et al.* (1989) showed in nursery trials that inoculation with pure culture can increase seedling growth as much or more than seeding into fertilized soil with a subsequent top-dressing with nitrogen. Out-planting of these seedlings showed better growth of inoculated seedlings, particularly in nutrient poor soils.

Both greenhouse media and nursery soils are routinely fumigated to control disease. Inoculation with *Frankia* is then necessary to produce nodulated stock because the indigenous populations are depleted by fumigation. Some soils may also have very low indigenous populations of *Frankia* (Houwens and Akkermans 1981).

Inoculation gives an advantage to seedlings very early in their growth. Much better growth in containers has been seen with inoculation at seeding than at 6 weeks growth or later (Stowers and Smith 1985).

We investigated methods for inoculating fumigated nursery soils to increase yield and nodulation of bare-root red alder seedlings. Pure-culture inoculum was applied, at seeding, either in solution or mixed with a peat-based carrier to protect the cells.

## **Materials and methods**

### *Soil*

The soil used was Nisqually loamy sand, at the Weyerhaeuser Mima Nursery in Washington. It is high in organic matter, with a KCl-extractible soil N of 25.5 mg kg<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N and 4.67 mg kg<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N. The soil was fumigated in the fall with 389 kg ha<sup>-1</sup> methyl-bromide/chloropicrin, injected at 15 to 20 cm, and retained in the soil for one week with a tarp. This treatment has been shown to result in *Frankia* population levels, at this site, below a detection limit of 10 infective units per gram soil using a red alder bioassay (Hilger *et al.* 1991). Plots were laid out in a 122-cm wide bed. Each plot was 61 cm long and was separated by a 15 cm buffer strip.

### *Inoculum preparation*

*Frankia* cells (strain ArI5) were cultured in BAP medium (Murray *et al.* 1984) in 6-L batches, stirred by bubbling with filter-sterilized air. The packed cell volume (pcv) was determined by spinning the cells down in 1.5 mL microfuge tubes at 14,000 rpm (relative centrifugal force of 16,000  $\times$  g) for 10 min. Appropriate amounts of a suspension were either mixed immediately with peat and refrigerated overnight for the peat-carrier treatments or brought up to volume in the field for the cell-suspension treatments. The peat mix (a commercial potting mixture of peat, bark fines, charcoal, and sand) was sieved (10 mesh) and autoclaved twice prior to the addition of *Frankia*.

The prepared seed-beds were inoculated, in late April, by distributing the appropriate inoculum over a plot and raking it in by hand to 3 to 5 cm depth immediately before seeding. The plots were then covered with Reemay (Ken-Bar Inc., 24 Gould St., Reading, MA 01867) to protect the seeds during germination. This was removed at five weeks after seeding, when the seedlings were well-established.

### *Experimental design*

A completely randomized block design was used, with 4 blocks and 8 treatments. Treatments were: control (1.3 L of H<sub>2</sub>O m<sup>-2</sup>), nodule homogenate (1.3 g fresh nodules in 1.3 L of H<sub>2</sub>O m<sup>-2</sup>), three levels (10-fold) of *Frankia* cells (3.4, 34, and 340  $\mu$ l pcv in 1.3 L of H<sub>2</sub>O m<sup>-2</sup>), and equal levels of *Frankia* in peat mix (1.3 kg wet m<sup>-2</sup> at 1.4 kg H<sub>2</sub>O kg<sup>-1</sup> dry matter). A trial of the peat mix carrier was performed a

second year including an unamended control and identical levels of *Frankia* in a smaller amount of peat mix (0.32 kg wet m<sup>-2</sup> at 1.3 kg H<sub>2</sub>O kg<sup>-1</sup> dry matter).

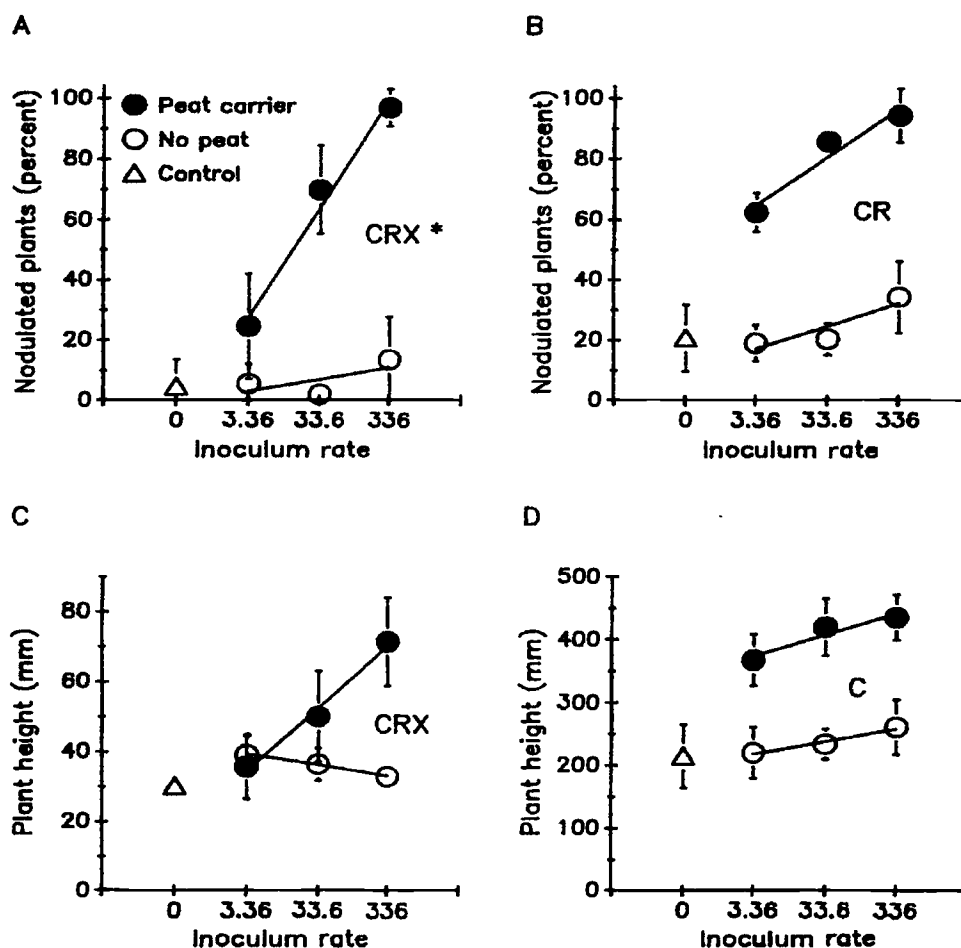
### *Experimental method*

At mid-season (the end of July), the seedlings were counted and height and percentage of seedling nodulated were determined by uprooting every 10<sup>th</sup> seedling counted. Separate counts were kept for nodules in the first 35 mm of root and for nodules below that depth. At lifting (late January), seedlings were counted and sorted in three size-classes for subsampling (10% of total). Length of stem, root collar diameter (rcd), dry weights for root and stem, and numbers of nodules above and below 35 mm depth were determined on this stratified sample. The size-classes were: high grade (>288 mm height and >4 mm rcd), low grade (>192 mm height and >3 mm rcd), and culls (those that failed to meet low grade standards).

### **Results**

At midseason, a far greater proportion of the plants were nodulated in the first 35 mm of root when peat-carrier was used (Fig. 2.1A). Plant height also increased in the two higher inoculation rates with peat-carrier as compared with treatments without carrier (Fig. 2.1C). Nodules in the upper 35 mm of soil were considered separately from those in the lower root because they would more likely have resulted from inoculation. These upper nodules were probably initiated earlier and thus had more

effect on the initial growth of the plant. The increase in inoculation success with carrier was less dramatic when the whole root was considered (data not shown).



**Figure 2.1.** Amount of inoculum applied with and without carrier is compared with plant height and proportion of plants nodulated to 35 mm at midseason (A and C) and harvest (B and D). The units for inoculum rate are:  $\mu\text{l pcv m}^{-2}$  soil surface. The x-axis is nonlinear, and the lines are from regressions of the means for the three inoculum rates indicated against the response variables. Bars indicate standard deviations. Where the bars are not visible, they are smaller than the symbol. The control treatment was an application of water in the same amount as the no-peat treatments. \*The letters between the lines indicate a significant ( $p > 0.01$ ) effect for carrier (C), rate (R) and interaction of carrier and rate (X), respectively.



At harvest, the differences between rates of inoculum in an inoculum types (carrier vs. no carrier) were less apparent. The carrier treatments produced larger seedlings than the other treatments (Fig. 2.1D). All measurements of size (height, root collar diameter, stem weight, and root weight) responded similarly to the treatments. The application of inoculum without carrier had little effect on growth, except for the highest inoculation rate cell suspension, which approached the lowest rate in peat carrier. The peat treatments produced many more nodulated seedlings than the other treatments (Fig. 2.1B). At harvest, the peat inoculum produced almost ten times more nodules in the upper 35 mm of fumigated soil than the equivalent rates in cell suspension.

The nodule homogenate treatment was not different from the control ( $p=0.05$ ) for any of the size parameters. In contrast to the midseason observations, no interaction between use of carrier and amount of inoculum was seen for most parameters at harvest, although the response to both variables was apparent.

**Table 2.1.** Nodulation success and seedling growth in 1989 experiment.

<u>Inoculum</u>		<u>Midseason</u>		<u>Harvest</u>	
<u>Carrier &amp; Level</u>	<u>(<math>\mu\text{l pcv m}^{-2}</math>)</u>	<u>Seedlings nodulated</u>	<u>Height</u>	<u>Seedlings nodulated</u>	<u>Height</u>
		<u>in 35 mm depth (%)</u>	<u>(mm)</u>	<u>in 35 mm depth (%)</u>	<u>(mm)</u>
None	0	1 a*	26 a	7 a	121 a
Peat	3.4	2 a	26 a	10 a	110 a
Peat	34	15 b	27 a	34 b	157 b
Peat	340	95 c	67 b	100 c	370 c

\* Values in the same column with the same letter are not significantly different (Fischer protected Duncan's mean separation test,  $p < 0.05$ )

The carrier treatments produced significantly more high-grade, bare-root seedlings than the other treatments, but yield of high-grade seedlings did not increase directly with increasing rates of inoculum in the peat carrier treatments. Plots with the high rate of *Frankia* in peat-carrier showed little difference in yield from plots with the intermediate rate.

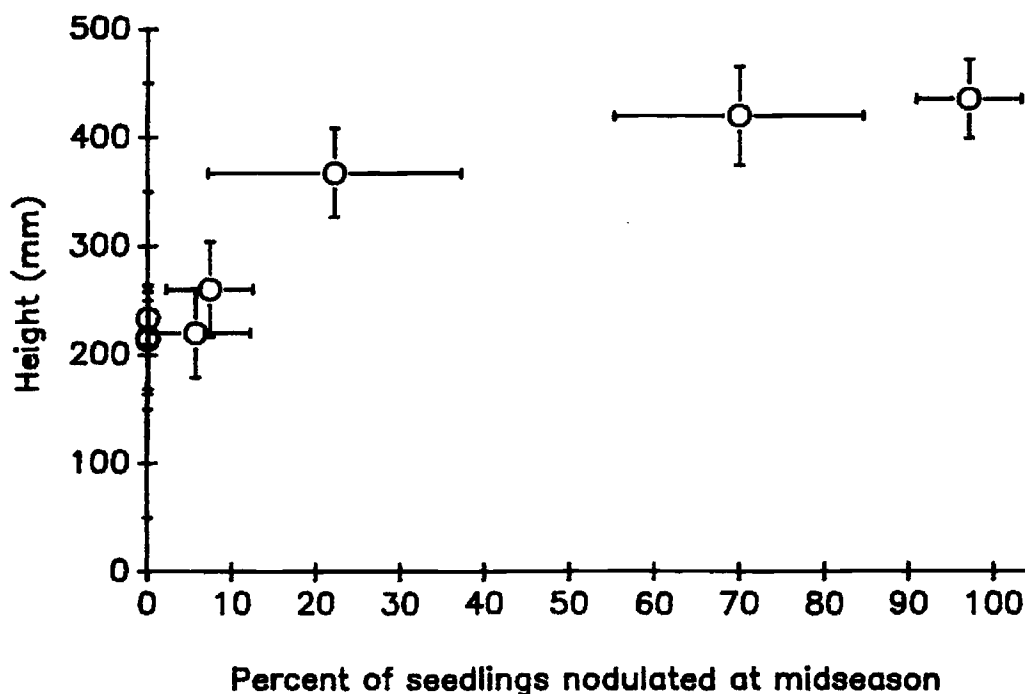
In the second year, at both midseason and harvest, the two highest rates showed responses in proportions of plants nodulated (Table 2.1). However, only the highest rates of inoculum resulted in seedlings of comparable height to the previous year. At harvest, the control seedlings were one-third of the dry weight of those the year before (data not shown). Blackened areas of necrosis were observed on the stems and lower leaves, due to a plant pathogen later identified as *Septoria* spp. Mean heights for both the control plots and those at the highest inoculation rate were decreased, presumably by this effect.

## Discussion

Nodulation due to *Frankia* inoculum was greatly enhanced by use of peat-carrier, particularly early nodulation as seen at midseason. At harvest, seedling size reflected this early nodulation. Around 30-40% nodulation was sufficient to reach most of the maximum height for the 1988 growing season (Fig. 2.2). An early-season check of nodulation rates may be possible to determine if conditions are sufficient for high yield. The choices of remedial actions are limited, but with further research,

such actions may be important to economical production of bare-root red alder seedlings.

The effectiveness of the carrier in promoting nodulation is most likely due to increased survival and vigor of the inoculum during seedling germination. Primary factors limiting survival are desiccation and solar irradiation (abiotic factors) and predation (biological attack) (Acea and Alexander 1988). Soil conditions (*e.g.*, pH) may reduce infectivity of viable cells, but the amount of carrier we used (320 g dry wt. m<sup>-2</sup>, mixed to 5 cm depth) would not be sufficient to significantly alter the physical or chemical properties of the bulk soil, consequently the effect of the carrier must be due to other factors. Mixing the inoculum into the soil (as well as the use of the Reemay mulch during germination) would limit the effects of irradiation and dessication, though these would no doubt take their toll. The biological interactions of *Frankia*, however, could be quite different for the naked cells than for the cells stabilized in the sterile peat mix. The predation rate for the unprotected cells is likely to be high relative to cells applied in an organic medium that would be capable of sequestering them through a variety of ionic linkages.



**Figure 2.2.** The level of sufficient nodulation at midseason is evaluated against plant height at harvest, 1988 trial. Bars indicate standard deviations. Where the bars are not visible, they are smaller than the symbol.

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### Chapter 3

#### **Dual-inoculation Increases Plant Growth with *Frankia* on Red Alder (*Alnus rubra* Bong.) in Fumigated Nursery Beds.**

K.J. Martin<sup>1</sup>, Y. Tanaka<sup>2</sup>, and D.D. Myrold<sup>1</sup>

<sup>1</sup> Department of Crop and Soil Sciences, Oregon State University, Corvallis, OR 97331-2213, USA, <sup>2</sup> Weyerhaeuser Co. Western Forestry Research Center, 505 North Pearl Street, Centralia, WA 98531, USA

#### **Abstract**

Inoculation trials for red alder (*Alnus rubra* Bong.) bare-root seedling production were set up in fumigated nursery beds. *Frankia* inoculum was applied in a peat mix carrier, either as a single strain (strains ArI5 or AvcI1) or as a combination of the two strains at the same total rate of application. The plots were laid out in four blocks of four treatments: control, strain ArI5, strain AvcI1, and dual-inoculum. Plots were 61 cm by 122 cm with 15 cm buffer strips. Number, height, and percentage nodulation of the seedlings were determined at mid-season. At lifting, seedlings were counted and sorted into size-classes for subsampling. Number, size, dry weight, and degree of nodulation were determined on this stratified sample. *Frankia* DNA from nodules was analyzed by DNA fingerprinting. The controls were poorly nodulated and grew slowly. The single strain inoculum for ArI5 produced larger seedlings than for AvcI1, both at mid-season and at lifting. The dual-inoculum treatment produced larger seedlings than all other treatments. The AvcI1 single-inoculum nodules

primarily produced fingerprints identical to AvCI1, but nodule fingerprints from all other treatments were dominated by ArI5 patterns. The observed seedling growth advantage with dual inoculum, then, is not attributable to nodule occupancy differences.

## **Introduction**

Red alder (*Alnus rubra* Bong.) is a common hardwood species in the Pacific Northwest that forms a nitrogen-fixing symbiosis with the actinomycete *Frankia*. As nitrogen-fixing trees, alder have several useful applications: they may be used in land reclamation, as an economic crop, and as a laminated root rot resistant reforestation species. Red alder improves soil fertility and structure, which can increase the growth of a subsequent stand of Douglas fir (Tarrant and Miller 1963). Additionally, growing red alder is comparable economically with growing the dominant forestry crop in the Pacific Northwest, Douglas fir (Tarrant *et al.* 1994).

In nursery production of red alder seedlings, both greenhouse media and nursery soils are routinely fumigated to control disease before seedlings are planted. Inoculation with *Frankia* is then necessary to produce nodulated stock because the indigenous populations are depleted by fumigation. Inoculation gives an advantage to seedlings very early in their growth. Inoculation at seeding, rather than at 6 weeks or later, resulted in much better growth in containers (Stowers and Smith 1985).

Inoculation of alder with mixtures of effective *Frankia* strains has been shown to provide better seedling growth in pots than inoculation with single strains (Prat

1989). This stimulation was even seen in dual-inoculation with an effective and an ineffective strain in greenhouse experiments (Hahn *et al.* 1990). We studied this effect under nursery conditions, in conjunction with Weyerhaeuser's efforts at developing a red alder seedling production method. The identity of the *Frankia* in the nodules was also explored to determine if dual inoculation led to dual occupancy.

## Materials and methods

### *Experimental design*

A trial of *Frankia* inocula was performed in 1988 for bare-root seedling production in fumigated nursery plots (61 cm by 122 cm with 15 cm buffer strips). A completely randomized block design was used, with four blocks and four treatments. The treatments were: peat mix carrier control (1.3 kg wet m<sup>-2</sup> at 1.35 kg H<sub>2</sub>O kg<sup>-1</sup> dry matter), two single strain inoculum treatments using Ar15 or Avcl1 (336 µl packed cell volume (pcv) in the same peat mix carrier) or a combination of the two at the same total rate of application (168 µl pcv each strain in the same peat mix carrier). One µl pcv *Frankia* is approximately 10<sup>6</sup> nodulation units in pure culture (Hilger *et al.* 1991).

The soil used was Nisqually loamy sand, at the Weyerhaeuser Mima Nursery in Washington state. It is high in organic matter, with a KCl-extractable soil nitrogen of 25.5 mg kg<sup>-1</sup> NH<sub>4</sub><sup>+</sup>-N and 4.67 mg kg<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N. The soil was fumigated in the fall with 389 kg ha<sup>-1</sup> methyl-bromide/chloropicrin, injected at 15 to 20 cm, and the soil



was covered with a tarp for 1 week. This treatment has been shown to result in *Frankia* population levels, at this site, below a detection limit of 1 nodulation unit per gram soil using a red alder bioassay (Hilger *et al.* 1991).

### *Inoculum preparation*

A peat mix carrier was used to increase inoculation success (Martin *et al.* 1991). The peat mix (a commercial potting mixture of peat, bark fines, charcoal, and sand) was sieved (10 mesh) and autoclaved, allowed to rest 48 hours, and autoclaved again. *Frankia* cells (strains ArI5 and AvcI1) were cultured in BAP medium (Murry *et al.* 1984) in 6-L batches stirred by bubbling with filter-sterilized air. Standard pcv was determined by sedimenting the cells in 1.5-mL microcentrifuge tubes at 14,000 rpm (16,000 x g) for 10 min. Appropriate amounts of a suspension were mixed immediately with peat mix and refrigerated overnight (sterile water was added for the uninoculated control). For each strain, the same suspension was used for all relevant treatments.

### *Experimental method*

Plots were laid out in a 122-cm wide bed. Each plot was 61 cm long and was separated by a 15-cm unplanted buffer strip. The prepared seed-beds were inoculated in late April by distributing the appropriate inoculum over a plot and raking it in by hand to 3 cm depth immediately before seeding. Care was taken not to cross-contaminate the plots. The plots were covered with Reemay fabric (Ken-Bar Inc., 25

Walkers Brook Dr., Reading, Mass. 01867) to protect the seeds during germination. This was removed at 6 to 7 weeks after seeding, when the seedlings were well established.

At mid-season (the end of July 1988), the seedlings were counted and height and percentage of seedlings nodulated were determined by uprooting every 25th seedling counted. Separate counts were kept for nodules in the first 35 mm of root and for nodules below that depth. At lifting (late March 1989), seedlings were counted and sorted into three size-classes, and subsampled (approximately 10% of total seedlings). The size-classes were graded by root collar diameter (rcd): high grade (>288 mm height and >4 mm rcd), low grade (>192 mm height and >3 mm rcd), and culls (those smaller than low grade standards). Length of stem, rcd, dry weights for root and stem, and numbers of nodules above and below 35 mm depth were determined on this stratified sample. The data were analyzed by analysis of variance in a randomized complete block design and Student-Newman-Keuls (SNK) tests ( $\alpha = 0.05$ ) were performed on measures with significant treatment effects ( $\alpha = 0.01$ ).

Nodules from a subsample of these seedlings were frozen for PCR-RFLP analysis. DNA was extracted from nodules using a CTAB method (Baker and Mullin 1994). A portion of the glutamine synthetase gene, *glnII*, was amplified with published primers (Jamann *et al.* 1993) and restricted with *HaeIII*. Preliminary work showed that ArI5 and AvcI1 could be differentiated with this assay. An electrophoretic analysis of this restriction digest was used to determine which group or groups were present.

## Results

Differences were not significant among treatments with respect to seedling numbers, at midseason or at harvest. There were very few block effects as well. There were 3330 seedlings excavated at harvest and 301 were evaluated. Nodules in the upper 35 mm of soil were considered separately from those in the lower root because they would more likely have resulted from inoculation in this fumigated soil. Only one nodule was found in the upper 35 mm of soil for all 102 non-inoculated seedlings examined, compared with a total of five nodules lower in the soil. These upper nodules would also have been initiated earlier and thus have had more effect on the initial growth of the plant. In the entire experiment, at harvest, there were 1724 nodules greater than 10 mm diameter in the upper 35 mm of soil and 49 such large nodules lower on the root.

The control plots did not nodulate well and produced small seedlings ( $\alpha=0.05$ , SNK mean separation). ArI5 inoculum produced larger seedlings than AvcI1, although both resulted in nearly complete nodulation (Table 3.1). Dual-inoculation resulted in larger seedlings than inoculation with single strains. The ArI5 and dual-inoculum treatments resulted in a greater proportion seedlings nodulated than the AvcI1 treatment, though AvcI1 still had a much higher nodulation rate than the control.

**Table 3.1.** Inoculum Effects on Plant Nodulation and Allometry

<u>Inoculum</u>	<u>Seedlings nodulated (%)</u>		<u>Plant height (cm)</u>		<u>Plant dry weight (g)</u>
	<u>Midseason</u>	<u>Harvest</u>	<u>Midseason</u>	<u>Harvest</u>	<u>Harvest</u>
Control	1 (A)*	12 (A)	2.7 (A)	10.3 (A)	0.8 (A)
AvcI1	78 (B)	99 (B)	5.0 (B)	26.6 (B)	2.2 (B)
ArI5	97 (C)	97 (B)	7.8 (C)	35.3 (C)	3.1 (C)
AvcI1+ArI5	95 (C)	97 (B)	9.2 (D)	38.4 (C)	4.1 (D)

\* Values in a column with different letters are significantly different ( $\alpha = 0.05$ , SNK)

In the RFLP analysis, the ArI5-type pattern was most prevalent. Most of the nodules in the control plots gave this pattern. ArI5 had not been previously used as an inoculum in this bed but was used in beds nearby. A unique wild-type pattern was also seen in one control plot. In the dual-inoculation plots, the ArI5 type dominated (Table 3.2). To characterize the sensitivity of this test to the presence of the two strains, genomic, pure-culture DNA extracts for the two strains were mixed in various ratios and amplified by PCR. Even at a 3:1 ratio of ArI5 to AvcI1, this test gave primarily an AvcI1 type pattern with a faint ArI5-type pattern visible, in contrast to the relative scarcity of the AvcI1-type patterns in most of the treatments. The few mixed patterns seen from nodule extracts gave results very similar to those for the 3:1 ratio of ArI5 to AvcI1 pure-culture DNA.

**Table 3.2.** Distribution of RFLP patterns among inoculation treatments.

<u>Treatment</u>	<u>RFLP Pattern</u>				<u>Total</u>
	<u>ArI5</u>	<u>AvcI1</u>	<u>AvcI1/ArI5</u>	<u>Wild</u>	
	<u>(numbers of nodules)</u>				
Control	14 (82%)*			3 (18%)	17
AvcI1	1 (4%)	24 (89%)	2 (7%)		27
ArI5	26 (96%)	1 (4%)			27
Dual	36 (100%)				36

\*Numbers in parentheses are percent of nodules in treatment with specified pattern.

## Discussion

Our dual-inoculation treatment resulted in an increase in seedling growth and nodulation over either of our single-inoculum treatments. Prat (1989) saw a similar effect for two strains of *Frankia* inoculated on nine species of alder. Mean shoot height across all alder species was significantly higher for the combined inoculum than for either inoculum alone. A stronger effect was seen for incremental shoot growth (between the 45th and 60th days after inoculation) showing a significant effect for the mean of all alder species as well as for *Alnus rubra* and *Alnus glutinosa* analyzed individually.

A third strain in the study of Prat (1989) was associated with greater growth rates compared with the other single-strain inocula but showed no significant increase in effectiveness when combined with either, or both, of the other strains. Although

we found that significant differences in effectiveness could be detected between our strains as well as for the dual-inoculum treatment, the effectiveness of the individual strains may not be important to the enhanced growth with dual inoculum. Indeed, Hahn *et al.* (1990) found that co-inoculating alder with an effective and a *Nif*<sup>-</sup> ineffective strain produced larger seedlings than the effective strain alone.

If the effect of dual-inoculation is not dependent on the effectiveness of both symbionts, then it would seem likely that dual occupancy is not important either. This may explain our observation that in spite of a dual-inoculum effect, the nodules produced under dual-inoculation did not show dual-occupancy. Our RFLP analysis of the nodules did not show any nodulation by AvcI1 in the dual-inoculation treatment. Strain ArI5 was more effective than AvcI1 in single-inoculum treatments, and completely dominated AvcI1 in nodule occupancy in the dual-inoculation treatment. In the two single-inoculum treatments, where the inoculated strain would be expected to dominate, at least one nodule was occupied by the other strain, indicating strong nodulating ability at low relative population levels in both strains. This makes it all the more noteworthy that dual inoculation produced only ArI5 nodules. This treatment, which exhibited the least diversity in occupancy, also produced the largest seedlings.

Less than 2% of all nodules analyzed showed mixed ArI5/AvcI1 patterns. Because the nodules were only cleaned by rinsing with tap water before DNA extraction, it is possible that some contaminating DNA may have been present in the samples. Reports of dual occupancy have been rare considering the numbers of

nodules that have been analyzed to date (Murry *et al.* 1997, Dobritsa and Stupar 1989). If dual occupancy did occur, it is unlikely that it played a significant role in the increased yield effect for dual inoculation.

If the increased growth rate observed with dual inoculation was indeed an effect of the diversity in the inoculum as the data indicate, this effect was likely through some means other than dual occupancy. It is possible that the effect of mixed inoculum was most important before occupancy had been established. The infection process could conceivably have been enhanced, through complementary actions of the strains. One strain may induce a strong plant response while another excels at rapid exploitation of infection sites. In this experiment, the plant growth advantage in the mixed inoculum treatments was well established at three months after seeding. A more rapid infection process would have given these plants an advantage even before we characterized nodulation at midseason. The larger seedlings at midseason with dual inoculation indicate that this may be the case.

Further research on dual inocula will be necessary before these results could be applied to nursery management. The effects we have observed in this study are intriguing, but they need the support of similar studies to provide a clear picture of whether dual inocula will result in better growth in other years and locations.

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## Chapter 4

### Nodulation Potential of Soils from Red Alder Stands Covering a Wide Age Range

K.J. Martin<sup>1</sup>, N.J. Ritchie<sup>1</sup> and D.D. Myrold<sup>1</sup>

<sup>1</sup> Department of Crop and Soil Science, Oregon State University, Agriculture and Life Sciences Bldg. 3017, Corvallis, OR 97331-7306, USA.

#### Abstract

Red alder (*Alnus rubra* Bong.) stands in the Pacific Northwest are the common first stage in succession following disturbance. These stands are highly productive and contribute a large amount of nitrogen to the soils as a result of their nitrogen-fixing symbiosis with *Frankia*. As these alder stands age, the soils not only increase in total nitrogen, but concentrations of nitrate increase and pH decreases as a result of nitrification. The objective of this study was to determine how the nodulation capacity of *Frankia* varies as red alder stands age and if differences in nodulation capacity are related to changes in soil properties.

Nodulation capacity was determined by red alder seedling bioassay for soils from red alder stands in the Oregon coast range covering a wide range of ages. Six chronosequences each containing a young, intermediate, and older alder stand were sampled. Total nitrogen, total carbon, nitrate, ammonium and pH were measured on the same soil samples. These factors as well as alder stand characteristics were compared with nodulation capacity in an attempt to identify soil characteristics typical

in developing alder stands that most strongly affect nodulation potential in controlled bioassays.

Soil pH was most highly correlated with nodulation potential, with nitrate content also significant. Cluster analysis of the sites using these two variables produced two groups with distinctly different nodulation capacities. The higher nodulation capacity cluster was lower in nitrate and higher in pH than the majority of sites in the lower nodulation capacity cluster. There was general overlap in the age ranges for the two groups but the lower nodulation capacity cluster contained the oldest sites.

The results of this study suggest that nodulation capacity decreases as red alder stands age, which may have implications for the subsequent establishment of red alder stands on the same site.

## **Introduction**

*Frankia* are filamentous soil bacteria (Actinomycetes) that can induce and are active in nitrogen-fixing root nodules in a wide range of woody plants including red alder (*Alnus rubra* Bong.). Red alder is an economically important hardwood in the Pacific Northwest (Resch 1979) which depends on *Frankia* for its rapid growth (Martin *et al.* 1991). Evaluating the potential of soils to induce nodulation, therefore, is important in developing management practices for red alder.

As stands age, alder forest soils will typically increase in acidity, nitrogen (particularly nitrate) and organic matter content while decreasing in cation exchange

capacity (Bormann *et al.* 1994). Red alder litter is rich in nitrogen and releases ammonium to the soil as it degrades. Nitrification then converts ammonium to nitrate which provides the primary source of acidification in alder soils (Van Miegrot and Cole, 1985)

The factor most important in predicting the nodulation potential of a soil is pH. A positive correlation was first seen between infective units (IUs) of *Frankia* and pH (in the range 3.4 to 5.8) by Smolander *et al.* (1990) on hybrids of *Alnus incana* (Moench) and *Alnus glutinosa* (Gaertn) (Smolander and Sundman 1987, Smolander *et al.* 1988). They also observed that low pH has the potential to directly inhibit nodulation in solution culture (Smolander *et al.* 1988). As with plant culture pH, solution nitrate concentrations have also been shown to directly inhibit nodulation with concentrations above 2 mM leading to complete inhibition (Kohls and Baker, 1989).

In this study we examined the nodulating potential of soil from a subset of the Red alder forest sites described by Carlton (1988) in an attempt to identify soil characteristics typical in aging alder stands that most strongly affect nodulation potential in controlled bioassays.

## **Material and methods**

### *Experimental Design*

Six chronosequences were identified in alder forests in the Oregon Coast Range. Each of the chronosequences consists of a set of three alder sites, which are fairly similar with respect to slope, aspect, landscape position and climate (distance from the coast). The primary difference between sites within a set is that they cover a range of ages; alder stand ages ranged from 10-20 years to 40-90 years. These sites were characterized in the thesis work of Gary Carlton (1988) at Oregon State University. The chronosequences were set up to serve as blocks in statistical analyses if they were found to be significant, but otherwise the sites would better represent a range of stand ages without bias related to landscape positions and climatic conditions.

### *Soil collection*

All sites within each chronosequence were sampled on the same day. Soils were collected mid-summer from each site at 1 m intervals along a 10 m transect. Samples of approximately 5 cm diameter and 6 cm depth were collected by trowel and numbered sequentially. MPN bioassays were performed on pooled subsamples of successive sets of 3 samples, the last sample was not included in the plant bioassays.

### *Plant Bioassay MPN*

Dilution series were prepared from the soil samples in order to perform most probable number analyses (MPN) of *Frankia* propagules capable of nodulating alder. Ten-fold dilutions (starting at 0.1 g soil moist weight in 1 mL solution) were applied to axenic alder seedlings in 50 mL tubes containing nitrogen-free, quarter-strength, Hoagland's plant medium. In order to ensure there were no plant culture effects of soil pH, subsamples of the soils were titrated with 0.1M KOH to pH 6.5 and an equivalent amount of 0.1 M KOH was added to the initial soil dilution (5 g moist soil, plus 50 mL total of plant medium plus KOH). Moisture content of the soil was measured by oven drying and these data were used to calculate exact ratios of dry weight soil per volume of solution in each dilution. Seedlings were maintained in solution culture in a growth chamber with weekly changes of solution for 8 weeks as previously described (Hilger *et al.* 1991) after which they were evaluated for presence of nodules. Replicate seedlings within a dilution were scored as positive if nodules were found on their root systems. The resulting values for numbers of infective units, inducing nodules, per gram soil dry weight (gsdw) will be referred to as IUs.

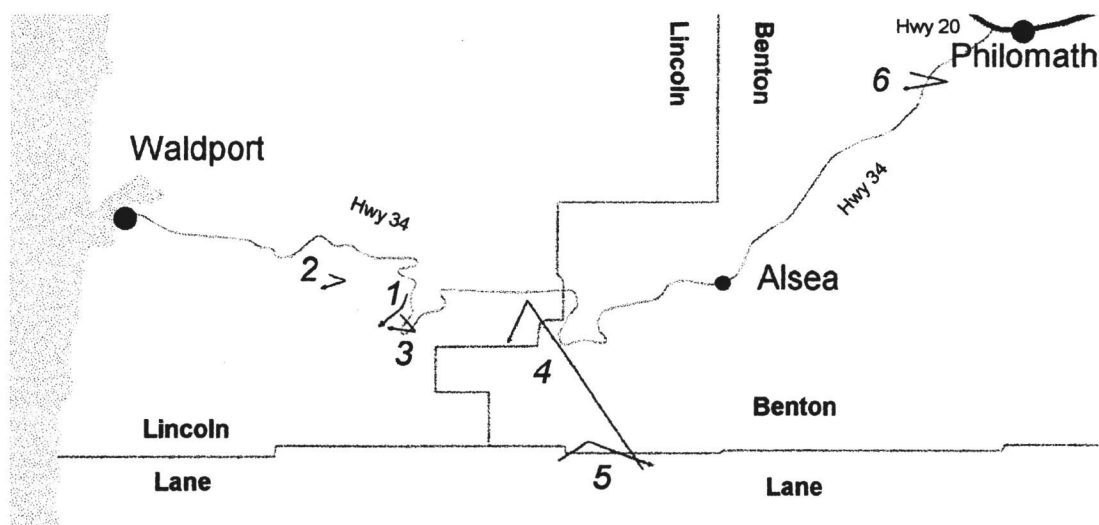
### *Site characterization*

A variety of soil characteristics were measured, including: total N, total C (by mass spectrometry) and  $\text{NO}_3\text{-N}$ , and  $\text{NH}_4\text{-N}$  (by Alpchem colorimetric solution chemistry). Characteristic of the alder stands (site index, trees/ha, dead/ha, d.b.h.,

basal area, height) were available as well (Carlton 1988). These would serve as cofactors to help to explain variability in the measurements of *Frankia* populations. Statistical analyses were performed using SAS (SAS Institute Inc., version 8). For cluster analyses, a each variable was multiplied by a constant that brought its variance within the range of 300-1000 (SAS manual, SAS Institute Inc.).

## Results

Chronosequences 1 through 3 were closely associated sets of alder stands in the area just south of Alsea River 14 to 19 miles from the coast. Chronosequence 1 had a greater slope and a more NE aspect than chronosequences 2 and 3, but otherwise they were quite similar and covered wide age ranges (see Table 4.1). Chronosequence 4 was much more geographically dispersed, ranging from Alsea River 27 miles inland south to Lobster Valley, and had very steep slopes. Chronosequence 5 was concentrated in the Lobster Valley area, it covered a narrow age range, but was included to represent low-slope bottomland. Chronosequence 6 also covered a narrow age range, but was included to represent riparian zones in the Mary's Peak area (see Figure 4.1).



**Figure 4.1** Locations of each set of alder stands is represented by a bent arrow connecting the three stands in order of age with the arrow head at the oldest stand.

One-way ANOVA of IUs showed no significant effect for the chronosequences (using either individual values or plot means), so the subsequent analyses were performed either on all individual analyses or on means of the three soil samples evaluated at each site. We used the antilog of the mean  $\log(\text{IUs})$  values for plot means to account for the apparent lognormal distribution of the *Frankia* propagules (IUs).



**Table 4.1.** Characteristics of the chronosequence alder stands.

<u>Stand number</u>	<u>Block number</u>	<u>Age (yrs.)</u>	<u>Elev. (m)</u>	<u>Slope (%)</u>	<u>Location (mi.inland)</u>	<u>Trees (perHA)</u>	<u>DBH (cm)</u>	<u>Height (m)</u>
4	1	32	90	44	19	1050	20.9	24
21	1	51	260	46	19	800	30.6	27.7
25	1	87	350	50	18	200	42.6	28.1
16	2	11	340	18	14	15300	4.1	9.7
39	2	31	240	30	16	1050	21	26.2
15	2	78	340	30	14	260	43.8	26
19	3	8	230	0	19	15600	3.5	9.6
40	3	28	30	0	21	1080	19.6	24.6
24	3	75	270	25	19	90	56.5	25.1
32	4	24	210	60	37	1500	15.1	20.6
6	4	31	120	50	27	1450	20.1	24.5
44	4	86	370	58	26	330	38.7	28.5
41	5	20	180	13	31	2500	12.1	16.6
42	5	29	90	0	34	1750	16.6	24.1
33	5	40	200	0	37	650	25.9	25.3
9	6	22	300	5	43	2350	12.6	20.3
22	6	28	270	25	45	1150	19.4	27.6
8	6	50	230	7	43	720	27.2	28.9

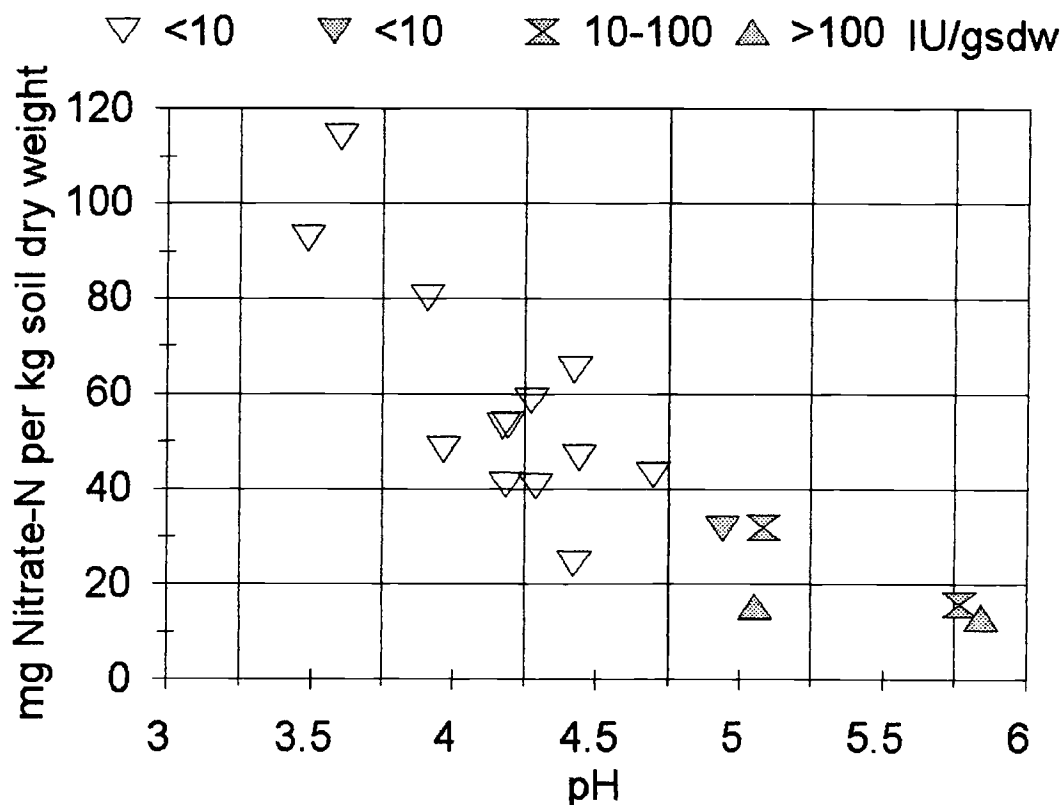
Data from the thesis work of Gary Carlton (1988), sorted by age within block.

A step-wise multiple regression indicated that only pH met the significance level ( $\alpha = 0.15$ ) for entry into a model predicting IUs for plot means. However, in linear regressions against IUs/gsdw, both pH and  $\text{NO}_3$  met a significance level of  $\alpha = 0.05$  in separate analyses. No other variable regressed separately against IUs met this level. Cluster analyses (Ward's Minimum-Variance Method, 2 clusters) also tended to show little effect with the inclusion of other variables.

A cluster analysis of soil nitrate and pH separated a small group of 11 samples with lower nitrate and higher pH, and a larger group of 43 samples where the reverse

was true. Average values for the two clusters were: 15 and 57 mg kg<sup>-1</sup> NO<sub>3</sub><sup>-</sup>, and pH 5.4 and 4.0 respectively. ANOVA analyses of the IUs for these clusters showed they were significant at the  $\alpha = 0.01$  level for both individual samples and plot means.

This cluster analysis separated a higher nitrate / lower pH (cluster 1) and a lower nitrate / higher pH cluster (cluster 2, see Table 4.2). The pH ranges of the 2 clusters did not overlap and only one of the soils in cluster 1 was in the nitrate range of cluster 2. All 19 alder stands in cluster 1 had fewer than 10 IU gsdw<sup>-1</sup>, whereas only one of five stands in cluster 2 fell below that mark (Figure 4.2). The stand with the lowest pH and the highest NO<sub>3</sub> content (#24) also had the lowest level of IU/gsdw. Although most of the stands in cluster 2 were in the mid to young age range, one stand was 50 years old. Cluster 1 covered a similar age range but included many stands well over 50 years of age.



**Figure 4.2.** Soil nitrate is compared with soil pH with symbols for alder stands indicating nodulation capacity. Empty symbols represent stands in cluster 1 and shaded symbols represent stands in cluster 2. Symbol shapes correspond to level of nodulation potential (IUs) as indicated.

Generally,  $\log_{10}(\text{IUs})$  gave only slightly better correlations with other variables than did IUs (Table 4.3). These correlations, where discernable, were negative, while correlations among pH,  $\text{NO}_3$ , and stand age were positive. Among the most highly correlated variables, pH was significantly correlated with  $\text{NO}_3$ ,  $\log_{10}(\text{IUs})$ , and IUs, though not very strongly in the last case. Nitrate also showed a weak, but significant, correlation with stand age (data not shown).

**Table 4.2.** Soil properties for clustered alder stands, sorted by cluster then age

Stand	Block	Cluster	Age (yrs.)	NH <sub>4</sub> -N (mg/kg)	NO <sub>3</sub> -N (mg/kg)	pH	%N	%C	IUs (/gsdw)
16	2	1	11	3.48	24.4	4.42	0.40	11.3	1.44
41	5	1	20	5.61	41.0	4.19	0.53	12.9	2.79
32	4	1	24	11.7	40.7	4.29	0.61	12.7	0.68
40	3	1	28	28.4	53.3	4.18	0.82	14.1	2.89
42	5	1	29	6.04	46.7	4.44	0.65	10.9	7.25
39	2	1	31	14.0	58.6	4.28	1.07	24.6	1.48
6	4	1	31	11.2	53.6	4.19	0.45	18.7	2.21
33	5	1	40	7.61	80.1	3.91	1.39	26.7	3.20
21	1	1	51	2.78	43.0	4.70	0.54	11.4	1.45
24	3	1	75	27.2	114	3.60	1.91	50.5	0.61
15	2	1	78	18.3	92.7	3.49	2.28	61.1	1.74
44	4	1	86	2.13	65.1	4.42	0.93	18.6	3.82
25	1	1	87	1.66	48.4	3.96	0.63	24.6	1.15
19	3	2	8	3.11	15.4	5.06	0.20	4.57	226
9	6	2	22	6.86	31.7	4.94	0.60	9.85	2.15
22	6	2	28	2.81	13.2	5.84	0.31	8.61	393
4	1	2	32	1.68	15.9	5.77	0.32	4.41	40.7
8	6	2	50	8.83	32.0	5.08	0.48	8.12	15.3

**Table 4.3.** Correlations among soil characteristics

	Correlation strength (R-squared)							
	log(IUs)		IUs		pH		NO <sub>3</sub>	
Age	0.12	<0.05*	0.08	>0.25	0.19	<0.10	0.41	<0.01
NO <sub>3</sub>	0.38	<0.10	0.23	<0.05	0.70	<0.01		
pH	0.64	<0.01	0.40	<0.01				

\* Significance of correlations ( $\alpha$  level for Fisher's statistic)

## Discussion

It has often been observed that lower pH soils produce fewer *Frankia* nodules.

Because pH and nitrogen are typically controlled in nodulation bioassays, the effect of soil chemistry on nodulation potential is likely attributable to either lack of viable

propagules or inhibition (other than by pH or  $\text{NO}_3^-$ ). Any inhibitory organics in the bioassay solutions from the soil would need to have been active at less than 0.1 g soil dry weight per plant and would likely have been proportional to organic matter content of the soil. Because the correlation between IUs and total carbon were poor there are no indications of such inhibitory compounds.

We have shown that among the factors we measured (soil pH, total N, total C, and  $\text{NO}_3\text{-N}$ , and  $\text{NH}_4\text{-N}$ ) and available data on alder stand allometry and age, only pH and  $\text{NO}_3$  were identifiable as significant factors in predicting soil nodulation potential. Soil pH and  $\text{NO}_3$  are well known to be strongly impacted as alder stands age. However, stand age did not explain much of the variation in pH and less of the variation in  $\text{NO}_3$  content. Any effect of these two factors on nodulation potential may be independent of alder stand development.

Smolander and Sundman (1987) examined the nodulation potential of eight soils from under pine, spruce, and birch as well as one from an alder stand. Their data indicate a positive correlation between pH and IUs with an  $R^2$  of 0.66 over the pH range of 3.4 to 4.4. Although all of the soils we studied were from alder stands and many of their soils had not had alder in the vicinity for periods of 20 to 80 years, the pattern we saw was very similar. Sharma and Ambasht (1988) found that the energy cost to the plant of nitrogen fixation in a 7 year old stand was only 66% of that for a 17 year old stands of Himalayan alder, and 55% of that in a 56 year old stand. It would appear that the strong selective pressure that the symbiosis confers on the two participants early in alder stand development may be lost in older stands. Whatever

the increases in population size gained by *Frankia* in early symbiosis with alder, *Frankia* persist beyond that point with relatively small advantages compared to free-living soil populations and with notable disadvantages late in stand development.

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## Chapter 5

### Conclusions

#### Summary

This collected work on the *Frankia* - red alder symbiosis begins with an applied experiment showing that use of a carrier with inocula was important to efficient establishment of nodules on alder nursery seedlings. From there it moves on to ecological studies aimed at elucidating the effects of the soil environment and *Frankia* populations on the ability of soils to induce nodule formation. In the first experiment, showing the utility of peat carrier protection of inocula in nursery trials, an equally important result was the strong connection between early nodulation and seedling size. This provides nursery managers with a powerful tool for evaluating alder seedling growth potential.

We followed this work with an evaluation of the nature of nursery inocula, specifically concentrating on the use of mixed inocula with more than one *Frankia* strain. We found a clear indication that such mixtures produced larger seedlings. In order to better understand this effect, DNA typing was used to determine which strain or strains occupied the nodules in the single and dual inocula treatments. Interestingly, we found no evidence that the dual inoculum effect was the result of dual occupancy of the nodules, an effect that may have been explained by complementary behavior of the strains in the functioning nodule. Evidently, the dual inoculum had its effect



outside of the nodules. Because early nodulation is such a strong predictor of seedling size, we hypothesize that the effect of dual inoculation may arise from a much more rapid infection process. The greater seedling size for the dual inoculum without a difference in nodulation rates at midseason would mean, under such a scenario, that the higher nodulation rate under dual inoculation had already disappeared by the time we counted nodules at three months, very early in seedling growth.

In the final study we looked at the ability of forest soils to nodulate axenic, solution-grown, red alder seedlings in relation to characteristics of the alder stand where the soils were sampled. I also pursued a molecular analysis of the soil *Frankia* populations, attempting to quantify total number of genomes as well as numbers for any subtypes identified. This work, while advancing the state of the art in soil DNA characterization, did not produce useful results for this study. The soil pH had the strongest correlation with nodulation capacities of the soils, with soil nitrate also showing some importance as a predictor. The soils could be grouped by these two factors to provide a good prediction of which soils would produce the most nodules, yet neither of these factors could directly affect the seedling in solution culture, where the chemistry was controlled. Some other factor which varies with pH and nitrate could cause the difference in nodulation, but this agent would either need to be active at the low levels present in the soil dilutions used as inocula or be within the *Frankia* themselves. It is interesting that the alder-*Frankia* symbiosis itself produces such conditions, potentially limiting the ability of the symbiosis to regenerate through the formation of new nodules.

As with other organisms with unique capabilities, there is an inclination to place strong emphasis on the nitrogen fixing symbiosis of *Frankia* with actinorhizal plants when formulating hypotheses regarding *Frankia* autecology. Clearly, this is a valid inclination with regard to the important role *Frankia* play in nitrogen acquisition for younger plants, most particularly in soils with poor fertility. Red alder would not succeed nearly so well in colonizing sites without the *Frankia* mutualism. As could be seen in the nursery experiments, nodulation by *Frankia* contributed significantly to the growth of the alder seedlings, even in relatively fertile nursery soils.

In spite of this highly beneficial mutualism to provide *Frankia* with a strategy for proliferation through growth within the nodules, it appears that for much of the ecological history of *Frankia* other strategies are as important. A parallel could be drawn to the ecology of denitrifying bacteria in the soil. Martin *et al.* (1988) found that these organisms, while capable of respiration under anaerobic conditions (in contrast to most other soil organisms) were highly competitive in aerobic soil conditions. The denitrifying mode of respiration, for most denitrifiers, may serve only as an auxiliary mode to persist in greater numbers under limiting conditions, while they thrive best in direct competition with the aerobic soil microbial community. Apparently the metabolic load of carrying this ability through evolutionary time was sufficiently offset by its advantages. Similarly, *Frankia* go through periods of very high population levels in symbiosis which could later aid in dispersal through time and space. This may be all that is necessary to offset the genetic costs of maintaining the symbiosis, while their primary mode of survival may be as free living soil microbes.

## Recommendations for Future Research

The above discussion is predicated on the assumption that *Frankia* populations are significantly increased by the symbiosis. Though this seems likely, we know relatively little about free-living *Frankia* populations. This is where some of the most intriguing questions arise. What are the population levels in a variety of soils, and how do they relate to nodulation capacity? What is the diversity of free-living soil populations and what part do the *Frankia*-like isolates that are not capable of inducing nodulation play in this relationship? *Frankia* are an excellent model for studies related to the huge range soil bacteria that cannot be isolated, and can only be studied thanks to the recent advances in molecular biology. *Frankia* have clear economic importance but are very difficult to isolate from soil. Unlike other organisms in the soil which we are just becoming aware of through these new molecular techniques, some portion of *Frankia* populations proliferate in plant nodules which gives us a window into the soil populations. With *Frankia* we have the challenge of characterizing soil populations we cannot culture, but about which we already have a great deal of information.

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## **APPENDIX**

## Appendix

### Gel Purification of Soil DNA Extracts

David D. Myrold, Kendall J. Martin, and Nancy J. Ritchie  
Department of Crop and Soil Sciences, Oregon State University, Corvallis, OR.

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#### Introduction

Many methods have been developed for extracting nucleic acids from soils or other environmental samples. They can be grouped into two general categories: (i) cell separation followed by lysis (Holben *et al.* 1988, Steffan *et al.* 1988, Torsvik 1980) and (ii) direct lysis of cells *in situ* (Creswell *et al.* 1991, Hilger and Myrold 1991, Ogram *et al.* 1987, Porteous and Armstrong 1991, Rochelle and Olson 1991, Slenska and Klingmuller 1991, Smalla *et al.* 1993, Steffan *et al.* 1988, Tsai and Olson 1991, Van Elsas *et al.* 1991). Releasing DNA from cells is only the first step; additional purification must be performed in order to produce DNA of sufficient quality for subsequent enzymatic reactions. Several approaches have been tried, including the incorporation of polyvinylpyrrolidone (PVPP) (Hilger and Myrold 1991, Holben *et al.* 1988, Ogram *et al.* 1987), selective precipitation of DNA (Holben *et al.* 1988, Ogram *et al.* 1987), hydroxyapatite columns (Torsvik 1980), gel filtration (Tsai and Olson

1992), and gel electrophoresis (Hilger and Myrold 1991, Rochelle and Olson 1991, Van Elsas *et al.* 1991).

Gel purification is based on the different electrophoretic mobilities of DNA and humic materials. Humic materials move much faster than high molecular weight DNA when both are run under standard conditions for agarose electrophoresis. Agarose gel purification had been used by Hilger and Myrold (1991), Rochelle and Olson (1991), and Van Elsas *et al.* (1991). Rochelle and Olson (1991) used 1 g soil samples and in-gel lysis to extract DNA, however, the DNA extracted was not of sufficient quality for use with restriction enzymes. Hilger and Myrold (1991) and Van Elsas *et al.* (1991) used preparative agarose gel electrophoresis and produced DNA that could be cut by restriction enzymes or amplified by the polymerase chain reaction (PCR). An alternative electrophoresis approach, which incorporates polyvinylpyrrolidone (PVPP) into the agarose gel, and thereby results in the retarded migration of humic materials, has also been reported (Young *et al.* 1993).

The following procedures are a modification of the direct lysis method reported by Hilger and Myrold (1991). Its main features are: (i) enzymatic cell lysis, (ii) gentle extraction methods to isolate relatively intact DNA of high molecular weight, and (iii) agarose gel electrophoresis for purification of DNA from contaminating soil humic materials. The method has been used successfully to isolate DNA from a wide range of soils. Total DNA extracted has ranged from 5 to 27  $\mu\text{g DNA g}^{-1}$  soil. Extraction efficiency has been estimated to range from nearly 100% in low organic matter, sandy soils to about 10% in soils with significant amounts of clay and organic matter. The

isolated DNA is suitable for PCR (polymerase chain reaction) amplification (Myrold and Huss-Danell 1994).

In principle, the following procedure should work in connection with most direct DNA extraction protocols, however, this should be confirmed on a case by case basis, because soils vary in their amounts and types of humic materials and extraction methods produce DNA of differing sizes and degree of purity.

### Procedures

**Table A.1.** Materials for gel purification of DNA

<u>Autoclaved or new sterile</u>	<u>per sample</u>
Stirring rods (bamboo cooking skewers work well)	2
Cotton tipped swabs	1
15 mL screw cap centrifuge tubes (polypropylene)	2
1.5 mL microcentrifuge tubes (polypropylene)	4
Plastic transfer pipettes	3
Siliconized wellformers (gel well)	1
5 mL plastic beakers (gel block)	1
Gel molds (gel block)	1
250 L pipette tips	2
1 mL wide-bore pipette tips	6
5 mL pipette tips	2
Dialysis membrane (Spectrapor 132678, 2.5 cm wide, 15 cm long, MW cutoff 14,000-16,000; prepared according to [8])	1
Clips for dialysis tubing	2
Centrifuge and rotors for 15 mL screwcap tubes	
Microcentrifuge	
70 °C water bath	
Electrophoresis apparatus (20 x 25 cm gel tray) & power supply (250 V, 7.5 amp)	

The following procedures have been developed for purifying crude soil DNA extracts of about 1 mL volume generated from 5 g of soil. It is typically most convenient to process six soil samples at a time, because this is the capacity of a standard 20 x 25 cm gel mold. Table A.1 lists the materials and Table A.2 describes the reagents used in this procedure. Two modifications of the gel purification method have been developed: the gel well method for soils low in organic matter, and the gel block method for high organic matter soils. Figures A.1 and A.2 show features of the gel purification methods.

**Table A.2.** Reagents for gel purification of DNA

Ethanol (100% and 70%) at 20 °C

TE (10:1) (Tris EDTA; 1x is 10 mM Tris, 1 mM EDTA, pH 8.0) (Sambrook *et al.* 1989)

TE (10:0.1) (Tris EDTA; 1x is 10 mM Tris, 0.1 mM EDTA, pH 8.0)

Phenol: chloroform: isoamyl alcohol (25:24:1) [8]

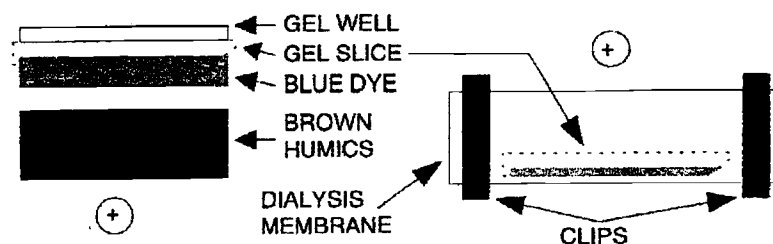
2-butanol (100%, also known as secbutanol)

TAE (TrisAcetateEDTA; 50x stock is 2 M Tris-acetate, 0.05 M EDTA, pH 8.0) (Sambrook *et al.* 1989)

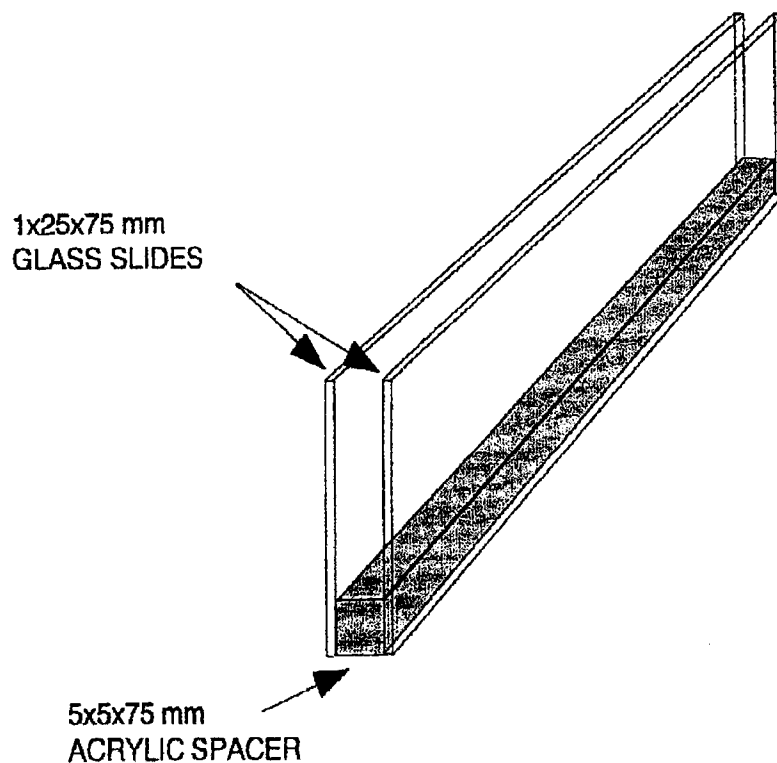
Ammonium acetate (10 M) (Sambrook *et al.* 1989)

Glycerol (100%, autoclaved)

Loading buffer (10x Type II; 0.42% bromophenol blue, 0.42% xylene cyanol FF, 25% Ficoll Type 400) (Sambrook *et al.* 1989)



**Figure A.1.** Schematic representation of the separation of humic materials from DNA during agarose gel electrophoresis and subsequent step of electroelution of DNA from an agarose gel slice. The brown humic materials migrate more quickly towards the anode than the blue dye (xylene cyanol) and DNA. The DNA typically migrates only a short distance out of the well (or gel block) with smaller DNA extending just into the light blue dye band. RNA co-migrates with the humic materials.



**Figure A.2.** Diagram of a gel block mold, which consists of two 1 x 25 x 75 mm glass microscope slides glued to a 5 x 5 x 75 mm acrylic spacer.

## Gel Well Purification

1. Precipitate the crude soil DNA and dissolve the pellet in 800  $\mu\text{L}$  1x TE (10:1). Set tube in 70 °C water bath until the pellet is completely dissolved (15-30 min). Transfer DNA solution to a 1.5-mL microcentrifuge tube using wide-bore pipette tip. Add 50  $\mu\text{L}$  glycerol (100%) and 20  $\mu\text{L}$  10x Loading Buffer, and mix gently by inversion. Spin down drops and store on ice until loaded on gel. *Time: 45 min.*

2. Prepare an agarose gel (1% agarose with 1x TAE buffer) with six, 1-mL capacity wells (see Note 1). After the gel has set, place the gel tray into the electrophoresis device and fill the device with TAE buffer until the buffer just reaches, but does not flow over, the top of the gel. Remove any TAE that has moved into the wells after the well formers have been removed. Carefully load samples into empty wells using a widebore 1-mL pipette tip and mop up any spills with a clean tissue. Run gel at 240 V for 5 min then at 120 V until brown humic material is separated from the blue dye nearest the well and the dye is about 1 cm from the well (about 30 min). Turn off power and collect buffer from the well with a transfer pipette and place in a labeled 15 mL polypropylene tube (store at 4 °C). Cut out gel slice (about 0.81 cm width) including well edge as one side and extending to the blue, xylene cyanol dye front (Fig. A.1). Cut a notch at one end to aid in orienting the gel slice later. *Time: 1h 30 min.*

3. With gloved fingers, drop the gel slice into TAE buffer filled dialysis tubing (15 cm long, clamped at bottom). Pour off excess buffer until tubing looks about half full, squeeze buffer towards the top until all air is removed, then clamp. Place dialysis bag in electrophoresis chamber so that the gel slice is pushed to one side and oriented



the same way it was when run (Fig. A.1). Dialysis bag should be submerged but not floating. Run at 240 V for 1 h, terminals reversed so that the DNA will migrate out of the gel slice and accumulate against dialysis tubing wall. Do not touch or rearrange tubing during this process. Briefly (30 s) reverse leads to detach DNA from dialysis tubing, then turn off current. *Time: 1h 30 min.*

4. Massage the dialysis tubing wall where the DNA has stuck. Using transfer pipette, draw up the buffer and gently rinse the inside of the dialysis membrane. Repeat three times. Collect the buffer from the dialysis tube and pool with the buffer from the gel well. Rinse the gel slice and inside of the dialysis tube with 1 mL of sterile water and pool wash with buffer in the tube. Total volume should be 5 to 8 mL. *Time: 30 min.*

5. Extract with 2-butanol to reduce volume of the DNA solution (see Note 4). Add one volume of 2-butanol, gently invert until emulsion forms (about 20 times), centrifuge (2,000 x g, 1 min), carefully pipette off the butanol (upper) phase, and repeat these butanol extractions until the volume of DNA solution is 1.5 mL. Depending upon the sample, three to six extractions may be needed to reduce a 5 to 8 mL sample to the desired 1.5 mL. *Time: 1 h.*

6. Extract with one volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). Mix gently by inversion until emulsified, and centrifuge (2,000 x g, 5 min). Carefully transfer upper phase to a new 15 mL tube using a widebore pipette tip, being careful to avoid the white material at the interface. Add 1 mL 1x TE (10:1) to the lower layer, mix gently by inversion, and centrifuge (2,000 x g, 5 min) again. Pool the

upper phase with the first and extract with one volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). Mix gently by inversion until emulsified, and centrifuge (2,000 x g, 5 min). Transfer upper phase to a new 15 mL tube and reextract lower phase as described above. Pool upper phases. The volume is now 3 to 4 mL. Reduce volume to about 400  $\mu$ L with 2-butanol as described in step 5 and transfer to a 1.5 mL microcentrifuge tube. Add 0.25 volume (about 100  $\mu$ L) 10 M ammonium acetate and two volumes (about 1.0 mL) ice-cold 100% ethanol, mix gently by inversion and store at 20 °C for 2 h (or at 70 °C for 20 min). Centrifuge (14,000 x g, 30 min, 4 °C). Pour off ethanol and rinse pellet with 70% ethanol. Dry pellet under vacuum at room temperature. Dissolve pellet in 100 to 250  $\mu$ L of 1x TE (10:0.1) or sterile water (Note 5). Store at 20 °C. *Time: 2 to 3 h.*

### **Gel Block Purification**

1. Precipitate the crude soil DNA and dissolve pellet in 800  $\mu$ L 1x TE (10:1). Set tube in 70 °C water bath until completely dissolved (15-30 min). Transfer DNA solution to 5 mL plastic beaker using a wide-bore pipette tip. Add 60  $\mu$ L ethidium bromide (200  $\mu$ g mL<sup>-1</sup>) and 20  $\mu$ L Loading Buffer, and dilute to 1.5 mL with 1x TE (10:1). Gently swirl and return mixture to 70 °C water bath. *Time: 7 h.*

2. Coat the bottom of the gel molds (see Note 3) with 3% agarose (low melting point). When firm, transfer 1.5 mL of the molten (70 °C) 3% agarose solution to each of the samples in the 70 °C water bath. (Do one sample at a time.) Mix the solution with a stirring rod and quickly transfer to the gel mold. Let the slice set in the freezer

(20 °C) for 10 min. Gently push the slice out of the mold using four microscope slides glued together. When all slices are in position in the 20 x 25 cm gel mold, gently cover with 1% agarose (about 500 mL) containing  $0.5 \mu\text{g mL}^{-1}$  ethidium bromide which has been cooled to 50 °C. It is important that the agarose solution is cooled to 50 °C, otherwise humic materials from the gel blocks will diffuse into the gel. Run gel at 240 V for 5 min and then at 120 V until the brown humic material is separated from the light blue dye closest to the original gel slice. Different samples will “clean up” at different rates. View gel under UV illumination and cut out the gel slice containing the fluorescing DNA. In most cases, this slice is the original gel slice that was transferred from the mold. *Time: 2 h.*

3. With gloved fingers, drop gel slice into TAE buffer filled dialysis tubing (15 cm long, clamped at bottom). Pour off excess buffer until tubing looks about half full, squeeze buffer towards the top until all air is removed, then clamp. Place dialysis tubing in the electrophoresis chamber, orienting it so that the 3% bottom coat is pushed to one side of the membrane and facing the H electrode and there is room for elution. Dialysis bag should be submerged but not floating. Run at 240 V for 1 h, terminals reversed. DNA will migrate out of the gel slice and accumulate against dialysis tubing wall. Do not touch or rearrange tubing during this process. Briefly (30 s) reverse leads to detach DNA from dialysis tubing, then turn off current. *Time: 7 h 30 min.*

4. Massage the dialysis tubing wall where the DNA has stuck. Using a transfer pipette, draw up the buffer and gently rinse the inside of the dialysis membrane.

Repeat three times. Collect the buffer from the dialysis tube. Rinse the gel slice and inside of the dialysis tube with 1 mL of sterile water and pool wash with buffer in the tube. Total volume should be 5 to 8 mL. *Time: 30 min.*

5. Extract with 2-butanol to reduce volume of the DNA solution. Add one volume of 2-butanol, gently invert until emulsion forms (about 20 times), centrifuge (2,000 x g, 1 min), carefully pipette off the butanol (upper) phase, and repeat these butanol extractions until the volume of DNA solution is 1.5 mL. Depending upon the sample, three to six extractions may be needed to reduce a 5 to 8 mL sample to the desired 1.5 mL. *Time: 1 h.*

6. Extract with one volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). Mix gently by inversion until emulsified, and centrifuge (2,000 x g, 5 min). Carefully transfer upper phase to a new 15 mL tube using a widebore pipette tip, being careful to avoid the white material at the interface. Add 1 mL 1x TE (10:1) to the lower layer, mix gently by inversion, and centrifuge (2,000 x g, 5 min) again. Pool the upper phase with the first and extract with one volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1). Mix gently by inversion until emulsified, and centrifuge (2,000 x g, 5 min). Transfer upper phase to a new 15 mL tube and reextract lower phase as described above. Pool upper phases. The volume is now 3 to 4 mL. Reduce volume to about 400  $\mu$ L with 2-butanol as described in step 5 and transfer to a 1.5 mL microcentrifuge tube. Add 0.25 volume (about 100  $\mu$ L) 10 M ammonium acetate and

two volumes (about 1.0 mL) ice-cold 100% ethanol, mix gently by inversion and store at 20 °C for 2 h (or at 70 °C for 20 min). Centrifuge (14,000 x g, 30 min, 4 °C). Pour off ethanol and rinse pellet with 70% ethanol. Dry pellet under vacuum at room temperature. Dissolve pellet in 100 to 250  $\mu$ L of 1x TE (10:0.1) or sterile water (see Note 5). Store at -20°C. *Time: 2 to 3 h.*

### **Conclusions**

Gel purification is a gentle procedure that keeps genomic DNA intact and does not noticeably shear the DNA. Thus, DNA of high molecular weight can be obtained. Generally the yield of DNA is quite good, with recoveries approaching 100% in a low organic matter sandy soil (Myrold and Huss-Danell 1994). Somewhat lower yields would be expected for samples high in organic matter. DNA purified by gel electrophoresis has been used successfully for hybridization and PCR amplification and is likely to be suitable for other molecular methods, such as restriction enzyme digests and cloning.

### **Notes**

1. All containers, solutions, and supplies should be sterile and DNase free.
2. For the gel well procedure, 1-mL well-formers can be made from three 1 x 25 x 75 mm microscope slides stacked and glued together with epoxy. Siliconize and boil before using, handle with gloves, tape to support and hold in position during

use. A 20 x 25 cm gel can be used to process six samples, with two wells per row and three rows 8 cm apart.

3. For the gel block procedure, gel block molds can be formed from two 1 x 25 x 75 mm microscope slides glued with epoxy to a 5 mm spacer.
4. Butanol extraction removes water only, salts become concentrated. If EDTA (from TAE buffer) has a final concentration greater than 10 mM, it will co-precipitate with DNA during the subsequent step. The removal of water is not linear; the entire DNA sample can be lost from over-extraction with 2-butanol. This extraction also removes ethidium bromide.
5. DNA should be colorless and free from humic contaminants, however, a slight yellow color usually does not inhibit the PCR reaction following dilution of the DNA sample. If further clean-up is required, precipitate with PEG (polyethylene glycol) as follows: Add an equal volume of PEG/NaCl (20% PEG 8000, 2.5 M NaCl), incubate at 37 °C for 15 min, centrifuge at 10,000 x g for 5 min, pipette off supernatant, wash pellet with 200  $\mu$ L cold 80% ethanol, vacuum dry and dissolve the pellet in 10:0.1 TE.

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