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

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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 inocula. 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-inocula 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. Thus, the observed seedling growth advantage with dual inocula was not attributable to nodule occupancy differences.

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Abbreviations

pcv: packed-cell volume; rcd: root collar diameter; SNK: Student-Newman-Keuls multiple comparison test; PCR: polymerase chain reaction; RFLP: restriction fragment-length polymorphism; CTAB: cetyltrimethylammonium bromide

1. Introduction

Red alder (*Alnus rubra* Bong.) is a common hardwood species in the Pacific Northwest that forms a N_2-fixing symbiosis with the actinomycete *Frankia*. As N_2-fixing trees, alder have several useful applications: they may be used in land reclamation, as an economic crop, and as a reforestation species resistant to laminated root rot. 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 (Hilger et al., 1991). 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.

2. Materials and Methods

Experimental design

A trial of *Frankia* inocula was performed 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^{-2} at 1.35 kg H$_2$O kg^{-1} dry matter), two single-strain inoculum treatments using ArI5 or AvcI1 (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 10^6 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 inorganic N of 25.5 mg-N kg^{-1} NH$_4$$^+$ and 4.67 mg-N kg^{-1} NO$_3$$^-$. The soil was fumigated in the fall with 389 kg ha^{-1} 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, MA 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), 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. The following spring at lifting (late March), 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, *gln*II, was amplified with published primers (Cournoyer and Normand, 1994) and restricted with *Hae*III. 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.

3. Results

Numbers of seedlings did not differ significantly among treatments at mid-season or at harvest. We excavated 3,330 seedlings at harvest and 301 were evaluated. Nodules in the upper 35 mm of soil were considered separately from those in the lower root system 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. For the entire experiment at harvest, there were 1,724 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 seedlings that were significantly smaller than those in inoculated plots (Table 1). ArI5 inoculum produced larger seedlings than AvcI1, although both resulted in nearly complete nodulation. Dual inoculation resulted in larger seedlings than

inoculation with single strains. The ArI5 and dual-inocula treatments resulted in a greater proportion seedlings nodulated than the AvcI1 treatment, though AvcI1 still had much higher nodulation than the control.

In the PCR-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 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 1. Inoculum effects on plant nodulation and size. Values in a column with different letters are significantly different ($\alpha = 0.05$, SNK).

Treatment	Nodulation (%)		Plant height (cm)		Plant dry weight (g)
	Mid-season	Harvest	Mid-season	Harvest	Harvest
Control	1a	12a	2.7a	10.3a	0.8a
AvcI1	78b	99b	5.0b	26.6b	2.2b
ArI5	97c	97b	7.8c	35.3c	3.1c
AvcI1+ArI5	95c	97b	9.2d	38.4c	4.1d

Table 2. Distribution of RFLP patterns among inoculation treatments. Numbers of nodules with a given pattern with percentage of nodules within a treatment given in parentheses.

Treatment	RFLP Pattern				
	AvcI1	ArI5	AvcI1/ArI5	Wild-type	Total
Control		14 (82%)		3 (18%)	17
AvcI1	24 (89%)	1 (4%)	2 (7%)		27
ArI5	1 (4%)	26 (96%)			27
AvcI1+ArI5		36 (100%)			36

4. 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-inocula treatment, the effectiveness of the individual strains may not be the primary factor leading to the enhanced growth with dual inoculation. Indeed, Hahn et al. (1990) found that co-inoculating alder with an effective and a Nif^- 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-inocula effect, the nodules produced under dual inoculation did not show dual occupancy. Our PCR-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 cleaned only 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 3 months after seeding. A more rapid infection process would have given these plants an advantage even before we characterized nodulation at mid-season. The larger seedlings at mid-season with dual inoculation indicate that this may be the case.

Further research on dual inoculation 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 inoculation will result in better growth in other years and locations.

Acknowledgments

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