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INOCULATION OF green alder [*Alnus crispa*] WITH *FRANKIA*-ECTOMYCORRHIZAL FUNGAL INOCULANT UNDER COMMERCIAL NURSERY PRODUCTION CONDITIONS

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ABSTRACT

We examined the feasibility of producing container *Alnus crispa* (Ait.) Pursh (Betulaceae) seedlings (green alder) inoculated with a pure culture of *Frankia* sp., Brunchorst strain AvcI1 and an ectomycorrhizal fungus, *Hebeloma crustuliniforme* (Bull. ex st. Amans) Quél. in a commercial nursery setting. Alders are actinorhizal plants that fix atmospheric nitrogen in a symbiotic association with *Frankia* species and can also form mycorrhizal associations. *Frankia* inoculation significantly increased seedling biomass, number of nodule lobes, nodule weight, and plant nitrogen content of green alder at the end of nursery culture compared with control or "*Hebeloma* only" treatments. Improved seedling growth, root nodulation, and nitrogen nutrition achieved in this study was attributable to *Frankia* inoculation, suggesting *Frankia* inoculation in nursery may be beneficial for the production of superior alder seedlings to use in reclamation work. Actinorhizal plants have the potential to enhance plant establishment on disturbed sites and to improve soil fertility and stability. Seedlings inoculated with *Hebeloma* only or in combination with *Frankia* did not show any visible ectomycorrhizal colonization, suggesting *H. crustuliniforme* may not be compatible with green alder under these experimental conditions. This study demonstrated the suitability of producing large-scale inoculated alder seedlings in commercial nurseries without altering regular nursery operations.

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KEY WORDS

growth and root nodulation, inoculation, seedling production, container nursery, nitrogen fixing

NOMENCLATURE

Plants: USDA NRCS (2007)
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Alders (*Alnus* P. Mill. [Betulaceae]) can grow in ecologically extreme and disturbed sites and have the ability to improve soil fertility and stability (Brunner and others 1990; Hibbs and Cromack 1990; Wheeler and Miller 1990; Yamanaka and others 2002). Alders are actinorhizal plants that fix atmospheric nitrogen in nodules by symbiotic association with actinomycetes of the genus *Frankia*. Many actinorhizal plants are also capable of forming a mycorrhizal association, thereby developing a dual symbiosis (Godbout and Fortin 1983; Miller and others 1991; Gardner and Barrueco 1995; Huss-Danell 1997) and increasing the success of these plants under disturbed soil conditions (Chatarpaul and others 1989; Gardner and Barrueco 1995). The soils of degraded sites are frequently low in available nutrients, mycorrhizal fungi, and other beneficial microorganisms (Cooke and Lefor 1990). The interplanting of nitrogen-fixing alder plants to contribute both carbon and nitrogen to degraded soils is considered a potentially useful technique in soil restoration and land reclamation (Yamanaka and others 2002; Martin and others 2003).

A number of strategies have been used to deal with degraded areas, including selecting low-nutrient–demanding species, using atmospheric nitrogen fixing species in single or mixed cultures, and improving the soils with organic materials (Moffat 2000). In general, there can be a high reduction or elimination of indigenous mycorrhizal, actinorhizal populations and other soil ecosystem components when a site has been highly degraded by mining or other activities (Visser 1985; Malajczuk and others 1994; Moynahan and others 2002; Bois and others 2005). Symbiotic association can be re-introduced via inoculation of nursery seedlings with appropriate symbionts and transplanting inoculated seedlings onto the reclamation sites (Wheeler and others 1991; Lumini and others 1994; Bois and others 2005).

Although actinorhizal plants are excellent candidates for contributing a larger amount of fixed nitrogen to improve soil quality for rapid revegetation and land restoration, these plants are not fully exploited for reclamation of Canadian composite tailings and tailings sand areas. Composite tailings and tailings sands are by-products of oil extraction from the oil sands in Alberta and are highly saline-alkaline in nature, lack important biological components, and are very low in organic matter and nutrients. Therefore, in such difficult situations, early stages of seedling establishment are extremely difficult unless seedlings have already formed microbial-root associations (Berry and Torrey 1985). One way to achieve quality seedlings is to select the right plant species for a particular site and then inoculate the target seedlings with efficient fungal and actinorhizal cultures (Landis and Dumroese 2006).

In many nurseries, production of alder seedlings in both container growth media and nursery soils is subjected to regular fumigation to avoid diseases before growing seedlings (Martin and others 1991, 2003). Such practices often restrict microbial-root associations. Therefore, in order to produce nodulated alder seedlings, inoculation with *Frankia* species is recommended and found beneficial (Périnet and others 1985; Lumini and others 1994). Traditionally, crushed-nodule homogenates were used to nodulate the alder seedlings for experimental purposes or field plantations (Akkermans and Houwers 1979; Monaco and others 1981). Pure culture inoculum was found, however, to be superior to crushed-nodule homogenate in many studies and has several advantages over crushed-nodule inoculants (Périnet and others 1985; Stowers and Smith 1985; Wheeler and others 1991). Basic techniques for growing pure cultures of *Frankia* species and for inoculating alder seedlings have already been reported (Lalonde and Calvert 1979). In Quebec, the feasibility of large-scale production of inoculated alder seedlings using a pure culture of *Frankia* species was demonstrated and used for a land reclamation and revegetation program (Périnet and others 1985). Unfortunately, in Alberta, the feasibility of inoculating alders with selected pure cultures of *Frankia* and the benefits of nursery inoculation for improved alder seedling production under a commercial nursery setting has not been demonstrated. To use alder plants for the revegetation of Canadian oil sands disturbed lands, we need a large-scale production of seedlings and adequate nodulation during seedling growth in nursery with selected strains of *Frankia* species. Nursery managers and owners are reluctant to adopt any inoculation program and hesitate to make any changes in their regular nursery schedules. To be convincing, what is required is to demonstrate the suitability of adopting an inoculation program in a commercial nursery setting and its benefit to nursery and field operations. Our study objective was to test the effectiveness of a pure culture of *Frankia* species combined with an ectomycorrhizal fungal inoculum on the nodulation, growth, and nitrogen nutrition of green alder when reared under an operational nursery production system.

MATERIALS AND METHODS

The Nursery

Alnus crispa (Ait.) Pursh (green alder) seedlings for this research trial were grown in the Bonnyville Forest Nursery, Bonnyville, Alberta (N 54°16.407'; W 110°47.784'), a commercial forest seedling nursery. The nursery greenhouse is made of heavy gauge extruded aluminum and iron frame, and covered with double polyethylene. The polyethylene is long lasting and provides high light transmission for plants with increased safety and insulation value. The greenhouse is equipped with many automated and improved technologies for applying fertilizer and water, and to control temperature, humidity, and pest infestation. The green alder seeds for this

experiment were obtained from local seed collections in the Cold Lake area of Alberta.

Frankia Inoculum Preparation

We used a *Frankia* species (strain AvcI1) that was originally collected from root nodules of *Alnus crispa* located in Atikokan, Ontario (Baker and others 1980). The *Frankia* isolate was cultured in sterile liquid QMod B medium for 6 to 8 wk at 27 °C (81 °F). The stock cultures were washed in phosphate-buffered saline solution and fragmented using a 20-gauge needle and syringe. The *Frankia* liquid culture was produced at the University of Sherbrooke, Quebec, and shipped to Bonnyville Forest Nursery for inoculation. *Frankia* was applied as a liquid suspension to seedlings. The packed cell volume (pcv) was determined by spinning the sample homogenate in microcentrifuge tubes at 14,000 rpm for 10 min. The stock culture was diluted with NaCl solution to achieve a final concentration of 1.5 µl pcv per 5 ml.

Ectomycorrhizal Inoculum

Our ectomycorrhizal inoculum (ECM) was a pure liquid mycelial slurry inoculum of *Hebeloma crustuliniforme*. The standard procedure used to produce pure liquid inoculum involved growing mycelia under aseptic conditions using a modified Melin-Norkans (MNN) liquid medium containing glucose for 4 wk (Marx and Bryan 1975). The fungal culture was homogenized in a Waring blender for 15 to 30 s to produce homogenized mycelial slurry. The mycelial slurry was then resuspended in fresh MNN medium containing 2g/l of glucose and allowed to grow for several days. Before inoculating seedlings, the excess nutrient medium was discarded from the slurry, and the mycelial mass was rinsed with distilled water to remove any excess nutrients. Then the homogenized slurry was mixed with distilled water to achieve a final concentration of about 5 x 10^5 viable propagules/ml inoculum for inoculation.

Seedling Culture and Inoculation

Seedlings were raised in a commercial nursery environment using the regular nursery production system. The seedlings were grown in Styroblock® planting containers of 112 cavities of 80 ml capacity containing non-sterile peat: vermiculite growing media (4:1, v:v). We sowed 5 to 8 seeds directly on the surface of the growing media and covered them with a thin layer of white quartz sand (1.2 to 4.5 mm in size). The containers were misted regularly with nursery water to maintain the substrate moisture level required for germination. Two wks after germination, seedlings were thinned to one per cavity and transplanted to empty cavities, if necessary. Seedlings were not fertilized. Seedlings were watered regularly using the automated systems used for watering and fertilizing nursery crops. Therefore, seedlings received some residual fertilizer remaining in the watering system during regular watering throughout the growing period.

Frankia inoculation was performed when the seedlings had reached the 2-leaf stage using diluted *Frankia* suspension as described above. Each cavity received 5 ml (1.5 µl pcv per plant) of *Frankia* cell suspension, which was directly applied into the peat-vermiculite substrate at the plantlet collar. Four wk after *Frankia* inoculation, seedlings were inoculated with 5 ml of diluted ECM (*Hebeloma crustuliniforme)* inoculum as described above and delivered into the root zone using a disposable pipette. Control seedlings were not inoculated.

Experimental Design and Measurements

A completely randomized block design was used with 4 replicates per block. The treatments were: *Frankia* alone, *Frankia* + *Hebeloma crustuliniforme, Hebeloma* alone, and control. The Styrofoam® blocks were arranged in 4 blocks and the treatments were randomly assigned within each block. All data were subjected to analysis of variance and the significant differences were determined by using the least significant difference (LSD) test.

A portion of seedlings from all treatments were harvested 20 wk after onset of the experiment to determine the seedling growth, nodule formation, ectomycorrhizal formation, and tissue nutrient status. Three seedlings from each experimental unit (a total of 12 seedlings per treatment) were harvested randomly and growth was determined as shoot height and fresh and dry weight of shoot, root, and nodule. Dry weight was determined after oven drying at 70 °C (158 °F) for 48 h. Total nitrogen content in the ground plant samples was determined using the standard procedure described by Kalra (1998).

RESULTS

Seedling Nodule and Ectomycorrhiza Formation

Inoculation of green alder seedlings with *Frankia* AvcI1 in the nursery induced abundant nodule formation in all seedlings as compared with *Hebeloma* alone and the control treatment. Fresh and dry weights of the nodules were significantly greater when inoculated with *Frankia* alone and *Frankia* + *Hebeloma* as compared with the non-inoculated control and those inoculated only with *Hebeloma* (Figure 1). Seedlings that were not inoculated with *Frankia* (for example, control and *Hebeloma* alone treatments) exhibited some degree of nodulation, but the extent was low.

Seedlings inoculated with *Hebeloma* or *Frankia* + *Hebeloma* did not show any visible ectomycorrhizal colonization. Vegetative hyphae of this fungus were not observed within the root system. It was difficult to assess the ECM root colonization due to the compact nature of container-grown alder root systems. It appears, however, that ectomycorrhizal inoculation was not effective in this experiment. Although its impact was not statistically significant, and the ECM inoculation was not effective, the *Frankia* + *Hebeloma* treatment produced the highest amount of root nodules, both on a fresh weight and dry weight basis, compared with all other treatments. Compared with control seedlings, *Frankia* inoculation increased the fresh weight of nodules by 90% and 153% for *Frankia* and *Frankia* + *Hebeloma* treatments, respectively. The number of nodule lobes per plant was 286% and 271% greater with the *Frankia* and *Frankia* + *Hebeloma* treatments, respectively, compared with the control (Figure 1). Similarly, the dry weight of nodules was 138% and 231% greater for the *Frankia* and *Frankia* + *Hebeloma* treatments, respectively, when compared with control seedlings (Figure 1). We observed no difference in nodulation between the *Hebeloma* only and the control. The photo above shows abundant nodule formation with inoculated seedlings due to *Frankia* inoculation.

Abundant root nodules collected from 20-wk-old *Frankia*-inoculated green alder seedling root systems.

Photo by Dr Ali M Quoreshi

Seedling Growth and Nitrogen Nutrition

Frankia inoculation resulted in significantly greater biomass production of green alder than those without *Frankia* inoculation or receiving only ECM inoculation. *Frankia* inoculation on green alder under a commercial nursery condition produced visibly larger and darker green seedlings when compared with the non-inoculated seedlings. Shoot height, root-collar diameter, and plant dry weight were significantly greater when inoculated with *Frankia* or *Frankia* + *Hebeloma* compared with the control and *Hebeloma* alone (Figure 2). Overall, shoot height and root-collar diameter were increased by 23% and 35%, respectively, when inoculated with *Frankia* or *Frankia* + *Hebeloma* as compared with control seedlings,

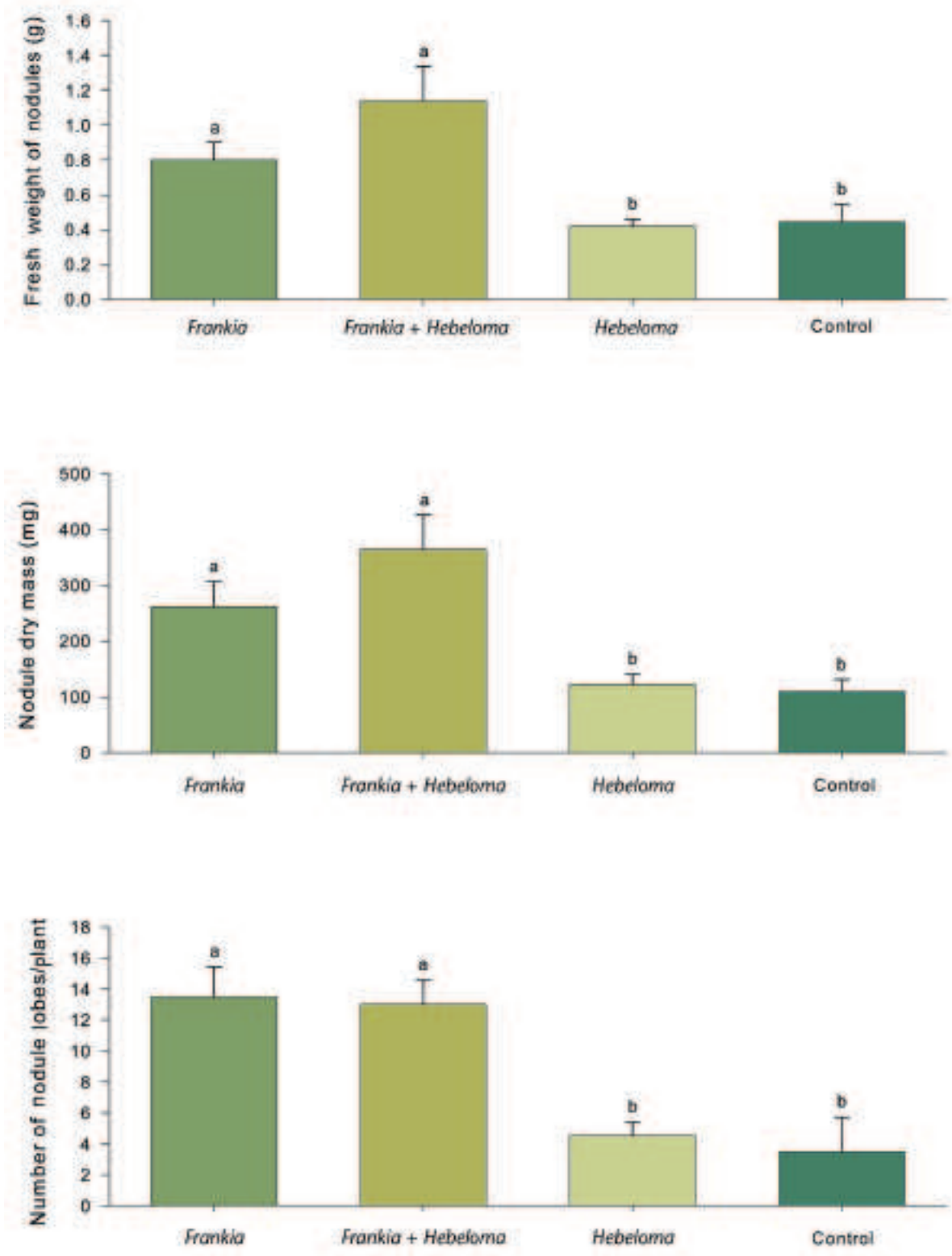

Figure 1. Effects of *Frankia* and *Hebeloma crustuliniforme* inoculation on fresh weight of nodules, nodule dry mass production, and number of root nodule lobes per plant of green alder seedlings grown under commercial nursery conditions for 20 wk. Each value is the average of 12 seedlings (3 seedlings per replication). Error bars represent standard error of the mean. Bars marked with different letters differ significantly according to LSD test at alpha = 0.05.

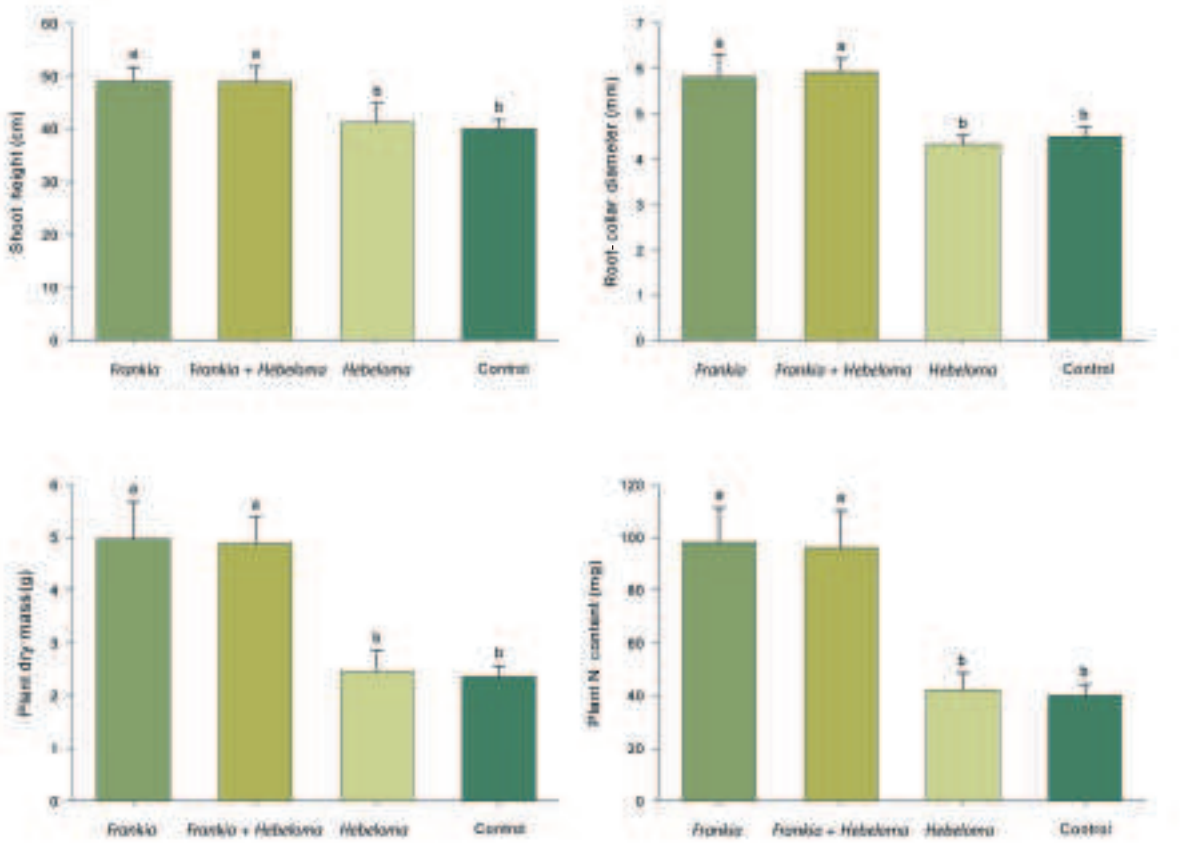

Figure 2. Effects of *Frankia* and *Hebeloma crustuliniforme* inoculation on shoot height, root-collar diameter, plant dry mass, and total plant nitrogen content of green alder grown under commercial nursery conditions for 20 wk. Each value is the average of 12 seedlings (3 seedlings per replication). Error bars represent standard error of the mean. Bars marked with different letters differ significantly according to LSD test at alpha = 0.05.

respectively. Consequently, the dry weight of the whole plant was increased by 110% and 107% for the *Frankia* and *Frankia + Hebeloma* treatments, respectively, compared with control seedlings. The photo on page 278 shows a general view of vigorous growth of alder seedlings inoculated with *Frankia.* At harvest, the total nitrogen (N) content of plants inoculated with *Frankia* or *Frankia + Hebeloma* was significantly higher than non-inoculated plants (Figure 2). Analysis of the total N content showed an increase of 106% and 118% when inoculated with *Frankia* or *Frankia + Hebeloma*, respectively, compared with the control and *Hebeloma* only treatments.

DISCUSSION

Growth, nodulation, and N content of alder seedlings were significantly increased by *Frankia* inoculation. Nodules were mostly located in the upper part of the root system at the root crown, and typically consisted of clusters with multiple lobes. These upper nodulation patterns suggest that nodule formation probably occurred early during seedling growth and thus exerted more of an effect on initial growth of the plant. Nodules on control seedlings occurred inconsistently on seedlings and appeared to be peripherally distributed on the root system. Nodulation of control seedlings probably occurred because of the presence of indigenous *frankiae* in the peat media. The low levels of nodulation of the non-inoculated seedlings observed in this study is representative of nodulation levels generally found on alder seedlings in this nursery at the end of the growing season. Our results show the advantage of inoculation with *Frankia* during seedling culture for improved seedling production.

The effectiveness of inoculation with the ECM fungus was not evident in this study, suggesting that *Hebeloma crustuliniforme* may not be compatible with green alder under these experimental conditions. Unlike other forest tree species, *Alnus* seems to establish associations with only a small number of ectomycorrhizal fungal species, indicating a high degree of specificity (Gardner and Barrueco 1995). Pure culture inoculations of ectomycorrhizas with different species of *Alnus* were tested in various laboratories (Molina 1981; Godbout and Fortin 1983; Brunner and others 1990; Miller and others 1991). Among the several fungal species isolated under various host trees, only a few of these fungi actually formed well-developed symbioses with *Alnus* (Molina 1981; Godbout and Fortin 1983). For exam-

ple, although the fungi *Alpova diplophloeus* and *Paxillus filamentosus* formed both mantle and Hartig net on *Alnus tenuifolia* Nutt. roots, *Hebeloma crustuliniforme* and *Gyrodon lividus* failed to form mantle or Hartig net (Brunner and others 1990). This result is similar to what we obtained in the present study. Miller and others (1991) found that *Alpova diplophloeus* and *Lactaria obscuratus* are the most common mycorrhizas with *Alnus* in Oregon. Several other associations were also reported with *Telephora americana, Cortinarius bibulous, Hebeloma crustuliniforme,* and *Paxillus involutus,* although the mycorrhizas were less developed in the latter 2 species. It is important to understand that results from laboratory synthesis of *Alnus*-mycorrhizal symbioses may not behave the same way under nursery and field conditions. These reports, however, indicate that the interrelationship among the mycorrhizal fungi, *Frankia,* and the higher plant is certainly very complex and highly host specific, and requires a greater understanding of the overall process.

It appears that the ECM-green alder combination in our study may not be sufficiently specific for the establishment of a successful symbiotic association. Reports suggest that ectomycorrhizal formation by *Alpova diplophloeus* improved the growth and phosphorus status of *Alnus rubra* Bong. (red alder) seedlings and emphasized the importance of actinorhizal development in manipulating red alder growth and ectomycorrhizal development (Yamanaka and others 2002). We suggest that *A. diplophloeus,* the best-known ectomycorrhizal fungus associated with the genus *Alnus* (Miller and others 1992; Gardner and Barrueco 1995), or *Russula alinicrispa* associated with green alder (Brunner and others 1992) should be considered for future dual inoculation studies.

The increase in N content observed in all inoculated plants suggests a more efficient symbiotic nitrogen fixation by the inoculated seedlings as compared with controls. These results clearly demonstrate that inoculation of *Alnus crispa* with *Frankia* during nursery seedling production significantly improved seedling growth, nodule formation, and nitrogen nutrition. Well-nodulated plants grew satisfactorily in containers in a commercial nursery environment without requiring a regular nitrogen fertilization schedule to be used by a nursery. Our control seedlings were relatively smaller suggesting need for regular fertilization for their optimum growth and development. It appears that container-grown actinorhizal green alders probably have most of their nitrogen demands met through symbiotic nitrogen fixation and reflected on their growth and nutrition. Our results demonstrate that watering every 2 to 3 d using the regular watering system, which may contain some residual fertilizer, was appropriate for growing alders and was compatible with early inoculation of the plantlets with *Frankia* species. Inoculation of container green alder with pure cultures of *Frankia* is promising under operational nursery production conditions without changing any

regular nursery operational schedules. Our results demonstrated a simple approach for producing more robust alder seedlings in commercial nurseries, which may be used for restoration of various difficult sites. Alder inoculated at the nursery could also be used for short-term biomass production because of their ability to fix atmospheric nitrogen and relatively better growth. Planting inoculated alders alone or in species mixtures has the potential to be a successful establishment vegetation in poor or reclaimed soils (Teissier du Cross and others 1984). Stowers and Smith (1985) suggested production of nodulated actinorhizal plants in the nursery improve planting stock over that of non-nodulated stock that is allowed to nodulate naturally after transplantation. Further research is needed to evaluate the benefit of these inoculated alder seedlings when outplanted in disturbed sites.

Our study has shown improved production of nodulated alder seedlings under a commercial nursery environment using *Frankia* AvcI1. Unfortunately, no commercial sources of inoculum are available in Canada. Crushing nodules and inoculating seedlings at the nursery is not recommended as an appropriate process for improved inoculation because nodulation may not be homogenous and the quality of the strain is unknown. Our ultimate aim is to produce high quality *Frankia* inoculum for nursery people. Symbiotech Research Inc, in collaboration with Sherbrooke University, is actively pursuing the goal of rendering this microorganism available to both industrialist and scientist through the use of state-of-the-art, scalable bioreactor culture techniques. The ultimate goal is large-scale production of *Frankia* species strains possessing specific traits selected for application needs; traits such as high nitrogen-fixing capabilities and tolerance to environmental stresses.

CONCLUSIONS

This study demonstrated that inoculation of container green alder with a pure culture of *Frankia* was feasible under commercial nursery settings without altering regular operational schedules. In this experiment, *A. crispa* inoculated with *Frankia* AvcI1 clearly showed enhanced seedling growth, number of nodule lobes, nodule weight, and nitrogen content. Consequently, *Frankia* inoculation resulted in improved alder seedling production. Our study demonstrated a simple technique for *Frankia* inoculation of container seedlings and production in a commercial nursery environment, which ensures the production of nodulated seedlings. This inoculation technique opens the potential for inoculating alder seedlings with superior strains adapted to the species and the planting site. Nursery managers, foresters, land reclamation experts, and tree planters are often concerned with seedling shoot size when evaluating seedling quality, ignoring one of the most important aspects of quality: root growth and its complement of root symbionts. Based on our nursery results, we suggest that *Frankia* inoculated green alder seedlings with abundant nodule formation are well prepared for revegetation and restoration of difficult sites. Upcoming research includes the study of the development of actinorhizal alders outplanted to Syncrude Canada oil sands residues in the Athabasca region of northern Alberta, Canada.

Frankia-inoculated green alder seedlings growing vigorously without nitrogen fertilization at Bonnyville Forest Nursery, Alberta.

Photo by Dr Ali M Quoreshi

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