Status on Commercial Development of *Burkholderia cepacia* for Biological Control of Fungal Pathogens and Growth Enhancement of Conifer Seedlings for a Global Market

M. S. Reddy[1]

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Abstract-Forestry is an extremely important industry in many countries. With an increasing demand for forest products, many forest companies and government organizations have turned toward more intensive management practices to increase productivity of forest lands. Seedling losses occur in conifer nurseries as well as on reforestation sites despite of the best efforts employed by nurserymen and foresters in disease control and site preparation. Fungal pathogens such as *Fusarium*, *Pythium*, *Rhizoctonia*, *Cylindrocarpon*, *Cylindrocladium* and *Botrytis* are widespread causing seedling losses in nurseries. These pests are also transported to field sites where they continue to cause economic losses by killing, stunting, or deforming transplanted seedlings. One of the most acceptable and environmentally-conscious approaches to solving these problems is the use of a naturally occurring microbial inoculant. We have assessed a microbial culture collection of approximately 500 strains of diverse origin for biological control of fungal pathogens and/or plant growth promotion of various types of conifer spp. under laboratory and greenhouse conditions. Variable results were obtained for most of the strains tested, except for one isolate which is a Burkholderia cepacia, strain Ral-3. For further product development, a proprietary liquid formulation was developed and used in product efficacy trials as a seed or root dip treatment on several conifer species at several locations in western Canada and the Pacific Northwest, USA. Storage stability of strain Ral-3 in commercial packages was maintained, with a viable population of about log 8-9 cfu/ml for over a year when stored at 5-201C. In most trials, strain Ral-3 showed significant suppressive effects on various soilborne fungal pathogens. Significant growth responses including survival, root and shoot biomass, height and caliper were observed. Strain Ral-3 is compatible with many seed treatment fungicides and with other cultural practices currently used in the forestry industry. Strain Ral-3 is also an active and aggressive rhizosphere colonizer of many conifer spp., such as white spruce, Douglas-fir, jack pine, Scots pine, cedar, and western hemlock. Possible mode of action and other data related to regulatory requirements will be discussed.

INTRODUCTION

Company Background Agrium Inc., is one of North America's largest integrated and diversified fertilizer companies. The Corporation is a major producer of nitrogen-based fertilizers and potash, and a leading marketer of the four primary nutrients vital to plant growth: nitrogen, phosphorus, potassium, and sulphur. It produces nitrogen fertilizers at four locations, two in Alberta, one in Texas and one in Nebraska; and potash at its mine and mill in Saskatchewan. Its net annual fertilizer production capacity is 2.9 million tons available for sale.

The company also operates a wholesale distribution, storage and marketing network in North America. At the wholesale level, the Corporation's fertilizers are purchased by a geographically-diverse group of approximately 1,500 customers in North America. Approximately one quarter of its potash production is sold offshore.

At the retail level, its subsidiaries Western Farm Services, Inc., (WFS) and Crop Production Services, Inc., (CPS) serve retail markets with a network of more than 200 outlets in the major agricultural areas of the western, upper midwestern and eastern United States.

Agrium Biologicals, a specialized group within the New Products R & D arm of Agrium Inc., is committed to the development and commercialization of microbial inoculants for use in agriculture and forestry as a method to increase the efficiency of fertilizer uptake and use, and to enable biological control of various crop diseases. This R & D unit is a natural extension to the fertilizer products and services offered by Agrium Inc., positioning the company for growth, innovation and profitability through the introduction and sale of leading edge, and environmentally responsible, new products and services.

Agrium Biologicals specializes in symbiotic, and non-symbiotic Plant Growth-Promoting Rhizobacteria (PGPR) and Biological Control Agents (BCA). Research is directed toward microbial inoculants able to directly promote plant growth and development through increased nutrient uptake, increased nutrient availability and suppression of plant pathogens.

Through the R & D efforts of the Biologicals group we have 3 successful biological products in the market. They are Rhizobium inoculants for legumes and are being marketed through Cargill in Canada and through WFS, CPS and Wilbur Ellis in the United States as RhizUD™ for Peas, Lentils and Beans.

Forestry

Forestry is an extremely important industry in many countries, including Canada (Reddy *et al. 1993*). With an increasing demand for forest products, foresters have turned toward more intensive management practices to increase productivity of forest lands. These methods include the breeding of forest trees with increased growth and superior wood characteristics, artificial regeneration of seedlings, control of competing vegetation, thinning stands to reduce competition between trees, fertilization to stimulate growth, and improved methods for harvesting and utilization of wood. Most of these practices are well understood and are used in modern forestry. Perhaps the most effective way of increasing productivity is the use of genetically improved material as planting stock. Unfortunately, this material is usually in short supply.

Over seven hundred million conifer seedlings are planted annually in Canada. It is imperative that seedling quality be at a high level to allow for successful reforestation of harvested land. Seedling losses occur in conifer nurseries as well as on reforestation sites. Fungal pathogens inciting seed and/or root diseases in nurseries include *Fusarium*, *Cylindrocarpon*, *Cylindrocladium*, *Pythium*, *Botrytis* and *Rhizoctonia*. Diseases caused by these pathogens may be important limiting factors in production of high quality seedlings in forest and conservation nurseries. All nurseries experience some losses to damping-off and root rot diseases, despite the best efforts at control. These losses may occur in several forms, the most obvious being dead and dying seedlings observed ill nursery beds. The economic loss represented by this type of seedling mortality may vary with the age of the affected seedlings. Diseases may also damage seed lings, making them unsuitable for planting. Damaged seedlings are thrown away (culled) during lifting and packaging. Some diseased seedlings may escape culling or remain symptomless at the time of lifting. In these cases, pests are transported to field plantings, where they continue to cause economic losses by killing, stunting, or deforming transplanted seedlings.

Chemical pesticides were initially formulated for effectiveness on many soil-borne pathogens. This broad spectrum efficacy often resulted in destruction of both beneficial and injurious organisms (Baker and Cook 1974). However, resistance to these chemicals can develop rapidly in pathogens. In recent years, problems with pesticide resistance, toxicity to non-target organisms, and environmental contamination have greatly reduced the desirability of chemical fungicides (Campbell 1989). Recent public and government involvement in banning chemicals used in agriculture and forestry will undoubtedly make the use of chemical fungicides difficult at best. Therefore, foresters and nursery managers need to exam me all alternatives for controlling fungal diseases.

Losses in forest productivity include poor seedling establishment and survival on reforestation sites due to factors other than disease. For example, root growth of transplanted seedlings is often limited, contributing to poor seedling health. Root system morphology is a major determinant of seedling success in the field. The goal of bareroot nursery managers is to produce high quality seedlings which can tolerate lifting, handling, and planting processes, and not only survive but grow competitively in the field. This goal is a challenging one to attain. No two nurseries are alike and within nursery variation in soil and microclimate may be as great as that among nurseries. Seedling grading has been controversial because no scientifically based procedure has been developed for identifying which seedlings in a nursery will be the most competitive in the field. The economic impact due to seedling losses on reforestation sites, regardless of the cause, is substantial since the approximate cost to plant a single seedling is $1.00. In addition, it often takes more than 5 years for seedlings to reach the "free to grow" stage. A major problem associated with this in white spruce is "growth check" which refers to the lack of growth in a seedling once it is planted in a reforestation site. In conifer nurseries, losses resulting from diseases and culling can be 15-25% in some years. This represents an annual loss of up to $45 million. Moreover, at reforestation sites poor seedling survival and establishment can result in an annual loss of $290 million.

Foresters and nursery managers need to examine all alternatives for controlling fungal diseases and reducing losses. One of the most acceptable approaches to disease control is the use of naturally occurring microbial inoculants to reduce or suppress the activity of fungal pathogens (Reddy 1991). Cook and Baker (1983) defined biological control as "the reduction or suppression of pathogen inoculum or its disease producing capacity by the action of one or more organisms, other than humans." There is a great potential to utilize beneficial microorganisms to reduce losses both in conifer nurseries and on conifer reforestation sites. The importance of microorganisms such as mycorrhizae for biocontrol of conifer seedling diseases and improving seedling growth is well established (Kropp and Langlois 1990; Harley and Smith 1983). Conifer seedling growth can also be stimulated by inoculating with strains of naturally occurring soil bacteria (Reddy et al. 1993; 1994; Chanway et al. 1991).

The rhizobacteria that exert beneficial effects on plant development are called plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). To date, most PGPR strains for which the mode of action has been investigated appear to enhance plant growth indirectly by reducing populations of deleterious microorganisms (Kloepper 1993). Direct growth promotion occurs when rhizobacteria produce metabolites (*i.e.* plant growth regulators) that directly promote plant growth without interacting with native microflora (Kloepper *el al.* 1989; 199 1; Lifshitz *et al*. 1987). Some direct acting PGPR strains can induce alterations in plant physiology and these changes may include increasing the host plant's defences to pathogen attack (Vanpeer el al. 199 1; Wei el al. 199 1). Disease reduction by PGPR may also occur as a result of competition, antagonism or parasitism. Weller and Cook (1983; 1986) showed that a *Pseudornonas* species isolated from *Fusarium* suppressive soils controlled take-all disease of wheat in greenhouse and field trials. They showed that the antagonism was due to the production of phenazine antibiotics (Brisbane *el al.* 1987). Howie and Suslow (1991) showed that a fluorescent pseudomonad produced antibiotics that Suppressed P. ultimum In cotton rhizospheres, decreasing disease incidence by 70% and increasing seedling emergence by 50%. Some strains that increased yields produced siderophores that bind Fe(III), making it less available to certain members of native microflora (Kloepper *el al.* 1980). Hydrocyanic acid (HCN) is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens (Schippers 1988). Biological control can also be achieved by the competition of rhizobacteria with other rhizosphere organisms for infection sites and siderophores. Pseudomonads that catabolize diverse nutrients and have fast regeneration times in the root zone are often suitable candidates for biological control by competition, especially against slower growing pathogenic bacteria (Weller 1985).

Many diverse groups of bacteria commonly inhabit nursery soil. Several of these species are antagonistic toward common soil-borne pathogens (Reddy and Rahe 1989; Reddy 1991; Reddy *et al* 1991; 1992; 1993; 1994). In our R & D program beneficial microorganisms specifically selected for forestry are being evaluated specifically for:

1. Suppression of damping-off and root rot pathogens of conifer seedlings.

2. Enhancement of conifer seedling germination, growth and survival.

Over the past several years of research using many bacterial isolates we have successfully identified a potential bacterial isolate for further product development and commercialization. We are pleased to present in this paper some of the product development related experimental results.

GENERAL USE RECOMMENDATIONS OF STRAIN RAL-3

Agrium's microbial inoculant contains naturally occurring, nonphytotoxic, nonpathogenic soil bacteria *Burkholderia cepacia* strain Ral-3. This strain was isolated from the root nodules of a soybean plant (cv. Braxton) grown in sandy loam soil at the E. V. Smith Experimental Station research site near Shorter, Alabama, USA. To maintain purity, cultures of strain Ral-3 are stored in a Kelvinator freezer at 80^{o}C in tryptic soy broth amended with 20% glycerol. Ral-3 has been identified in Agrium's laboratory by determining the Analytical Profile Index (API) 20 NE, OXI/ FERM TUBE and ampicillin sensitivity. Strain Ral-3 has also been identified in two other laboratories using fatty acid analysis. Base on these tests, strain Ral-3 was identified as *Burkholderia cepacia*.

Strain Ral-3 is commercially produced under fermentation conditions and is available in a liquid formulation. The concentration of the bacterium is approximately 109 viable cells per ml. Liquid inoculant is packaged in sterile 3L plastic bags which are placed in cardboard boxes for long-term survivability and ease of shipping. The shelf-life stability of this inoculant is at least a year when stored at temperatures of 30^{o}C (86 F) or less. Short-term exposure to 40^{o}C or below freezing temperatures does not have any adverse effects on the shelf-life stability of the inoculant.

The active component is antagonistic to several plant pathogenic fungi such as *Pythium, Fusarium, Cylindrocarpon, Botrytis,* and *Rhizoctonia,* thereby aiding in suppression of infection by these damping-off and root rot pathogens. This product can be used with most other silvicultural practices. The types and degree of responses observed may vary depending on environmental factors and management practices. Best results are achieved if the product is used according to the instructions provided on the label.

Use Instructions

This product can be easily applied to conifer seeds and seedlings in several ways.

Seed treatment

A volume of 300 ml of this product will treat 1 kg of conifer seed. Weigh the seed intended to be treated first. Place the seed in a plastic bag. Apply the product to the seed using the indicated volume and seal the bag. Shake the bag by hand until the surface of the seeds are evenly coated or moistened. Air dry the treated seed for 5 min. Treated seed must be planted within 5 days of inoculation. Store inoculated seed in a cool place (5 to 10^{o}C) away from heat and stress if not planting on the day of treatment. Planting on the day of treatment is recommended.

Fungicides

This product is compatible with Vitaflo-250, Captan, Thiram, Benlate, Baytan, Crown and Rovral. These fungicides may be applied to seed before inoculation with the product. Fungicide treated seed must be allowed to dry before treating with the product. Destroy unplanted treated seed in accordance with applicable municipal, provincial and federal statutes and guidelines.

Use a rate of **300 ml per kg seed** for the following conifer types:

White Spruce	Jack pine
Black Spruce	Scots Pine
Engelmann Spruce	White Pine
Douglas Fir	Slash pine
Loblolly pine	Longleaf pine

Seedling treatment

Seedlings can be inoculated with the product using one of several methods.

Boom irrigation for containered seedlings
If seedlings have not been lifted from growth containers, boom irrigation/injection system is the preferred method of inoculation. Inject the product into the boom irrigation system at a ratio of 1:100 (10 ml inoculant per 1000 ml water). Agitate the product continuously during injection to prevent clumping or settling of bacterial cells. Irrigate the seedlings until the plugs in the containers are completely saturated (i.e. dripping from bottom of blocks).

Portable sprayer for containered or bareroot seedlings
If a boom irrigation/injection system is not available, the product can be applied through a portable or backpack sprayer. Triple rinse the sprayer tank with water before use. Dilute the product at a ratio of 1:100 (1 ml in 100 ml of water) and fill-up the tank up to a desired volume. Spray onto seedlings until evenly irrigated 3-4 days before lifting. This method is applicable for both containered and bareroot seedlings.

Seedling dip for containered or bareroot seedlings
The product can also be applied directly to seedlings by the root dip method. Dip the bareroot seedling Plugs in a suspension after diluted to 1:10 in tap water (100 ml inoculant plus 900 ml water) and container stock in a suspension diluted to 1:100 (10 rnl inoculant plus 990 ml water) for a few seconds. This method is applicable either in the nursery or at tile plantation site.

Timing of application
Apply the product to seedling plugs preferably 1-3 days before lifting. Do not apply chemical pesticides to seedlings at the same time as the inoculant. The recommended time interval after pesticide treatment and before inoculation is 48 h. Inoculated seedlings can be lifted according normal to nursery practices. Treated seedlings can be planted or stored for winter hardiness

Product requirements for application to seedling plugs
Product uptake by the seedlings will vary depending upon seedling type (bareroot or container), container size, and moisture status of seedling plug at the time of application. For containered seedling stock use at the rate of 10-15 L of the product diluted 1:100 in water to treat approximately 100,000 seedlings. In case of bareroot seedling stock use at the rate of 10-15 L diluted 1:10 in water to treat approximately 100,000 seedlings.

Frequency of application
Only once at time of seeding, or only once at time of lifting or at time of planting of the seedlings.

The purpose of this report is to draw the attention of forest managers and researchers to the potential commercial value of incorporating microbials as seed or root inoculants to increase productivity in intensive forestry programs. Selection of this bacteria was based upon availability, ease of manipulation, wide geographic and host range, and demonstrated benefits to a wide variety of host trees.

Agrium Biological's team of research scientists and fermentation and formulation specialists work from a facility located in Saskatoon, Saskatchewan, Canada. Research on microbial Inoculants for conifers has been ongoing for the last six years to identify microorganisms capable of improving seedling field performance and promoting seedling emergence and growth in the nursery. Research trials conducted on reforestation sites have shown that seedlings treated with our microbial inoculant survive better and have improved root and shoot growth compared to untreated seedlings. Similar trials in commercial seedling nurseries have revealed that inoculants applied as a seed treatment can promote seedling emergence and enhance shoot and root growth.

COMMERCIAL BENEFITS

The shelf-life stability of strain Ral-3 maintained an acceptable level irrespective of its storage at various temperature regimes. The strain had initial populations of log 9 to 10 cfu/mi. After 12 months of storage at 5^{o}C, Ral-3 maintained a population of log 8 to 9 cfu/ mi. The antagonistic activity of strain Ral-3 retrieved from the packages after storage at the various temperatures was tested in vitro using a dual plate technique against *F. solani, R. solani*, and *C. destructans*. Strain Ral-3 significantly suppressed the radial growth of the fungi tested irrespective of its storage regimes.

Enumeration of a rifampicin marked strain of Ral-3 on various conifer seeds stored at PC showed that its populations were maintained at about log 4-5 cfu/seed for the entire sampling period, except on black spruce (Figure 1), when tested as seed treatment at a rate of 0.3 mi per gram of seed with a cell suspension of log 910 cfu/ml in a commercial liquid formulation.

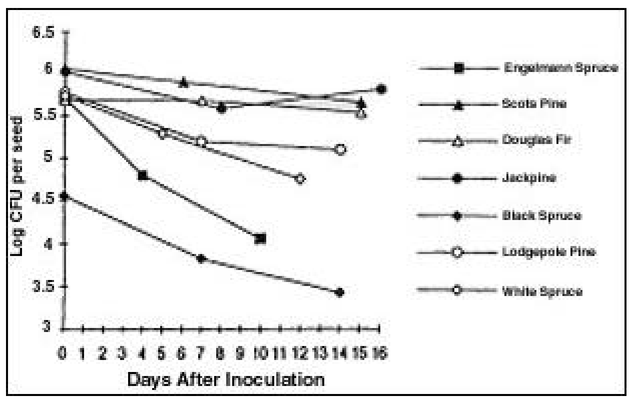

Figure 1. Shelf-life of strain Ral-3 on various conifer seeds.

Strain Ral-3 survived very well on white spruce seedling plugs when applied as a seedling dip at a rate of 6-8 ml/ plug and stored under commercial storage conditions (2 to 3^{o}C) for winter hardiness before being planted on reforestation sites (Figure 2). Figures 3 and 4 illustrate the colonization potential of strain Ral-3 on growing seedling rhizospheres when introduced either as a seed or seeding treatment on various conifer species.

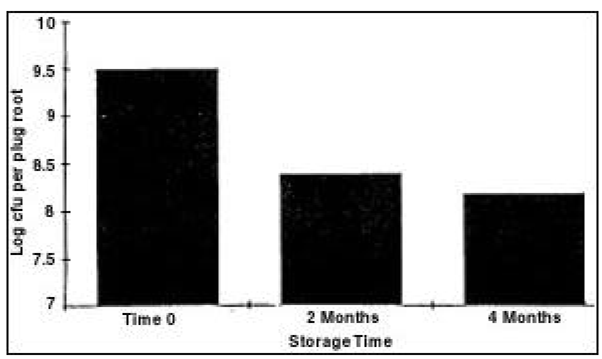

Figure 2. Shelf-life of strain Ral-3 on White Spruce seedling plugs stored under commercial conditions.

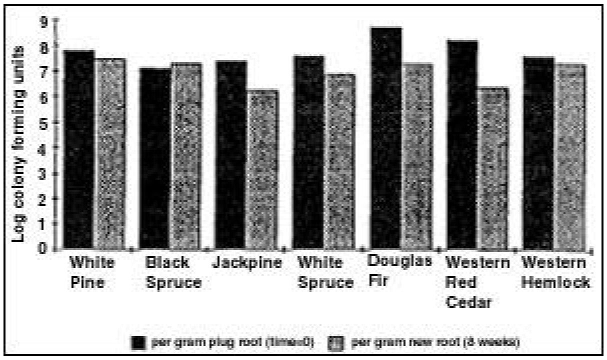

Figure 3. Colonization potential of Ral-3 on various conifer seedling rhizospheres under greenhouse conditions when applied as a seedling plug treatment.

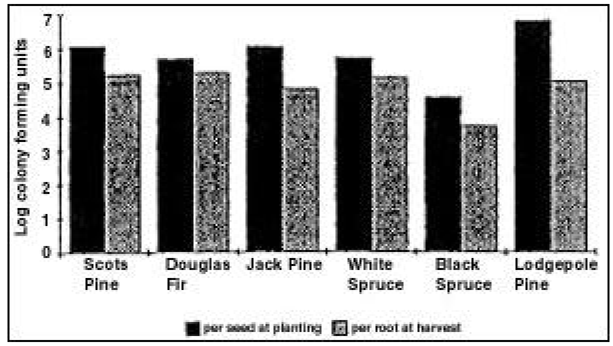

Figure 4. Colonization potential of Ral-3 on various conifer seedling rhizospheres when applied as a seed treatment.

When applied as a seed treatment strain Ral-3 reduced disease incidence caused by F oxysporum on Douglas-fir seedlings and improved healthy stand compared to the non-treated control (Figure 5). Similarly, strain Ral-3 reduced disease incidence and improved emergence of white spruce and jack pine seedlings (Figure 6). In many cases this strain also minimized symptom expression on roots infected with many fungal pathogens. As shown, (Figure 7) Ral-3 significantly reduced the fungal contaminants of western larch seed when cultured oil filter paper.

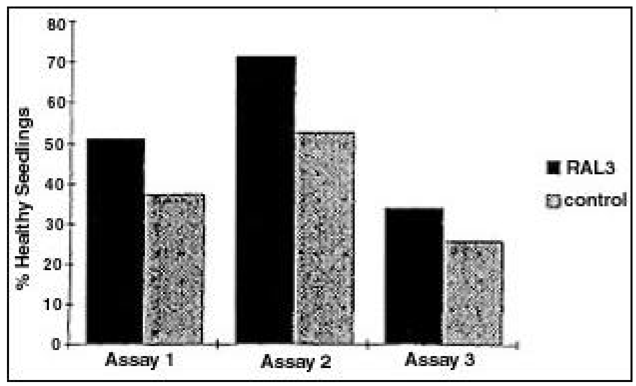

Figure 5. Healthy stand of Douglas-Fir seedlings grown in soil mix artificially infested with Fusarium oxysporum. Asterisks denote significant increases compared to control ($p \leq 0.05$).

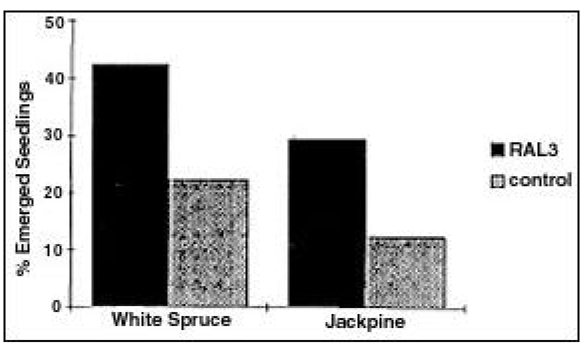

Figure 6. Influence of Ral-3 on the emergence of conifer seedlings grown in soil mix artificially infested with Fusarium oxysporum.

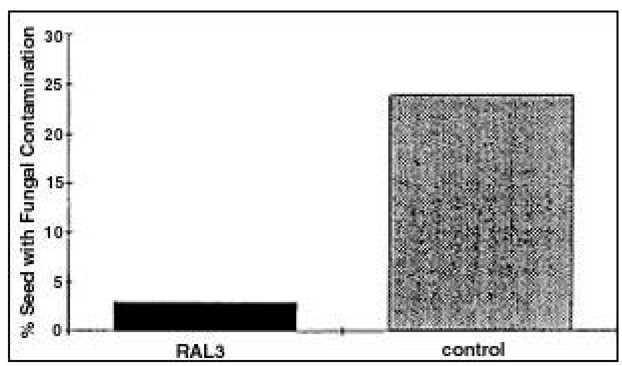

Figure 7. Reduction of fungal contamination on Western Larch seed. Asterisks denote significant difference compared to control to control (**$p \leq 0.05$).

Seedling emergence was assessed in several greenhouse nurseries for containered and bareroot seedlings. Strain Ral-3 increased the emergence in most of the cases compared to untreated control. Also the root rot symptoms, root plug quality, height and root collar diameter were evaluated at the end of the growing season. Strain Ral-3 had a significant influence on these parameters. These results suggests that the bacteria may be decreasing symptom expression by reducing infection or colonization of roots by pathogens.

Plant growth promotion field trials were conducted at several locations across Canada and the Pacific Northwest of United States on several conifer species using Ral-3 as a seedling plug treatment. Results of growth parameters measured such as root and total shoot biomass were significantly increased compared to untreated controls in most of the experiments conducted. For example, as shown in Figure 8, strain Ral-3 significantly increased survival of white spruce bareroot seedlings on a reforestation site in Saskatchewan by 19 to 23% when compared to nontreated seedlings. In addition, Ral-3 increased new shoot biomass of white spruce seedlings planted on reforestation sites (Figure 9).

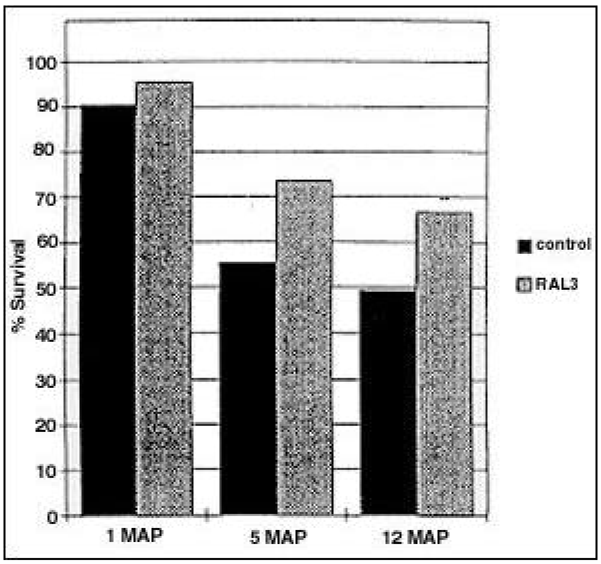

Figure 8. Influence of Ral-3 on survival of bareroot White Spruce seedlings on a reforestation site. Asterisks denote significant increases compared to control (*p≤0.10, **p≤0.05).

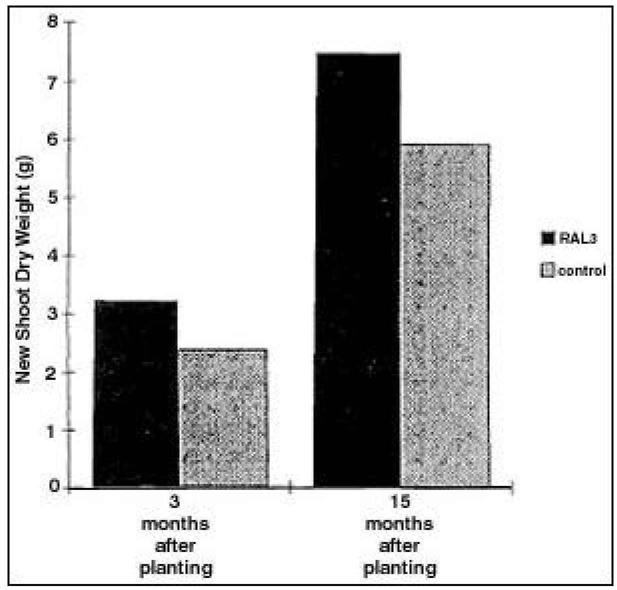

Figure 9. Influence of Ral-3 on White Spruce seedling new shoot growth on a reforestation site. Asterisks denote significant increases compared to control (p≤0.05).

Due to space constraints we are unable to discuss other product developmental activities such as scale-up of the product in commercial formulation, optimizing the delivery system either as a seed or seedling application, packaging of the product, storage conditions for the product etc.

DISCUSSION

Out of approximately 500 bacterial strains, Ral-3 has been selected for the ability to suppress *Fusarium, Rhizoctonia, Cylindrocarpon* and *Pythium* diseases and promote seedling growth . Inoculation of the strain onto Douglas-fir seed reduced the incidence of disease caused by these common fungal pathogens and increased the number of healthy seedlings in a commercial nursery. In tests at replant sites, root-dip inoculation with the strain increased new root dry weight, total plant biomass, and survivability of transplanted seedlings. Seedlings with more roots generally have increased incremental height and diameter growth and it is these seedlings that establish most successfully after transplant.

In natural environments, growth and yield of plants depends on the quantity and balance of water, minor nutrients, air, light, and heat, but are also subject to positive and negative influences of various rhizosphere microorganisms. Both direct and indirect mechanisms have been suggested to explain the positive influence of certain bacteria on plant growth. Hypothesized direct mechanisms are that bacteria elaborate substances that stimulate plant growth, such as nitrogen, plant growth hormones and compounds that promote the availability of phosphates in the root zone. A popular hypothesis for an indirect mechanism is that populations of various pathogenic and deleterious microorganism that affect the root system are reduced by the introduction of a beneficial organism via seed or root inoculation. Each of these hypotheses suffers from insufficient supportive data. Direct information about the activities and interactions of microorganisms in natural soil and plant root environments is technically difficult to obtain due to the complexity and variability of these environments. Regardless of the mechanisms of biological control or growth promotion, our results have implications for management within the forest industry. Seed inoculation with bacteria capable of stimulating emergence would have obvious benefits in reducing costs associated with poor seedling emergence in commercial nurseries. The inoculants may also be useful for the production of seedlings with higher root to shoot ratios. Our results are consistent with other studies that have shown new root growth to be extremely important in the establishment of outplanted conifer seedlings.

There are many opportunities for the application of microbial inoculants in forestry, but gaps remain in our knowledge of how factors such as soil type, soil moisture, soil pH, and silvicultural techniques affect interactions between microbial inoculants and plant roots. There is also a great deal to be learned about the interaction of microbial inoculants with mycorrhizae and other so] I biota. As we learn more about the ecology of these microflora, we may be able to establish the critical processes and specific roles performed by different microbes in maintaining sustainable forests.

The results obtained from this and other studies demonstrate that microbial inoculants can be used operationally in container and bareroot nurseries to significantly improve seedling quality. Our reforestation trials have shown that survival and establishment of seedlings can be significantly improved through treatment with bacterial inoculants. The cost of inoculating seed or seedlings with these microbes represents only a minor portion of the total tree planting expense and high seedling quality is an obvious key to successful reforestation. The technology developed through this pioneering project is being expanded to other host species, forest applications and geographic locations. Our goal is to make this technology available to nursery managers, foresters, Christmas tree growers, and other land managers for use in a sustainable forest management system.

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[1]*Agrium Biologicals, 402-15 Innovation Blvd., Saskatoon, Saskatchewan, CANADA, S7N 2X8; Tel: 306/975-3843; Fax: 306/975-3750; Email: mreddy@innovplace.saskatoon.sk.ca.*

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