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Nutritional Preconditioning and Ectomycorrhizal Formation of *Picea mariana* (Mill.) B.S.P. Seedlings

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Abstract

Ectomycorrhizal inoculated seedlings may improve forest plantation establishment by enhancing growth and nutrition of newly planted seedlings, but successful nursery colonization of planting stock is often incompatible with conventional fertilization practices because of toxic inhibitive effects. A new cultural technique for seedlings is examined, called "mycorrhizal nutrient loading" that integrates exponential high dose fertilization with ectomycorrhizal association without causing serious inhibition. Containerized black spruce (*Picea mariana* (Mill.) B.S.P.) were inoculated at sowing with *Hebeloma crustuliniforme* or *Laccaria bicolor* and fertilized with a complete nutrient solution conventionally at 12.5 mg N/plant and exponentially at 12.5, 25 or 50 mg N/plant representing conventional, loading and high loading application rates, respectively. At the end of nursery culture, exponential fertilization significantly stimulated ectomycorrhizal formation at higher rates (49-85%) than those of conventionally fertilized (22-26%) seedlings. Improved ectomycorrhizal colonization, even at high loading rates, was attributed to low initial nutrient additions and lower electrical conductivity levels maintained in the growing media under exponential fertilization. The gradual increase in nutrient delivery enabled the fungi to develop tolerance to high fertilizer inputs. Nutrient loading significantly increased N (51-135%), P (29-45%), and K (13-47 %) uptake of seedlings, reflecting progressive luxury consumption of nutrients. Mycorrhizal inoculation further elevated loading efficiency by stimulating plant nutrient uptake of N (9-20%), P (7-12%), and K (4-18%), demonstrating accumulation was more efficient with fungal colonization. Vector nutrient diagnosis revealed marked nutrient dilution under conventional fertilization, but induced steady-state nutrition under exponential fertilization that benefited sustained symbiosis. When outplanted on bioassays retrieved from two contrasting boreal forest (Feathermoss and Hardwood-Alnus) sites in northern Ontario, mycorrhizal nutrient-loaded seedlings outperformed conventional seedlings by enhancing dry matter production (45-92%), and increasing N (80-124%), P (89-129%), and K (72-106%) assimilation. The new growth drew on greater internal nutrient reserves built up by both exponential fertilization and mycorrhizal colonization in the nursery. Results were integrated into conceptual models demonstrating interactions of fundamental processes involved in mycorrhizal nutrient loading of trees and their resource utilization after planting in relation to different nutritional regimes. Given the current concern about adequate forest regeneration in Canada, nutrient loading in combination with mycorrhizal inoculation practices provides a potentially effective tool to improve early plantation establishment on boreal sites.

Keywords: Black Spruce, ectomycorrhiza, mycorrhizal nutrient loading, exponential fertilization, seedling nutrition

Chapter 1

GENERAL INTRODUCTION

Intensive silviculture is becoming increasingly important in the current world situation of shrinking forest resources and expanding human population. Forest managers are constantly looking for lower cost, and more convenient management strategies for successful forest regeneration. In Ontario, black spruce (*Picea mariana* (Mill.) B.S.P.) is one of the most abundant and important commercial tree species of the boreal forests (Carleton and MacLellan 1994), and is extensively planted for artificial regeneration. However, regeneration of harvested land is often difficult due to severe competition for resources between young black spruce seedlings and the natural vegetation. In addition, herbicide use as a traditional vegetation control method has been restricted due to strong public concern and environmental issues (Environics Research Group 1989, Wagner 1993). Consequently, seedling growth and survival on competitive sites is often low after planting (Margolis and Brand 1990). This situation has become more complicated by the recent trend of using only containerized seedlings for reforestation programs in Ontario. Container-grown seedlings are usually less competitive in the field due to their smaller size,

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restricted root system, and insufficient nutrient preconditioning (van den Driessche 1991, Timmer 1997). Relatively limited root contact of newly planted containerized seedlings with the soil may initially create water and nutrient stress, this being further complicated under competitive conditions, thus plantations may often fail (Nilsson *et al.* 1996). Furthermore, these seedlings may lack an important biological component — the mycorrhizal association — which increases seedling survival and growth by enhancing uptake of nutrients and water, extending root life, and protecting against pathogens (Harley and Smith 1983). There is, therefore, increasing pressure for high quality planting stock to improve growth and establishment of planted seedlings at various forest sites.

The production of superior planting stock is recognized by nursery managers as a high priority in forest regeneration programs. Peat moss or peat-vermiculite is commonly used as a substrate for containerized seedling production and fertilization is required for optimal seedling growth; it is regarded as an essential practice in forest nurseries. In Ontario, operational nurseries commonly follow conventional fertilization regimes, where fertilizers are delivered periodically to seedlings in equal amounts during the growing season. Growth of conventionally fertilized seedlings normally increases as the season progresses, but internal nutrient concentrations usually decline due to growth dilution. Steady-state nutrition, on the other hand, is characterized by dilution free, stable nutrient concentrations during exponential growth, a concept introduced by Ingestad *et al.* (1986). Steady-state nutritional conditions can be achieved by applying fertilizer in exponentially increasing amounts rather than conventionally.

Considerable effort has been made to increase planting success by physiological preconditioning of nursery seedlings (Dureya and Brown 1984). Successful production of superior planting stock is highly dependent on the fertilization regimes and nutrient levels used for seedling production (Timmer 1997). Efficient plantation establishment can be achieved through a number of independent strategies, including judicious harvesting and site-preparation practices; use of high quality physiologically preconditioned seedlings; control of natural vegetation competition; and pest and disease control (Nambiar 1990). Successful regeneration of black spruce may be accomplished by improving the pre-plant nutrient status of seedlings by building up internal nutrient reserves which increases competitiveness of seedlings over naturally occurring vegetation (Malik and Timmer 1996, Timmer 1997, Imo and Timmer 1998). Such nutrient preconditioning in the nursery is environmentally safe and cost effective because it can reduce reliance on extensive herbicide use for vegetation management, and post planting field fertilization. Effective nutrient preconditioning in the nursery, such as exponential nutrient loading, has been demonstrated with black and white spruce reared under steady-state nutrient cultures without building up soluble salt toxicity in the substrate (Timmer *et al.* 1991, Timmer 1997, McAlister and Timmer 1998), indicating potential for combining mycorrhizal inoculation in the seedling culture.

Traditional, intensive fertilizer and fungicide use in nursery stock production may inhibit ectomycorrhiza development because of a potential conflict between high levels of fertilizer supply and adequate mycorrhizal formation (Marx *et al.* 1977, Kropp and Langlois 1990). Inoculation of nursery seedlings with ecologically adapted ectomycorrhizal fungi may also enhance quality of planting stock by increasing mineral nutrient uptake, and should be an integral part of seedling production and nursery management practices (Villeneuve *et al.* 1991). Addition of mycorrhizae to nursery stock culture is a biological approach appropriate for overall forest health compared to chemical approaches. Several experiments have demonstrated that the use of non-mycorrhizal seedlings might limit regeneration success of spruce on both fertile and disturbed sites (Trappe 1977, Le Tacon *et al.* 1988). In general, a high level of nutrient availability, particularly N and P, reduced ectomycorrhizal development (Harley and Smith 1983). However, ectomycorrhizal formation was not restricted when seedlings were reared in a solution-based culture under steady-state nutrient conditions (Ingestad *et al.* 1986, Nylund and Wallander 1989). The challenge for forest scientists is to determine whether intensive fertilization and adequate mycorrhizal formation under steady–state conditions will occur in peat-based culture used for containerized stock production. I undertook this challenge; this thesis is an account of my research using black spruce as the test species.

Outplanting trials have shown superior field performance of exponentially loaded container stock over conventionally reared seedlings (Malik and Timmer 1995, 1996, 1998, Imo and Timmer 1998, 1999) on various forest sites. I postulate that seedling quality may be further improved by combining nutrient loading and mycorrhizal inoculation in the nursery, which would promote survival, growth and nutrition in the field. The primary objective of my research is to examine the compatibility of conventional and exponential fertilization regimes in relation to mycorrhizal formation of containerized black spruce, and to integrate steady-state nutrient culture and nutrient loading with ectomycorrhizal inoculation to precondition nursery stock. Another objective is to test the effectiveness and demonstrate the benefits of this integrated approach on performance of outplanted seedlings on different forest sites. Finally, a conceptual model is developed to systematically integrate the processes and mechanisms that may contribute to the positive response of mycorrhizal nutrient loading.

To address the research objectives, the paper is organized into seven separate, but related, chapters. The current chapter (Chapter 1) is a general introduction to the study. Chapter 2 provides a background and a review of the relevant literature on

research problems and needs for improving seedling quality for reforestation, fertilization techniques in forest nurseries, the role of mycorrhizae in forest ecosystems, and the relationships between nutrient supply, seedling nutrient status and mycorrhizae. The next three chapters (3 to 5) report on specific experiments used to test the research objectives. Chapter 3 reports on the compatibility of conventional and exponential fertilization regimes with ectomycorrhizal formation. An evaluation of the effect of nutrient loading on mycorrhizal association in the greenhouse is discussed. Chapter 4 reports on whether steady-state nutrition was achieved under the experimental conditions, and met the nutrient demand for growth, nutrient dynamics and sustained ectomycorrhizal development in response to nutrient loading. Chapter 5 reports on the growth and nutritional responses after outplanting on two contrasting forest sites, using pot bioassays. Chapter 6 is an integrated synthesis of experimental results and fertilization techniques obtained from information in chapters 3, 4, and 5. The synthesis is presented in the form of a conceptual model. Finally, chapter 7 presents general conclusions and suggests future research directions.

Chapter 2

LITERATURE REVIEW AND RESEARCH APPROACH

INTRODUCTION

The regeneration of forests is an essential component of silviculture and is considered to be one of the most crucial problems facing forest managers in Canada (Arnup 1996). Although natural forest renewal may be satisfactory for forest stands in certain instances, artificial regeneration is often necessary under particular silvicultural systems. Recently, forest regeneration in Ontario has become increasingly dependent on containerized planting stock as container-grown seedlings are less expensive to produce and plant, and possess a shorter nursery rotation when compared to bare-root stock (Timmer 1997). When planted, however, containerized seedlings are often younger and smaller, less nutritionally preconditioned, more lacking in mycorrhizae, and more highly sensitive to competing in adverse situations in the field than are bare-root stock (MacDonald and Weetman 1993). The increased reliance on container seedlings for regeneration on competitive and nutrient stressed sites has required forest scientists to develop high-quality planting stock in order to optimize field performance.

A seedling is considered of high quality when it meets survival and growth standards of performance on a particular planting site (Sutton 1982, Duryea 1985). Much effort has been devoted to enhancing reforestation success by physiological preconditioning of nursery seedlings (Duryea and Brown 1984). Increasing pre-plant nutrient status improves initial competitiveness of planted seedlings against natural vegetation, and enables efficient retranslocation of stored nutrients to new growth (Fife and Nambiar 1984, Malik and Timmer 1998, Imo and Timmer 1999). However, none of these nutrient-preconditioning studies has included mycorrhizae in the stock production process to enhance seedling quality further, although it is known that the presence of ectomycorrhizae can improve survival, growth, and nutrient uptake of seedlings (Harley and Smith 1983, Marx *et al.* 1977, Ruehle 1982). The reason for not including mycorrhizae in the cultural phase may be the inconsistency of adequate mycorrhizal development under existing nursery practice and a lack of sufficient demonstration of benefits of pre-plant inoculation after planting. Hence, better understanding of nursery cultural practices in relation to mycorrhizal development and seedling nutrition will provide the basis for developing superior nursery seedling, and will foster stronger links between stock production and plantation establishment (Glerum 1990).

The objectives of this chapter are: to review current information on the problems of successful reforestation of black spruce and possible solutions to enhance establishment of spruce plantations, to assess present fertilization practices and their significance in improving nursery stock quality, to describe the functions and importance of ectomycorrhizae in seedling nutrition, and to provide the rationale and study approach of the present research.

PROBLEMS WITH REGENERATION OF BLACK SPRUCE

Intense competition

Black spruce is one of the most common conifer species found in boreal regions of Canada. In Ontario, it is the single most valuable commercial tree for the pulp industry because of its abundance and high pulp quality (Arnup 1996). Black spruce accounts for approximately 48% of the total volume of wood harvested in Ontario annually (Campbell 1990). However, timber harvesting and regeneration practices over past decades have resulted in a decrease in relative abundance of black spruce within the boreal forest region of northeastern Ontario (Hearnden *et al.* 1993). After harvesting, replanting problems are common since regeneration of young black spruce on most logged areas is hampered by a number of factors, such as intense competition between natural vegetation and other hardwood species with black spruce (Jeglum 1983, Brumelis and Carleton 1988). Open, mesic environments quickly become revegetated, and intense competition occurs between black spruce seedlings, herbs, shrubs, and hardwoods for available soil resources and light. Consequently, in terms of stand dynamics black spruce is often replaced by hardwoods or by species of poor commercial value (Brumelis and Carleton 1988).

Competitiveness in the growing environment of newly planted containerized seedlings is acute given their small size and the fact that their root systems have relatively limited contact with the soil. Moreover,

containerized seedlings usually lack mycorrhizal association due to the intensive use of fertilizer and biocides in the nursery (Kropp and Langlois 1990). Consequently, restricted root systems of containerized seedlings due to limited container volume and the non-mycorrhizal nature of roots may have minimal exploitation of soil, creating a restriction for water and nutrient absorption from the soil resulting in plantation failure (Burdett *et al.* 1984, van den Driessche 1985, Burdett 1990).

Low soil fertility

Low nutrient status of the soil can also cause plantation failure in some boreal forests. Reforestation success depends on the ability of seedlings to capture resources quickly and on acclimatization to the planting environment (Perry *et al.* 1987). Although following disturbance to a site, such as fire or harvesting, availability of nutrients in soil is initially enhanced because canopy removal results in increased soil temperature and moisture levels (Binkley 1984); the same site may become relatively low in nutrients because of leaching if reforestation is delayed. Furthermore, competing natural vegetation in a logged area can decrease soil moisture, nutrient levels, light availability, and the soil temperature of planting sites resulting in a reduction of successful seedling establishment due to restricted resource availability.

Nutrient availability may also be limited on some reforestation sites, such as droughted sites where there may be only a short period for available soil moisture before the sites are subjected to drought conditions. If a seedling does not become established before drought conditions occur, it is unlikely to survive (Amaranthus and Perry 1987). Thus, inadequately nutritionally preconditioned seedlings, such as growth-diluted seedlings (Timmer 1997) or seedlings without mycorrhizal root systems may be highly susceptible to conditions of stress in the post-planting period. In the next section, I will discuss some silvicultural means to regenerate forests and discuss the rationale behind the approach of current research.

POSSIBLE SOLUTIONS TO IMPROVE SPRUCE REGENERATION

Fertilization at planting

Fertilization after outplanting is one method for increasing field performance of seedlings (Wagner 1993). Although field fertilization shows some promise in increasing outplanting performance due to the direct addition of nutrients (Burdett *et al.* 1984), broadcast fertilization often benefits the competing vegetation rather than the target species. It has been shown that N fertilization in the field can stimulate growth of non-crop vegetation, increasing competition for light, moisture and nutrients, and reducing crop tree uptake efficiency (van den Driessche 1991; Chang *et al.* 1996). Furthermore, field fertilization often damages recently planted seedlings due to the increased soluble salt effect in the soil (Smith *et al.* 1971). In a recent study (Staples *et al.* 1999), efficiency of applied N uptake, using ^{15}N, by planted white spruce and understory vegetation showed that white spruce seedlings utilized only a small proportion, i.e. <1% of the applied ^{15}N after two growing seasons, while the surrounding vegetation captured 6% of the ^{15}N. The result indicates field fertilization is not always efficient. However, effective results using field fertilization can be obtained if competition is controlled.

Herbicide application

Successful management of non-crop vegetation is one of the essential criteria for reforestation success in most North American forests (Walstad and Kuch 1987). The most effective and economical methods of controlling competing vegetation while minimizing soil disturbance involve chemical herbicide application (Reynolds and Roden 1996). In Canada, traditional vegetation management is mainly by the application of aerial herbicides (Campbell 1990). However, there is increasing public concern over the extensive use of herbicides in the forest, due to possible effects on wildlife (Wagner 1993, Buse *et al.* 1995) and the risk of exacerbating environmental pollution (Notnes 1991). Furthermore, forest policy makers have restricted herbicide use in several Canadian provinces (Wagner 1993). Consequently, there is a need for a suitable alternative to chemical vegetation control to improve plantation success.

Fertilization of nursery seedlings before planting

Fertilization practice in the nursery is another approach to conditioning seedling stock that can build up nutrient reserves in the seedling for field environments. Since early rapid growth of planted seedlings requires nutrients to be quickly absorbed, assimilated, and readily available to the actively growing parts (Margolis and Brand 1990), nutrient reserve build up in seedlings prior to planting may be effective in enhancing growth after planting. Although certain morphological characteristics are considered useful indicators of superior outplanting performance, the alteration of seedling physiological criteria in the nursery may be an important strategy for improving growth and nutrition during early seedling establishment in the field. Attempts are, therefore, being made to develop new methods of preconditioning seedlings to improve regeneration success. To ensure outplanting success, several researchers have experimented in manipulating seedling nutritional status in the nursery by using different cultural practices such as exponential fertilization and nutrient loading to meet the required demand of internal nutrients for a specific planting site (van den Driessche 1980, Thompson 1986, Timmer 1997). In my view, nutritional preconditioning of nursery stock should be the priority measure among silvicultural practice to enhance artificial regeneration, because this practice is less costly and more effective than all other practices.

Preconditioning of nursery seedlings

The availability of nutrients is a major contributor of

plant growth and development, and its manipulation and evaluation during seedling production have significance in enhancing seedling quality (van den Driessche 1991). In the last few decades, much research has focused on the improvement of nursery stock quality showing that physiologically preconditioning nursery seedlings can enhance outplanting performance (Duryea and Brown 1984, van den Driessche 1991). Nursery fertilization is a relatively inexpensive and easy way to manipulate forest stock quality and earlier studies of this technique have shown enhancement in seedling field performance (Mulin and Bowdery 1977, Margolis and Waring 1986, Gleason *et al.* 1990). A nutrient preconditioning technique, such as nutrient loading, in which high dose fertilization during nursery culture builds up internal nutrient reserves in nursery seedlings by inducing luxury nutrient consumption, is a relatively new approach to the culture of nursery stock. This technique can substantially increase growth and nutrition of seedlings after outplanting on competitive forest sites (Timmer 1997, Malik and Timmer 1995, 1996, 1998, McAlister and Timmer 1998). Nutrient loading is more effective under an exponential-based delivery regime, where nutrients are applied at progressive addition rates that correspond more closely with the exponential growth and nutrient consumption of seedlings (Timmer 1997, Xu and Timmer 1998, 1999). This technique has potential for further improvement in nutrient loading if mycorrhizal inoculations are introduced to stock culture, which may contribute to greater nutrient uptake, consequently higher nutrient reserves build up in seedlings.

Role of pre-plant nutrient reserves

Enhanced nutrient reserves in seedlings may play an important role in reducing planting shock to planted seedlings early in plantation establishment (van den Driessche 1985, Timmer and Munson 1991, McAlister and Timmer 1998). Several studies have reported that late season N fertilization in the nursery increased tissue N concentration without affecting seedling growth and enhanced field performance after planting due to earlier bud break (Benzian *et al.* 1974, van den Driessche 1988). Contrary to these reports, Burdett (1990) notes that in relation to experiments with Douglas-fir seedlings (van den Driessche 1980) there appears to be no firm evidence that loading stock with a super optimal nutrient concentration improves field performance. However, several recent experiments reported that nutrient loading during seedling culture significantly increases growth and nutrient uptake in plants by stimulating root and shoot development after planting (Gleason *et al.* 1990, Timmer and Miller 1991, Miller and Timmer 1997, Malik and Timmer 1996, 1998, Imo and Timmer 1998). Malik and Timmer (1998) and Xu and Timmer (1998, 1999) also demonstrated that improved competitiveness of nutrient-loaded seedlings after planting is due to the accumulation of increased nutrient reserves during nursery culture. These higher reserves become critical internal nutrient sources that are readily available for retranslocation to new growth soon after planting (Malik and Timmer 1996, 1998, Imo and Timmer 1999). The nutritional status of planted seedlings depends on several factors: pre-plant nutrient content, nutrient uptake after planting, and nutrient dilution resulting from growth, as well as the development of mycorrhizal symbiosis (Burdett 1990). However, retranslocation of nutrients is an important source for new growth at all stages of plant development (Nambiar and Fife 1991), and may occur in all physiologically active needles. Since tissue nutrient retranslocation is an essential growth driven process, it is expected that the manipulation of the build up of nutrient reserves in nursery seedlings by fertilization and mycorrhizal inoculation will enhance the early growth and development of planted trees in the field.

Unfortunately, none of these nutritional preconditioning studies has considered incorporating mycorrhizal associations during seedling culture. Although ectomycorrhizal fungi may already be present in most forests, seedlings planted on both routine reforestation sites and on disturbed land may benefit from pre-inoculation (Mikola 1973, Marx and Cordell 1987, 1988, Browning and Whitney 1993). Mycorrhizal plants have a greater ability to absorb and accumulate nutrients and may better utilize the nutrient poor sites common to boreal forests. Manipulation of mycorrhizal infection could, therefore, be a biological tool for improving seedling quality in the nursery and, subsequently, enhanced performance after planting

Nursery inoculation

Artificial inoculation of growing media with ectomycorrhizal fungi may be an effective approach for producing superior planting stock. Mycorrhizal inoculation can improve plant nutrient status by increasing the absorption capacity of root systems, enhancing uptake of both water and nutrients, and increasing survival and growth rates after planting (Harley 1969, Kropp and Langlois 1990). Apparently, nursery seedlings with mycorrhizal root systems are more biologically preconditioned than non-mycorrhizal seedlings and have greater ability to exploit the outplanting site for the resources required for growth. However, intensive fertilization in the nursery has always been a problem for mycorrhizal seedling production because the mycorrhizal fungi are commonly sensitive to higher nutrient supply and the latter may reduce the survival and activity of mycorrhizae, particularly early in the inoculation process (Marx *et al.* 1977, Kropp and Langlois 1990, Browning and Whitney 1992). Conventionally, containerized seedlings are usually reared with generous use of fertilizers to produce acceptable seedling size in a short period, resulting in the inhibition of the mycorrhizal association. Since substrate fertility is a critical factor in establishing mycorrhizal symbiosis, appropriate fertilization scheduling and dose rates that are compatible to mycorrhizal development are essential for a successful inoculation program. There is a need, therefore, for finding an effective fertilization

practice to optimize both ectomycorrhizal formation and seedling growth.

FERTILIZATION PRACTICES IN OPERATIONAL NURSERIES

Background

Fertilization is an essential practice for the production of quality planting stock in forest nurseries (van den Driessche 1984). This section will review the current fertilization practices followed in producing seedling stock in operational nurseries; it will also outline recent trends in modifying fertilization techniques in order to obtain nutritionally preconditioned planting stock. Although horticultural peat moss has desirable physical properties, a high cation exchange capacity, and the ideal pH necessary for containerized conifer growth (Tinus and McDonald 1979), the peat substrate is deficient in mineral nutrients for plant growth. Furthermore, these substrates generally lack mycorrhizal fungi (Trappe 1977). Fertilizer additions to the substrates are required to meet seedling nutrient requirements for optimum growth within a short period. Conventional practice in Ontario is to apply fertilizer in solutions through the irrigation water on a weekly basis at a constant fertilizer level or concentration. This type of fertilization practice is commonly referred to as a conventional regime (constant addition rates).

Conventional fertilization regime

Conventional fertilization practices are based on nutrient additions delivered in equivalent amounts as constant top dressing throughout the growing period (van den Driessche 1988) as illustrated in Figure 2.1.

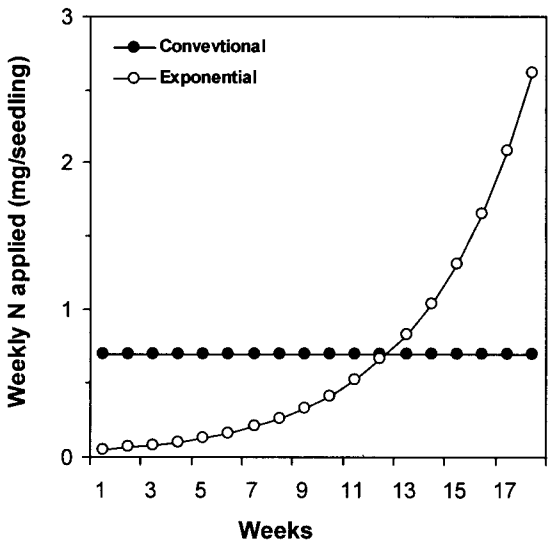


Fig. 2.1. Schedules of weekly fertilizer (N) addition with a common dose (25 mg/seedling) by seedling age applied as constant rate conventional, or progressively increasing dose rates applied as exponentially-based fertilization regimes during greenhouse seedling culture.(Adapted from Timmer and Miller 1991).

Although this schedule may induce increased seedling growth and nutrient accumulation in the nursery, it is not adequately matched with actual plant demand for nutrients during the seedling growth phase. Under constant rate fertilizer additions, seedlings may be exposed to relatively high nutrient levels at the start of the season due to the supply of nutrients at rates higher than those required for uptake and assimilation, which may result in early toxicity in seedlings. Furthermore, seedlings may suffer nutrient deficiency later in the season because of inadequate fertilization, resulting in declining internal nutrient concentrations (Fig. 2.2) due to growth dilution as the season progresses (Timmer *et al.* 1991, Imo and Timmer 1997). In theory, growth

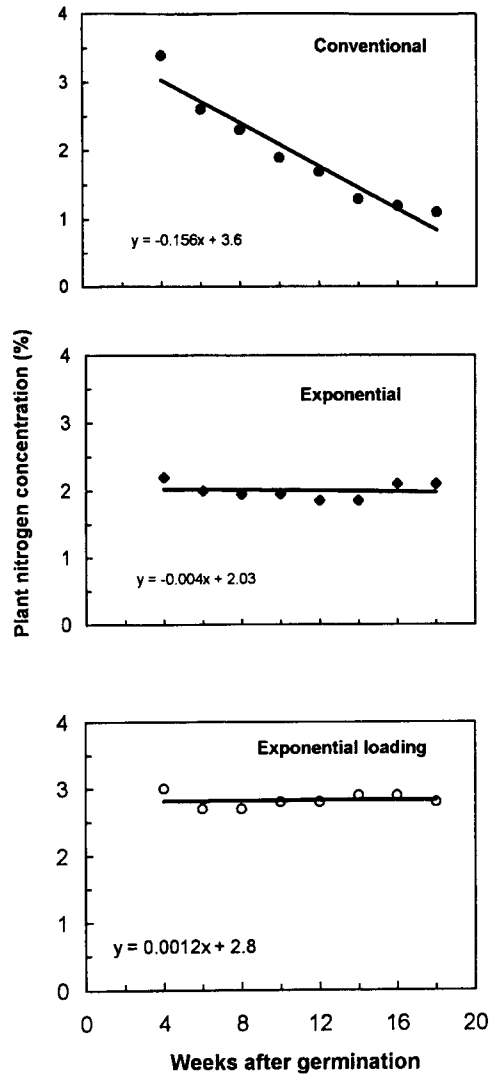


Fig. 2.2. Progression in tissue nitrogen concentration (%) of black spruce seedlings reared under conventional, exponential , and exponential loading regimes over one growing season. Typically, internal N concentrations declined steadily under conventional regime (dilution). Both exponential and exponential loading regimes induced steady-state nutritional conditions in plants, but at higher levels with loading dose. Data adapted from (Timmer 1997).

dilution occurs when nutrient concentrations decline because of growth levels higher than nutrient uptake. Conventional fertilization techniques widely used in nursery culture do not guarantee a balanced nutritional condition in seedlings due to non-synchronized growth and nutrient uptake. Hence, conventional fertilization regimes may not be a suitable way to precondition nursery seedlings. Rescheduling the fertilization regime according to seedling growth and nutritional demand may rectify declining internal nutrient concentrations in seedlings. Nutrient preconditioning (loading) of black spruce has been successfully demonstrated in previous studies, and found more effective when using exponential fertilization regimes rather than conventional regime (Timmer *et al.* 1991, Timmer 1997).

Exponential fertilization regime

In contrast to conventional regimes, exponential fertilization involves progressively increasing nutrient additions (Fig. 2.1) that correspond closely with exponential growth and nutrient consumption during the exponential growth phase of plants (Ingestad and Lund 1986, Ingestad and Ägren 1988) as depicted in Figure 2.3. Nutrient accumulation in seedlings fertilized exponentially matches dry mass production (Fig. 2.3), demonstrating close synchronization of plant nutrient consumption and growth when compared to constant

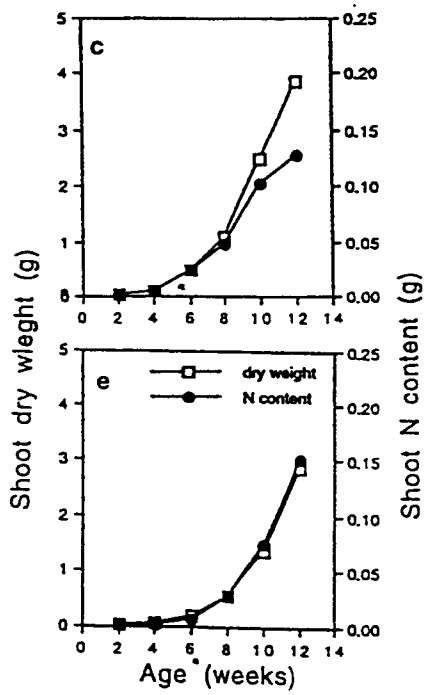


Fig. 2.3. Progression of dry matter production and N uptake of mesquite seedling shoots at constant top dressing (c) and exponential (e) fertilization regimes during the growing season. Data show N accumulation matched the growth increments under exponential regime, but not in constant top dressing conventional regimes (modified from Imo and Timmer 1992).

top dressing (conventional) nutrient supply techniques. Consequently, internal nutrient concentrations in plants are relatively stable or undiluted during the growing period (Fig. 2.2). Stable internal nutrient accumulation in the nursery conforms better with uptake pattern of naturally regenerated seedlings (Munson and Bernier 1993), indicating the advantages of exponential fertilization in the nursery to mimic field situations. Exponential nutrient additions, in which nutrients are applied incrementally at exponential rates, also improve ectomycorrhizal development due to controlled exposure of nutrient to symbiont as noted under hydroponic systems (Ingestad *et al.* 1986). In exponential fertilization systems, mycorrhizal fungi are exposed gradually to higher nutrient levels and nutrient supply corresponds more closely with exponential growth and nutrient consumption, hence internal concentrations are relatively stable inducing a steady-state nutritional condition in plants.

Steady-state nutrition

The concept of steady-state nutrition suggests that plants should be grown with stable internal nutrient concentrations free from nutrient stress (Ingestad and Lund 1986). To maintain stable nutrient concentrations in plant tissue during exponential growth, nutrients must be supplied at exponentially increasing amounts per unit time. In theory, steady-state or dilution-free nutrient concentration (n/W) in plant tissue over time (t), can be expressed by

$$d(n/W)/dt = 0 \qquad (1)$$

where, n and W are the amount of nutrient and biomass of the seedling, respectively.
Differentiation of equation (1) provides:

$$[W(dn/dt) - n(dW/dt)]W^2 = 0 \qquad (2)$$

which transforms to:

$$(1/w)\, dW/dt = (1/n)dn/dt \qquad (3)$$

In equation (3), the left and right side indicates relative rates of plant growth (R_G) and nutrient uptake (R_U), respectively,

$$R_G = R_U \qquad (4)$$

In hydroponic culture systems, the relationship of (4) was shown experimentally, and demonstrated that

$$R_G = R_U = R_A \qquad (5)$$

where R_A is the relative or exponential supply of nutrients. Since relative addition rates are strongly related to relative growth rates, the relative addition rate (R_A) can, thus, be used as the driving factor for plant growth and nutrition. Relative addition rates can be easily manipulated during seedling production to achieve steady-state nutrient conditions (Ingestad and Lund 1986) by fertilizing seedling with exponentially increasing amounts of nutrients during the exponential growth period.

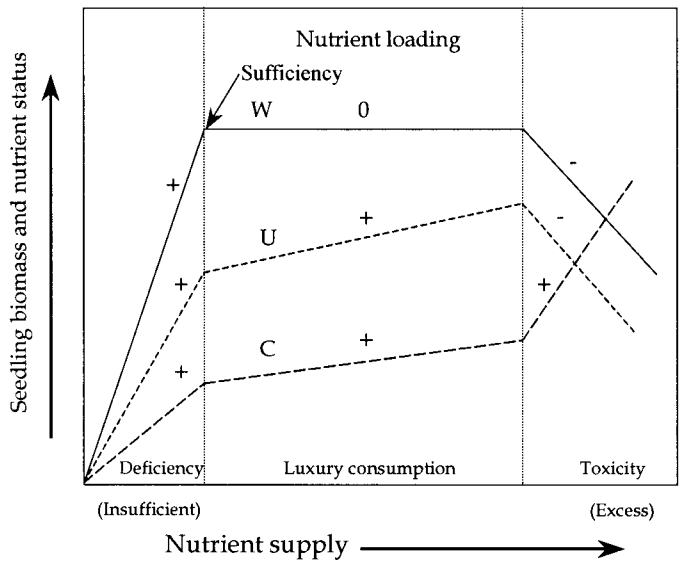
The recent adoption of steady-state fertilization in some commercial nurseries in Ontario has led to the

production of nutrient loaded planting stock, in which high dose fertilization builds up internal nutrient reserves to improve seedling quality for competitive forest sites using exponential delivery models (Timmer and Aidelbaum 1996). Several researchers have used Ingestad's concept of controlling relative addition rates to produce seedlings stock (Burgess 1990, Burgess 1991, McAlister and Timmer 1998, Xu and Timmer 1998) indicating that this approach can be utilized in a controlled and repeatable manner (Burgess and Peterson 1987). However, only a few researchers have incorporated this concept to mycorrhizal inoculation practices in nursery conditions. Peterson and Chakravarty (1994) have demonstrated several techniques for synthesizing ectomycorrhiza between known symbionts under both sterile and non-sterile conditions; however, these techniques are mostly suitable for physiological and molecular studies in laboratory conditions. Ingestad *et al.* (1986), and Kähr and Arveby (1986) have synthesized ectomycorrhiza using steady-state nutrient culture in hydroponic systems, but these systems are not applicable to peat-based culture in commercial forestry.

Steady-state nutrient principles were successfully incorporated into operational practices in the form of "exponential nutrient loading" (Timmer 1997), which integrates high dose fertilization with exponentially increasing nutrient additions during seedling culture to improve competitiveness of containerized seedlings. The advantages of this practice are reduced early salt build up in the growing media and a progressive increase in nutrient levels during the season (Timmer 1997). The incremental step-up in nutrient dose avoids early nutrient toxicity and can acclimatize plants to high fertilization during seedling culture. These features may be well suited for compatible culture of both mycorrhizal and nutrient loaded seedlings.

Nutrient loading

Exponential nutrient loading is a relatively new approach to conditioning planting stock that builds up the internal nutrient reserves of seedlings in the nursery. Studies have shown that nutrient loading during seedling culture significantly increases seedling growth and nutrient uptake on low fertility or competitive sites by stimulating root and shoot development after planting (Timmer and Miller 1991, Miller and Timmer 1997, Malik and Timmer 1996,1998, Imo and Timmer 1998). In this technique, high dose fertilization during nursery culture builds nutrient reserves in plants. Nutrient accumulation occurs whenever uptake exceeds demand inducing luxury consumption of nutrients (Chapin 1987) without significantly altering seedling size (Timmer and Munson 1991). Figure 2.4 is a generalized interpretation of the relationship between increasing nutrient supply and seedling biomass production (W), nutrient concentration (C) and nutrient content (U). The diagram shows that growth responses to increased nutrient supply follows a curvilinear relationship that can be characterized by three nutritional phases: deficiency, luxury consumption, and



Diagnostic interpretation	Change in plant		
	Growth (W)	Nutrient content (U)	Nutrient concentration (C)
Deficiency	+	+	+
Luxury consumption	0	+	+
Toxicity	-	-	+

Fig. 2.4. Generalized concept of nutrient loading showing the relationships between nutrient supply, and plant growth (W), nutrient content (U) and nutrient concentration (C). At each phase, changes in growth, nutrient concentration and uptake can be identified as either increasing (+), no change (0), or declining (-). (Modified from Timmer 1997).

toxicity (Timmer 1997). Fertilizers are usually applied to the sufficiency level, where crop production is maximized (Fig. 2.4). Seedling growth (W) below the sufficiency level is generally restricted by insufficient nutrient availability and is known as the deficiency range. Nutrient supply beyond the sufficiency level is considered inefficient because seedling biomass is not increased significantly, although nutrient uptake (U) can still be enhanced due to luxury uptake. Hence, nutrient supplementation above the sufficiency level induces loading before it reaches excessive levels, where nutrient levels become toxic.

Although nutrient loading can be achieved by both the conventional and exponential addition of fertilizer during seedling production, exponential delivery techniques based on steady-state nutrition are more effective for loading (Timmer *et al.* 1991). Exponential fertilization occurs in a progressive fashion, hence the buildup of nutrients in growing media is therefore gradual, minimizing possible toxicity to seedlings and allowing plants to develop tolerance to higher nutrient levels (Timmer 1997). Since this technique is associated with careful and controlled fertilizer use to avoid toxicity and nutrient imbalance, the approach may also be compatible with mycorrhizal formation in the nursery. Fertilizer input starts low and increases incrementally to high levels, hence avoiding early nutrient toxicity to the fungal inoculum, and may allow fungi to adapt to higher nutrient levels. Combined mycorrhizal inoculation with nutrient loading practice during seedling culture may increase nutrient reserve in seedlings while maintaining an effective mycorrhizal association. I have called this new approach "mycorrhizal nutrient loading". As mycorrhizae enhance resource acquisition of plants, mycorrhizal nutrient loaded seedlings may be better preconditioned for outplanting, and can function more effectively on nutrient stressed or competitive sites that restrict tree growth because of competition.

FUNCTION OF MYCORRHIZAE IN FORESTS

Background

Mycorrhizae play a fundamental role in plant nutrition of forests. Before applying ectomycorrhizal techniques to forest regeneration practices, it is necessary to understand the ecophysiological functions of the symbiosis between plant and fungus, their relationships and the factors affecting the formation of ectomycorrhizae. This section will present a background on how mycorrhizae affect the mineral nutrition of conifer seedlings, how cultural practices influence mycorrhizal formation in the nursery, and how pre-plant inoculation influences seedling growth and nutrition in the field.

Mycorrhizae have long been recognized for their widespread occurrence in both natural and managed forests. Mycorrhizal associations play an essential role in absorbing nutrients, affecting growth and water uptake, providing protection from pathogenic fungi, and competing with other microorganisms for resources in boreal and temperate forests (Harley and Smith 1983, Amaranthus and Perry 1987, 1989, Kropp and Langlois 1990, Smith and Reid 1997). The tree, in turn, supplies the fungal symbiont with carbohydrate for its growth and development (Melin and Nilsson 1957). The ubiquitous nature of the plant-fungus relationship is composed of several components in the forest systems.

Components of mycorrhizal symbiosis

The essential components of mycorrhizal symbiosis can be illustrated in diagram form (Fig. 2.5), in which the relationships between plant-soil-mycorrhizal fungi and their interactions are evident. When plants are in association with a mycorrhizal fungus, nutrient uptake

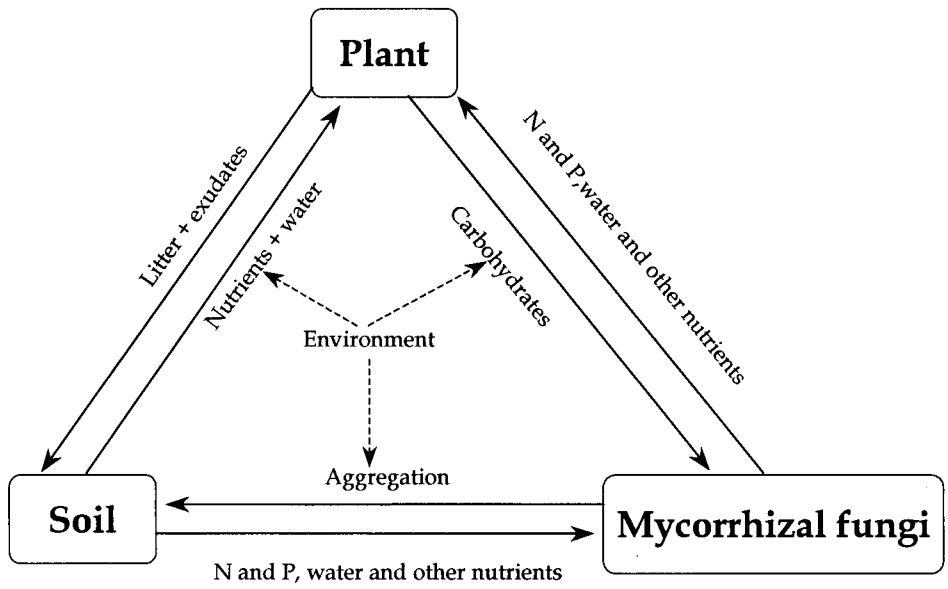


Fig. 2.5. Mycorrhizal symbiosis showing the relationships between the three essential components of the association and their interactions. (Modified from Raman and Mahadevan 1996).

occurs via two systems, the fungal network and the root itself. This bi-directional nutrient uptake pattern exemplifies the efficient nutrient acquisition by mycorrhizal plants.

According to Trofymow and van den Driessche (1991), mycorrhizas of conifers can be divided into three types on the basis of morphological characteristics: ectomycorrhizae, endomycorrhizae, and ectendomycorrhizae. However, in general, mycorrhizas are classified into seven types (Smith and Read 1997). These are VA (vesicular-arbuscular mycorrhizas), Ectomycorrhiza, Ectoendomycorrhiza, Arbutoid, Monotropoid, Ericoid, and Orchid mycorrhiza. In conifers, ectomycorrhizae have received the most attention from plant scientists probably because they can be recognized by the naked eye and some of which can be grown in pure culture (Trofymow and van den Driessche 1991). Mycorrhizal fungi are considered the primary interface between roots and forests soils, and are involved in various important functions such as enhancing nutrient uptake, maintaining soil structure, buffering against drought, and protecting plants from toxic environments (Sutton and Sheppard 1976, Lynch and Bragg 1985). Initial recognition of the biological requirement of forest trees for ectomycorrhizal association occurred when attempted establishment of pines in the tropics and East Africa routinely failed unless ectomycorrhizal fungi were introduced to these trials (Marx 1991). There is no doubt that in a forest ecosystem, mycorrhizae play an important role in seedling nutrition and for the successful establishment of seedlings, especially in those soils low in mineral nutrients.

Importance of mycorrhizae in seedling nutrition

Nitrogen nutrition

Of the primary nutrients required by trees for normal growth and development, N appears to be the most important in increasing forest productivity. Nevertheless, N is one of the most limiting nutrients for the productivity of boreal and temperate forests in the Northern Hemisphere (Van Cleve *et al.* 1981, Ellengberg 1988, Tamm 1991). It is important, therefore, to know the processes in the soil systems that determine how nutrients are readily mobilized, assimilated and transported. Soil microbial activity is of great significance in the utilization of both organic and inorganic N. Besides microbial decomposition by various microorganisms, ectomycorrhizae play a significant role in plant nutrition by absorbing nutrients and making available the nutrients needed for the growth and development of trees (Carrodus 1967, France and Reid 1983, Martin *et al.* 1986). Consequently, mycorrhizal plants accumulate more nutrients than non-mycorrhizal plants as reflected in Figure 2.6, in which mycorrhizal plants accumulated much higher N than non-mycorrhizal plants. Nutrient uptake from the soil by the ectomycorrhizal root systems involves many factors relating to the physical and biological components of the soil system (Finley *et al.* 1992). Regardless of these factors, the ectomycorrhizae result in greater absorption area (see Photo plate 2.1), which allows trees to extract nutrients from soil in a more efficient manner by the mycelial network.

The uptake efficiency of a root system is defined in the context of increased surface area for absorption (Hatch 1937) and increased effectiveness of the absorption system (France 1980) because of ectomycorrhizal development. The hyphae of mycorrhizal fungi act initially as organs of nutrient accumulation and then the fungal sheath acts as a nutrient storage organ (Harley and Smith 1983, Harley 1989). It is possible that this ability for nutrient storage in fungal sheaths will be an advantage in nutrient loading. Mycorrhizal infection may, at times, increase the rate of nutrient accumulation (during seedling culture) beyond that which can be utilized for biomass production by inducing luxury accumulation of nutrients, and serve as a storage organ of internal nutrient reserves (Koide 1991). These internal reserves can be utilized immediately after planting for seedling growth and development, allowing mycorrhizal plants to outperform non-mycorrhizal plants. However, the traditional view of increased absorptive area of mycorrhizal roots is not the only advantage of the symbiotic association, but may have other functions in the N metabolism of forest plants.

It is becoming evident that fungal symbionts play an integral role in the N metabolism of forest trees (Clement *et al.* 1977) by making more readily utilized forms of nutrients. Specific mycorrhizal associations may also alter the efficiency of N assimilation and enable conversion of N compounds into more readily utilized forms (Martin *et al.* 1987). It has been suggested that mycorrhizae may be associated with the breakdown of soil organic N (Read *et al.* 1989).

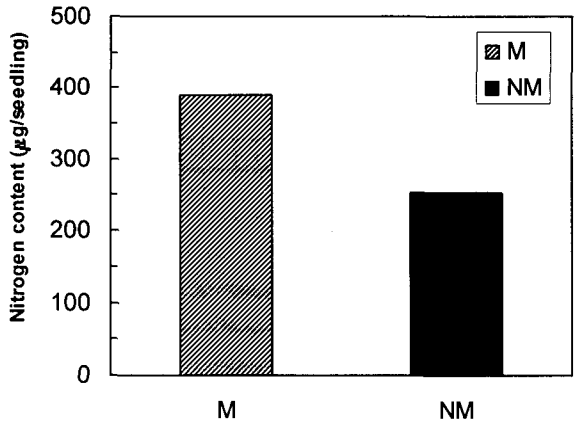


Fig. 2.6. Histogram showing the total N contents of *Pinus contorta* grown on Media containing ammonoiun as sole source of nitrogen. Mycorrhizal (M) Plants take up more N than non-mycorrhizal (NM) plants due to greater absorbing surface area and efficient N metabolism capacity associated with mycorrhizal plants. (Adapted from Finlay et al. 1992).

Ectomycorrhizal fungi are known to be efficient in using different amino acids in pure culture (Abuzinadah and Read 1988, Quoreshi *et al.* 1995). Compared to non-mycorrhizal root systems, mycorrhizal associations may result in changes in a metabolic event, such as alteration in free and bound amino acid levels in the mycorrhizal plants (Krupa *et al.* 1973; Martin *et al.* 1986; Dell *et al.* 1989). These free amino acids may be stored in plants as reserves that can be readily available, when required, for growth; these readily available reserves are similar to the surplus nutrient storage concept in plants suggested by Chapin (1990). Other studies have shown that ectomycorrhizal fungi can release proteinase enzymes to degrade protein from the soil litter and can make the production of available N for assimilation (El-Badaoui and Botton 1989, Leake and Read 1989).

Phosphorus nutrition

Mycorrhizae also affect P nutrition of the host plant. Earlier studies indicate that the absorption of P is enhanced by mycorrhizal systems, more so in fact than other nutrients (McComb and Griffith 1946, Stone 1950). Phosphate is the major form of P available for uptake by plants. It is relatively insoluble in soil solution, and therefore not readily transported by mass flow (Nye and Tinker 1977). Since mycorrhizal root systems can explore the bulk of the soil by their greater absorptive area beyond the root and root hairs, additional P is captured by the hyphae, stored in the fungal vacuoles as polyphosphate, and transported to the host. Besides this increase in P acquisition by the hyphal network, production of extracellular acid phosphatases and phytases as coenzymes may also catalyze the release of P from organic complexes in the soil (Ho 1989, Hilger and Krause 1989). Since mycorrhizae can enhance seedling nutrition, use of mycorrhizal fungi can be of major significance to artificial regeneration of forest, particularly nutrient stressed and where the planting site lack of abundant mycorrhizal inoculum. The importance of ectomycorrhizae to artificial regeneration has been demonstrated under diverse field conditions in the USA as reflected in Photo plate 2.2, showing that pine seedlings with abundant pre-plant ectomycorrhizae exhibit dramatically greater survival and growth rates than non-inoculated seedlings.

Nursery cultural conditions and regulation of ectomycorrhizal development

Despite the well-known nutritional advantages and the ubiquitous nature of mycorrhizal associations in natural ecosystems, seedling growers are reluctant to incorporate mycorrhizal inoculation programs in forest nurseries (Kropp and Langlois 1990). Reasons for this reluctance include a lack of general agreement among researchers in understanding the most suitable fertilization schedules for inoculation of mycorrhizal fungi under real nursery conditions. As nursery fertilization conditions may have significant influence on the regulation of mycorrhizal development and function of the seedlings, finding a simple, easy, and uniform method of producing ectomycorrhizal seedlings is necessary for enhancing seedling quality.

Of the various factors affecting ectomycorrhiza formation in container nurseries, fertilizer supply and substrate fertility appears to be the most important. It is commonly acknowledged that high concentrations of N and P in the growth media prevent the formation of ectomycorrhiza (Mikola 1973, McFall *et al.* 1988, Kropp and Langlois 1990). The inverse relationship between soil fertility and ectomycorrhizal infection has long been recognized (Bjorkman 1942). Several researchers have also reported that a high level of mineral availability, particularly N and P, can reduce and finally inhibit mycorrhizae (Richards and Wilson 1963, Dumbroff 1968, Harley and Smith 1983). Containerized seedling production usually involves generous applications of fertilizer and biocides for optimal growth in a short period. Such cultural conditions result in restricted mycorrhiza formation in the nursery (Gagnon *et al.* 1995). Inoculum viability may be decreased because the inoculum used for containerized seedlings is not accustomed to abrupt high nutrient levels early in the growing process (Holden *et al.* 1983). The inhibitory effect of N has been interpreted as an indirect reaction to reduced sugar concentration in the roots. Bojrkman (1970) and Marx *et al.* (1970) suggest that the level of soil fertility controls the availability of carbohydrate in short roots and that, in turn, regulates ectomycorrhizal development. In a one investigation (Nylund and Wallander 1989), *Pinus sylvestris* seedlings were grown under steady-state nutritional conditions in a semi-hyrdoponic system and inoculated with ectomycorrhizal fungi. It was found that free access to a balanced concentration of mineral nutrients did not inhibit mycorrhiza formation. Nylund and Wallander (1989) also report that there was no effect of the nutritional regime on root or shoot carbohydrate content. Formation of adequate mycorrhiza in hydroponic culture where growth is not restricted by nutrient availability has also been reported earlier (Ingestad *et al.* 1986, Kähr and Arveby 1986). Most of these mycorrhizal studies were conducted under steady-state nutrition regimes and are based on hydroponic or semi-hydroponic cultural systems; they may, therefore have limited applicability to nursery and field situations. The challenge for researchers is to integrate intensive fertilization under a steady-state nutrition regime while combining it with mycorrhizal inoculation, where seedlings may build up tolerance to higher nutrient levels, and, therefore, benefit from both nutrient loading and ectomycorrhizae after planting.

Fungal tolerance

Experimental evidence suggests that several mycorrhizal fungal species show substantially increased sporocarp production after high N additions; this would indicate that mycorrhizal fungi are adaptable to increased levels of N additions (Ohenoja 1978, 1988). Nursery mycobionts are generally found more tolerant

of the high fertility and moisture levels normally used in the nursery (Marx and Artman 1979). Furthermore, with VA mycorrhizae, long-term P fertilization of pasture soils results in the development of P tolerance among the indigenous fungi (Hayman 1982). These results demonstrate the potential for mycorrhizal fungal tolerance to elevated nutrient supply. Effective symbiotic associations may be achieved under exponential nutrient loading regimes where the fungal symbiont is exposed to gradually increasing nutrient levels. The fungus may adapt to high nutritional regimes once initial infection is established, and sustained symbiosis is attained under steady-state seedling nutrition. Sustained mycorrhizal symbiosis in seedlings is possible as long as the tree supplies the fungal symbiont with carbohydrate for its growth and development (Melin and Nilsson 1957), suggesting the importance of stable carbohydrate relationship for successful mycorrhizal association.

Carbohydrate relationship with mycorrhizae

Mycorrhizal symbiosis not only enhances the uptake of nutrients in plants but also influences host carbohydrate status. Competition between the fungus and the roots for photosynthates may be a major factor responsible for the typically larger shoot:root ratio in mycorrhizal plants (Berta *et al.* 1990). The impact of this change on host plant growth depends on various soil and environmental factors and, in particular, on the fertilization practices followed in the nursery (Kropp and Langlois 1990).

It can be expected that if the mineral nutrition of seedlings is not adequately controlled, seedlings may show variable and undefined relative growth rates (Nylund and Wallander 1989), where growth is not synchronized with nutrient uptake of seedling. Consequently, the growth and nutrient content of conventionally reared seedlings increased as the season progressed, but nutrient concentration declined due to growth dilution (Imo and Timmer 1997), that may negatively affect carbohydrate status of plants. In contrast, steady-state nutrition achieved by exponential fertilization can ensure stable growth, and possibly maintain stable carbohydrate relations in seedlings (Ingestad and Khär 1985, Warining *et al.* 1985, Ingestad and Lund 1986). Several researchers have suggested that sustained ectomycorrhizal development is possible where seedling growth is not restricted by nutrient availability using solution culture (Khär and Arveby 1986, Ingestad *et al.* 1986, Nylund and Wallander 1989).

The presence of ectomycorrhizae is often associated with elevated rates of net photosynthesis (Reid *et al.* 1983, Rousseau and Reid 1990). Ectomycorrhizae could influence the CO_2 assimilation capacity of the host plant via enhancement of P and N nutrition in the host plant. Rousseau and Reid (1990) report that photosynthetic rates in loblolly pine increase as a result of mycorrhizal association and suggest that an elevated photosynthetic rate is related to improved P nutrition. However, mycorrhizal associations may also increase the sink activity of the root system (Harley and Smith 1983). Several researchers have suggested that increased net photosynthetic and translocation rates in mycorrhizal plants are due to greater sink-strength created by mycorrhiza formation (Reid *et al.* 1983, Conjeaud *et al.* 1996). Nylund and Wallander 1989, Wallander and Nylund 1991 noted no significant effects of nutritional regimes on internal carbohydrate concentrations. Therefore, it is suggested that nutrient supply may negatively influence mycorrhiza formation, which is not mediated through effect on carbohydrate concentration in plants.

CONCLUSIONS

A new approach is required to integrate mycorrhizal technology to planting stock production, such as exponential fertilization combined with fungal inoculation. It is apparent from the review that manipulating seedling nutrition in the nursery by adopting appropriate fertilization schedules, such as exponential fertilization and exponential nutrient loading, can lead to the development of nutritionally preconditioned nursery stock. However, container seedlings are traditionally produced in operational nurseries using fertilization techniques that are convenient for nursery logistics and for optimal growth of seedlings, but not for optimal development of mycorrhizae in trees. These regimes often induce declining nutrient concentration in plants due to growth dilution of nutrients since fertilizer supply is not synchronized with plant growth and nutrient consumption during seedling culture. Moreover, conventional fertilization practices are usually associated with high initial substrate fertility, the peat substrates usually lack mycorrhizal inocula. Consequently, root systems of containerized seedlings often lack sufficient mycorrhizae (Molina 1979, Langlois and Fortin 1987). Thus, artificial inoculation of the growing media by mycorrhizal fungi is required to meet the biological requirement of seedlings for successful plantation establishment. The challenge is to develop cultural systems that are compatible with intensive fertilization and mycorrhizal inoculation, so that seedling can benefit from both nutrient loading and mycorrhizae for reforestation program.

STUDY APPROACH

This study approach is to integrate exponential nutrient loading and mycorrhizal inoculation under steady-state nutrition in peat-based seedling culture for the commercial production of ectomycorrhizal planting stock. The first step of this research is to test the compatibility of exponential fertilization regimes and ectomycorrhizae formation at different dose levels. The second step is to investigate whether steady-state nutritional condition in plants occurs in peat-based culture, and to elucidate the relationships between exponential nutrient supply, seedling growth, nutrient uptake, and mycorrhizal development. Subsequently,

the intention is to test these seedlings on different site conditions to assess the benefits of nutrient-loaded mycorrhizal seedlings for reforestation. A greenhouse bioassay approach was undertaken to test these seedlings so that uniform growing conditions would minimize micro-climate effects due to local topography in the field. The ultimate aim is to demonstrate that mycorrhizal nutrient loading may further improve loading efficiency in the nursery, and enhance competitiveness of seedlings by reducing nutrient and moisture stress on outplanting.

Chapter 3

EXPONENTIAL FERTILIZATION INCREASES NUTRIENT UPTAKE AND ECTOMYCORRHIZAL DEVELOPMENT OF BLACK SPRUCE SEEDLINGS*

Summary

Intensive fertilization may inhibit adequate mycorrhizal development for forest nursery stock production. Containerized black spruce (*Picea mariana* [Mill.] B.S.P.) seedlings exposed to four fertilization regimes (one conventional and three exponential at 12.5, 12.5, 25 and 50 mg N seedling^{-1}, respectively) and two ectomycorrhizal inoculations (*Hebeloma crustuliniforme* (Bull. Ex St-Amans) Quel. and *Laccaria bicolor* (R. Mre.) Orton) were grown from seed to assess mycorrhizal formation and nutrition of young trees under intensive greenhouse culture. Exponentially increasing fertilizer additions stimulated ectomycorrhizal development (49-85%) compared with conventional constant-rate fertilization (22-26%). Exponential fertilization also increased seedling N (13-34%) and P (5-18%) uptake, although dry matter production was reduced (17-25%) at the lowest exponential addition rate. Ectomycorrhizal inoculation did not affect seedling biomass, but increased uptake of N (6-17%), P (5-20%) and K (4-18%) demonstrating potential for nutrient loading by fungi. Higher ectomycorrhiza formation found under exponential fertilization regimes was attributed to lower initial nutrient levels maintained in the growing media. Results indicate that high exponential fertilization combined with fungal inoculation may be effective for producing both nutrient loaded and ectomycorrhizally infected planting stock.

INTRODUCTION

The production of vigorous seedlings for subsequent outplanting is a key factor in nursery stock production. Inoculation in the nursery with suitable ectomycorrhizal fungi may benefit planting stock production by improving field survival and early growth on both highly disturbed sites and routine reforestation areas (Holden *et al.* 1983, Villeneuve *et al.* 1991, Browning and Whitney 1993). However, intensive fertilizer and biocide use in nursery culture to enhance seedling growth and control disease may inhibit mycorrhizal development because of toxicity to fungi (Molina 1979, Langlois and Fortin 1982). Peat moss or peat-vermiculite, a common substrate for containerized seedling production, generally lacks viable mycorrhizal inoculum because these fungi do not persist long without suitable host-supplied substrates (Hacskaylo 1973). Nevertheless, cultural practices may create environmental conditions that encourage certain ectomycorrhizal fungi (e.g., *Thelephora terrestris* and E-strain fungi (Ursic *et al.* 1996)), even in intensively managed nurseries (Castellano and Molina 1989, Marx 1991), but these conditions are usually ecologically different from those prevailing in the field. Consequently, when outplanted even in highly fertile soils, seedlings may incur delayed growth and development without ecologically adapted mycorrhizae (Trappe 1977).

Soil fertility influences seedling growth and carbohydrate content of roots as well as mycorrhiza development (Marx *et al.* 1977). There is a potential conflict between the generous use of nutrients to produce container seedlings both of acceptable size and abundant ectomycorrhizal development (Browning and Whitney 1992). Nutrient shortages and surpluses are detrimental for ectomycorrhizal formation suggesting that there may be an optimum requirement of these nutrients (Beckjord *et al.* 1985, Gagnon *et al.* 1988, 1991). Conventionally in greenhouse culture, fertilizers are delivered periodically to seedlings in equal amounts (as "constant-rate" topdressings) during the growing season. The operational dose applied to black spruce container stock ranges between 10 and 13.2 mg N/seedling for the entire growing season (Bigras and D'Aoust 1992, Calmé *et al.* 1993; Timmer and Aidelbaum 1996). Although these levels do not appear to be high for seedling production, they may impair mycorrhizal inoculum function in the rhizosphere (Ekwebelam and Reid 1983, Gagnon *et al.* 1995). Particularly early in the growing season, high nutrient inputs reduce survival of fungal inoculum or limit capability to initiate further infection (Ruehle and Wells 1984, Gagnon *et al.* 1991). Constant-rate addition schedules may result in early buildup of nutrients and later nutrient deficiency, since nutrient additions are poorly synchronized with relative growth rate and nutrient demand of seedlings during the exponential growth phase of the greenhouse rotation (Timmer *et al.* 1991, Imo and Timmer 1992). Attempts have been made to optimize nutrient supply for both ectomycorrhiza formation and seedling growth using different fertilizer delivery models and dose rates (Ruehle and Marx 1977, Shaw *et al.* 1982, Danielson *et al.* 1984*a*, 1984*b*, Ruehle and Wells 1984, Gagnon *et al.* 1987, 1988, 1995), but nutrient delivery regimes are mostly based on constantly increasing rather than

* Reproduced from Canadian Journal of Forest Research, Quoreshi, A.M., and Timmer, V.R. Exponential fertilization increases nutrient uptake and ectomycorrhizal development of black spruce seedlings. 28, 674-682, 1998, with permission from CJFR.

exponentially increasing addition rates.

Most of these attempts achieved adequate mycorrhizal development at low fertilizer application levels but at a cost of smaller seedling size and nutrient uptake, exemplifying the problem of seedling sensitivity to low nutrient inputs and symbiont intolerance to high nutrient exposure. The problem may be avoided by using fertilizer delivery techniques that are compatible to both ectomycorrhiza formation and seedling growth. Ingestad *et al.* (1986) demonstrated that ectomycorrhiza formation was not inhibited when seedlings were reared under "steady-state" nutrient culture in which plants were grown with constant internal nutrient concentrations free from nutrient stress. This was achieved by applying nutrients at exponential rather than conventional (constant) addition rates. Exponential fertilizer delivery involves progressively increasing nutrient applications that correspond closer to the relative growth rate of seedlings during their exponential phase of growth (Ingestad and Lund 1979). Besides inducing steady-state nutrient uptake, this schedule avoids high initial rhizosphere fertility levels that may cause toxicity and restrict ectomycorrhiza development. Gradual acclimatization of the symbiont to higher nutrient inputs may also be conducive to increasing fungal tolerance to high fertilizer applications. Formation of adequate mycorrhizae where seedling growth was not restricted by nutrient availability has been reported by others (Kähr and Arveby 1986, Nylund and Wallander 1989); however, these studies were based on hydroponic or semi-hydroponic cultural systems not applicable to conventional nursery operations.

Another technique to improve nursery stock for outplanting performance is exponential nutrient loading (Timmer and Aidelbaum 1996, Timmer 1997), which involves high fertilizer inputs delivered progressively to build up plant nutrient reserves as steady-state luxury consumption of nutrients during greenhouse culture. Nutrient loaded seedlings have exhibited superior outplanting performance and increased competitiveness with neighboring vegetation (Timmer and Munson 1991, Malik and Timmer 1995, 1996). Since ectomycorrhizal systems enhance nutrient uptake, loading efficiency in the nursery may be improved by combining mycorrhizal culture with nutrient loading practices, thus reducing the need for high fertilizer inputs. The primary objectives of this study were to examine the compatibility of conventional and exponential fertilizer regimes with mycorrhiza formation of containerized black spruce seedlings, and to evaluate exponential nutrient loading practices in relation to mycorrhizal association and seedling growth. The findings may aid development of effective fertilizer practices for producing both nutrient-loaded and ectomycorrhiza-infected black spruce seedlings.

MATERIALS AND METHOD

Growing conditions and seedling establishment

Black spruce seedlings were grown in the greenhouse for 20 weeks using 110 cm^3 capacity Spencer-Lemaire Rootrainers® containers (Spencer-Lemaire Industries Limited, Edmonton, Alta.) arranged in 50-cavity trays as shown in Photo plates 3.1 and 3.2. Containers were uniformly filled with an autoclaved sphagnum peat moss and vermiculite (10:1 by volume) mix with or without ectomycorrhizal inoculum. The seeds were obtained from a commercial grower, North Gro Development Inc., near Kirkland Lake, Ont. Seeds were surface-sterilized with 30% H_2O_2 for 15 min and washed three times with sterilized water before sown at three seeds per cavity. Seedlings were grown at ambient air temperatures ranging between 18°C and 25°C and relative humidity levels between 65 and 85%. Supplementary light was provided with sodium vapor lamps to ensure an extended 20-h photoperiod with a light intensity of 250 µmol. $s^{-1}.m^{-2}$. The seeds were misted daily during the 10-day germination period, and each cavity was thinned to a single seedling. The seedlings were irrigated manually when needed, using spray nozzles to maintain the growing media close to container capacity (Timmer and Armstrong 1989) to avoid moisture saturation and possible nutrient loss due to leaching. The trays were rotated in position every 2 weeks to minimize edge effects.

Fungal inoculum and inoculation

Two species of ectomycorrhizal fungus, *Hebeloma crustuliniforme* (Bull. Ex St-Amans) Quel. and *Laccaria bicolor* (R. Mre.) Orton, generally native to black spruce forests in Canada, were tested for this experiment. These species were selected because they (*i*) adapt well to planting sites (Trappe 1977, Navratil 1988); (*ii*) have the ability to grow rapidly in pure culture and withstand manipulation for commercial inoculum production (Marx 1991); and (*iii*) are known to assimilate nitrate, ammonium and organic N sources for their growth requirements (Ahmad and Hellebust 1991, Martin *et al.* 1994, Quoreshi *et al.* 1995) that is considered beneficial for establishment on a wide range of sites. The live vegetative mycelial inoculum was obtained from Plant Health Care Inc., Pittsburg, PA 15238, U.S.A. It was maintained as a pure culture, and grown aseptically on a vermiculite carrier, a solid inoculum (for detailed procedure see Appendix 1). The inoculum was leached in a 150-µm sieve with distilled water to remove excess nutrients and stored at 4° C before inoculation. Sphagnum peat moss used as growing media was steam sterilized twice using an autoclaving bag (FISHERbrand:01-815A) to prevent possible contamination. The autoclaved peat moss was thoroughly mixed with the solid inoculum at a rate of 10:1 (by volume) before filling the containers. Instead of a vermiculite inoculum, a similar volume of sterilized vermiculite moistened with sterilized water was used as a control treatment.

Fertilization regimes

The four fertilizer regimes tested increasing amounts of nutrients applied conventionally or exponentially for

18 weeks during the growing season (Fig. 3.1). The lowest dose (12.5 mg N/ seedling per cavity), representing the operational level used by commercial growers for black spruce production (Bigras and D'Aoust 1992, Calmé *et al.* 1993), was applied both conventionally (12.5C) and exponentially (12.5E). The higher dose rates (25 or 50 mg N/seedling per cavity) representing low (25E) or high (50E) nutrient loading levels were delivered exponentially. Fertilizer treatments started 2 weeks after germination to avoid injury to young germinants and promote early inoculum infection. Weekly applications were supplied as a commercial water-soluble fertilizer mixture (Plant Products as 20:10:20 N-P_2O_5-K_2O plus micronutrients). Scheduled measured nutrients were applied by hand to the growing media for each cavity using a plastic measuring cup. Although fertilizer doses varied with treatments, all seedlings received the same volume of solution (5 mL) per application.

The delivery schedule of the conventional regime consisted of repeated applications of fertilizer at a constant addition rate (0.69 mg N/cavity/seedling per week) as topdressings. The delivery schedule for exponential fertilization was based on the following exponential function described by Ingestad and Lund (1979) and Timmer and Armstrong (1987):

$$[1] \quad N_T = N_S (e^{rt} - 1)$$

where N_T is the selected amount of N to be added in the total number of applications (18), N_S is the initial amount of N in the seedling (0.2 mg) determined at the beginning of the fertilization, t is the number of fertilization periods, up to and including, the current period, and r is the relative addition rate needed to increase N_S to a final level $N_T + N_S$. Once r was determined for the 18 fertilizer applications, the amount of N to be added on a specific day (N_t) was calculated from the following equation:

$$[2] \quad N_t = N_S (e^{rt} - 1) - N_{t-1}$$

where N_{t-1} is the cumulative amount of N added up to and including the last fertilization. The weekly application rates for each treatment are shown in Fig. 3.1; the linear and curvilinear trends clearly distinguish between constant and exponential delivery schedules.

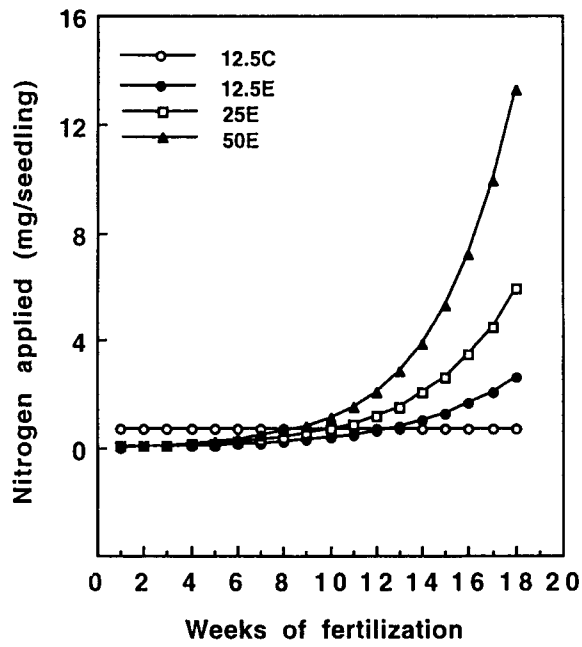


Fig. 3.1. Weekly N application schedule of black spruce seedlings grown under conventional (12.5C) and exponential (12.5E, 25E, and 50E) regimes.

Experimental design and statistical analysis

An experiment with four fertilization regimes (F) and three inoculation (I) treatments was set up according to the protocol for a factorial complete randomized block design using four replications. The experimental unit was composed of 20 seedlings per treatment for a total of 960 seedlings in the experiment (4 blocks x 4 fertilization x 3 inoculation x 20 seedlings). The fertilization regimes consisted of a conventional (12.5C) treatment (12.5 mg N/seedling) and three exponential (12.5C, 25E, and 50E) treatments (12.5, 25, and 50 mg N/seedling). The three inoculation treatments were: (*i*) *H. crustuliniforme*; (*ii*) *L. bicolor*; and (*iii*) an uninoculated control (no fungus). The seedling trays were grouped in four blocks (replications), containing the 12 treatments that were randomly distributed within the block. Seedling growth and nutrient data were evaluated by analysis of variance (ANOVA). Treatment means were separated by a least significant difference (LSD) test using a SAS statistical package (SAS® Institute Inc. 1989). The general linear model for the analysis was:

$$[3] \quad Y_{ijk} = \mu + ß_i + F_j + I_k + (FI)_{jk} + \varepsilon_{ijk}$$

where Y is the dependent variable, μ is a constant, ß is block, F is fertilization, I is inoculation, FI is fertilization x inoculation interaction, ε is the error and the levels of factors were replication $i = 4$, fertilization $j = 4$, and inoculation $k = 3$. Treatment interaction means were also tested for significance after ANOVA by orthogonal contrasts. These contrasts were (*i*) fertilization: 12.5C (conventional) versus 12.5E (exponential), (*ii*) fungal inoculation: control versus (*Hebeloma* + *Laccaria*), *Hebeloma* versus *Laccaria*, and (*ii*) fertilizer x fungal inoculation interaction: 12.5C (conventional) versus 12.5E (exponential) x control versus (*Hebeloma* + *Laccaria*), 12.5C (conventional) versus 12.5E (exponential) x *Hebeloma* versus *Laccaria*.

Seedling harvesting

The seedlings were harvested at the end of a 20-week growing period. The root system was washed free of the potting mixture and shoot length and root-collar diameters were measured for each seedling. Five of the

20 seedlings per experimental unit were examined for the percentage of ectomycorrhizal formation using a standardized procedure (Danielson *et al.* 1984). Three or more lateral roots were taken from each plant from the top, middle and bottom of the root system. Each lateral was cut into 3-cm segments of which 15-20 segments per treatment were randomly selected. Ectomycorrhizal infection was rated on 300-400 short roots using a dissecting microscope (25x magnification). Seedling root systems were examined for degree of ectomycorrhiza formation by visual estimation based on morphological characteristics (color and texture). The presence of Hartig net was also verified microscopically from a random sample of short roots treated with a fixing solution formalin-acetic acid-alcohol (FAA), sectioned, and stained with 0.1% (w/v) cotton blue in 10% (v/v) lactophenol/water. Ectomycorrhizal infection was characterized by the presence of fungal hyphal networks around short roots as well as lateral roots that formed a complete to moderately developed external mantle or sheath. The formation of Hartig nets was characterized by the presence of growing mycelia between the epidermal and cortical root cells. The percentage of ectomycorrhizal short roots (PESR) was calculated according to the method described by Beckjord *et al.* (1985).

$$[4] \quad PESR = [ESR / ESR + NSR] 100$$

where ESR and NSR were the number of ectomycorrhizal and non-ectomycorrhizal short roots per treatment, respectively. This value was used as an indicator of ectomycorrhiza formation. The remaining 15 seedlings per treatment were separated according to root and shoot components, and then pooled and oven-dried at 70°C for 48 h for biomass determination. Shoot and root dry material was ground separately for subsequent chemical analysis. The samples were wet ashed in a block digested at 380°C, using a H_2SO_4 - H_2O_2 mixture solution (Lowther 1980). The resulting digest was determined for N by the Technicon Auto Analyzer II Industrial Method No. 154 -71 W (Eastin 1978), for P by the molybdenum blue method (Allen 1974), and for K by atomic absorption (Model Perkin-Elmer 3100). The electrical conductivity (EC) of saturated aqueous extracts of growing media from both inoculated and uninoculated seedlings was measured at weeks 8 and 20 to assess soil nutrient status early and late in the growing season. Peat samples were saturated with distilled water and suction filtered as described by Timmer and Parton (1984). The EC was measured directly on the clear extract using a Metrohm 660 conductivity meter.

RESULTS AND DISCUSSION

Ectomycorrhiza formation

Microscopic examinations of seedling root systems confirmed that inoculation of black spruce seedlings at sowing with both inoculum types resulted in ectomycorrhizal formation. For inoculated seedlings, a network of dense fungal hyphae formed a thick mantle around short roots revealing a distinct Hartig net. In most cases, the ectomycorrhizal roots were typically devoid of root hairs. The uninoculated seedlings had developed root hairs, but were free of ectomycorrhizae. ANOVA (Table 3.1) revealed no significant difference (p = 0.20) in ectomycorrhizal development between inoculum types for all treatments (Fig. 3.2). Conventionally fertilized seedlings inoculated with either *H. crustuliniforme* or *L. bicolor* formed only 26% and 22% of their short roots ectomycorrhizal, respectively, values not considered sufficient for improved seedling outplanting performance (Marx and Cordell 1988). Exponential fertilization significantly increased (p = 0.0001) ectomycorrhizal formation (49-85%) compared to conventional fertilization (22-26%) for both fungal species. Short roots of the exponentially fertilized seedlings inoculated with *H. crustuliniforme* were 85, 77, and 49% ectomycorrhizal when fertilized at 12.5, 25, and 50 mg N, respectively. Inoculation with *L. bicolor* showed similar root infection rates (82, 70 and 51%) at these dose rates (Fig. 3.2). The infection levels were considered adequate for promoting outplanting performance (Holden *et al.*1983, Marx and Cordell 1988).

The infection results demonstrate the advantage of employing exponential fertilization practices over conventional fertilization to promote mycorrhizal formation in containerized planting stock (p = 0.0001), although higher loading rates reduced ectomycorrhizal development somewhat (15-40 %). Despite this dose effect, infection levels in the high loading treatment (50E) were twice those of the conventionally fertilized (12.5C) seedlings. Equivalent nutrients applied exponentially (12.5E) resulted in four fold increases in ectomycorrhizal development as compared to conventional treatments. Thus, mode of delivery rather than nutrient dose seemed crucial for the production of

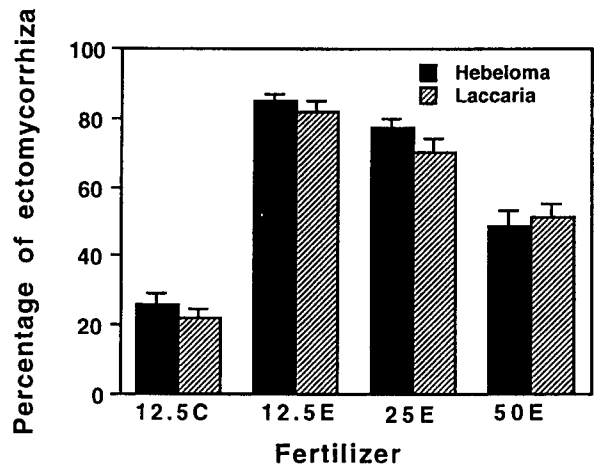


Fig. 3.2. Percentage of ectomycorrhizal short roots of black spruce seedlings inoculated with two ectomycorrhizal fungi and fertilized under conventional and exponential fertilization regimes after 20 weeks of growth. The values are means of four replicates (n = 15) with vertical bars representing standard error of the mean.

Table 3.1. ANOVA table (p > F) for growth parameters, shoot dry weight (SDM), root dry weight (RDM), total dry weight (TDM), shoot to root ratio (S/R), % ectomycorrhiza (%ECTO), and N, P, and K concentration and content of 20-week-old black spruce seedlings grown in the greenhouse under four fertility regimes and two fungal inoculation treatments.

Growth parameters and percentage of ectomycorrhiza

Source of variation	df	*P* > F				
		SDM	RDM	TDM	S/R	% ECTO[a]
Block	3	0.2281	0.0086	0.0638	0.0130	0.0778
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001
12.5C vs 12.5E	1	0.0004	0.0001	0.0001	0.0001	-
Fert E linear	1	0.0001	0.0184	0.0001	0.0001	-
Fert E quadratic	1	0.0001	0.5040	0.0004	0.0223	-
Fungus (1) [a]	2	0.2187	0.6721	0.2497	0.4656	0.1968
Fungus C vs (H+L)	1	0.3091	0.9538	0.4221	0.2451	-
Fungus H vs L	1	0.1551	0.3773	0.1445	0.6879	
Fertilizer x Fungus (3) [a]	6	0.3137	0.9168	0.4987	0.3214	0.5350
12.5C vs 12.5E x C vs (H+L)	1	0.5501	0.4282	0.4823	0.1682	-
12.5C vs 12.5E x H vs L	1	0.2623	0.9171	0.3454	0.3629	-
Fert E linear x C vs (H+L)	1	0.9086	0.6857	0.8122	0.6411	-
Fert E linear x H vs L	1	0.0342	0.6907	0.0627	0.3571	-
Fert E quadratic x C vs (H+L)	1	0.3484	0.5622	0.3355	0.8113	-
Fert E quadratic x H vs L	1	0.8030	0.3416	0.8878	0.0483	-
Error[2] (21) [a]	33	(1021.17)	(200.54)	(1567.31)	(0.2097)	(32.52)
Total (31) [a]	47					

Concentration and content of N, P, and K in seedlins

Source of variations	df	*P* > F					
		Concentration			Content		
		N	P	K	N	P	K
Block	3	0.0686	0.0766	0.0874	0.9086	0.7886	0.0414
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0003	0.0001
12.5C vs 12.5E	1	0.0001	0.0001	0.0001	0.0001	0.0181	0.1592
Fert E linear	1	0.0001	0.0001	0.7977	0.0001	0.0001	0.0001
Fert E quadratic	1	0.0986	0.2722	0.0006	0.0002	0.0025	0.7675
Fungus	2	0.0001	0.0001	0.0110	0.0025	0.0163	0.3696
Fungus C vs (H+L)	1	0.0001	0.0001	0.0083	0.0008	0.0003	0.1856
Fungus H vs L	1	0.4351	0.0030	0.1249	0.4100	0.2478	0.6396
Fertilizer x Fungus	6	0.0001	0.0182	0.0001	0.0511	0.4904	0.0062
12.5C vs 12.5E x C vs (H+L)	1	0.0212	0.0018	0.0163	0.1552	0.3032	0.1529
12.5C vs 12.5E x H vs L	1	0.0754	0.0796	0.3868	0.5153	0.4930	0.7377
Fert E linear x C vs (H+L)	1	0.1006	0.4354	0.7108	0.0885	0.6904	0.6359
Fert E linear x H vs L	1	0.0323	0.0115	0.0001	0.4642	0.0565	0.0181
Fert E quadratic x C vs (H+L)	1	0.2478	0.9220	0.0042	0.8220	0.8102	0.0067
Fert E quadratic x H vs L	1	0.1307	0.4989	0.0842	0.5412	0.9034	0.2165
Error	33	(0.0088)	(0.0001)	(0.0054)	(0.6930)	(0.0117)	(0.2481)
Total	47						

Note: Bold values under (df) indicate the single degree of freedom for the contrasts. 12.5 C and 12.5 E denote operational dose of N fertilization (mg /seedling) applied conventionally and exponentially, respectively. Fungal treatments C, H and L refer to control (no fungus), *Hebeloma*, and *Laccaria*, respectively. Fert E linear and Fert E quadratic indicate linear and quadratic relationship for exponential fertilization. [a] Applies only to the analysis of ectomycorrhizal infection.

Table 3.2. NOVA (p > F) for electrical conductivity of the growing media of *Hebeloma*- and *Laccaria*-inoculated treatments and control (no fungus) measured 8 and 20 weeks after germination.

		P > F					
		Week 8			Week 20		
Source of variations	df	**Hebeloma**	**Laccaria**	Control	**Hebeloma**	**Laccaria**	Control
Block	3	0.0502	0.1481	0.0510	0.4006	0.1434	0.3970
Fertilization	3	0.0014	0.0003	0.0006	0.0001	0.0001	0.0001
Error (Mean Square)	9	(895.45)	(695.30)	(642.88)	(20982.5)	(10509.2)	(12357.2)
Total	15						

Note: Each fungal treatment was analyzed separately.

ectomycorrhizal seedlings. Lower (p = 0.0014 for *Hebeloma*, p = 0.0003 for *Laccaria*, and p = 0.0006 for control) soil EC occurring early (week 8, see Table 3.2. Fig. 3.3) in the growing season may have benefited initial infection of exponentially fertilized seedlings more than conventionally fertilized seedlings. Relatively abundant mycorrhizae (Fig. 3.2) found at much higher (p = 0.0001 for all fungal treatments) EC levels at the end of the fertilization period (week 20) suggest that gradual and progressive nutrient enrichment from exponential fertilization may enable the fungalmycelium to develop tolerance mechanisms to high nutrient conditions (Hayman 1982, Wallander 1995). Ohenoja (1978, 1988) has noted substantial increase in fungal fruiting body production after high N fertilization, indicating that these fungi have the potential to adapt to elevated N environments by adjusting physiological function.

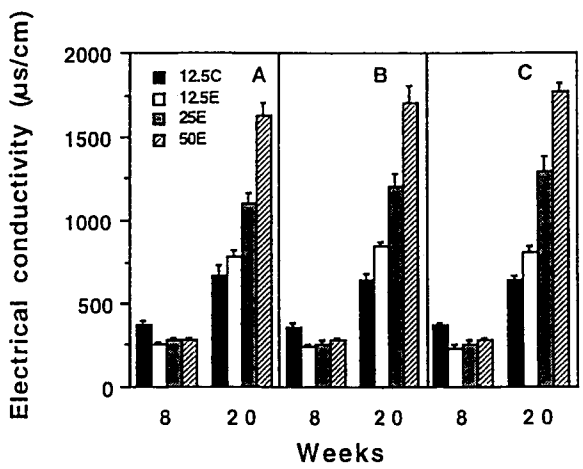


Fig. 3.3. Electrical conductivity of saturated aqueous extracts of peat growing media from black spruce container seedlings reared under a conventional and three exponential fertilization regimes and three inoculation types: (A) control, (B) *Hebeloma*, and (C) *Laccaria*. All values are means of four replicates with vertical bars representing standard error of the mean.

Seedling development and growth characteristics

Seedling survival after 20 weeks in the greenhouse was 100% and no visual symptoms of either nutrient stress or diseases were observed, indicating that ectomycorrhizal inoculation or nutrient loading treatments did not markedly influence initial seedling growth. There was no significant difference in seedling biomass productivity (p = 0.22 for shoot, p = 0.67 for root, and p = 0.25 for total dry weight) and in shoot:root ratio (p = 0.46) between control plants and plants inoculated with either *H. crustuliniforme* or *L. bicolor* for each level of N fertilization (Tables 3.1 and 3.3). Total biomass increased linearly (p = 0.0001) with increased exponential fertilization for all fungal treatments. However, reduced shoot (p = 0.0004) and root (p = 0.0001) biomass was observed under low-dose (12.5 mg N) exponential rather than conventional fertilization regardless of the fungal treatment, reflecting the lack of significant fertilizer x fungus interactions for all growth parameters. The growth reduction was probably the result of exponential fertilization rather than ectomycorrhizal treatment because of low initial nutrient supply. Early in the rotation, roots of the young seedlings have not fully occupied the container cavity, hence may not sufficiently absorb the small amount of nutrients applied exponentially (Imo and Timmer 1992). The growth inhibition was not evident with nutrient loading presumably because of comparatively high initial application levels. Further research is needed to modify or adjust low-dose exponential fertilization regimes to overcome initial growth stunting without reducing ectomycorrhizae formation of seedlings.

Shoot:root biomass ratio was significantly smaller in conventionally fertilized seedlings when compared with exponentially fertilized seedlings (p = 0.0001, Tables 3.1 and 3.3). Reduced shoot:root balance coupled with

increased nutrient dilution (particularly for N and P) probably reflects the strategy of allocating proportionally more growth to roots than shoots (Ericsson 1995) to counter late-season nutrient limitation associated with conventional fertilization schedules (Timmer 1997).

Seedling nutrient status

The main effect of fertilizer treatment on seedling nutrition (Table 3.1) was significantly higher for N (p = 0.0001), P (p = 0.0001) and K (p = 0.0001) concentration as well as for N (p = 0.0001), P (p = 0.0003) and K (p = 0.0001) content indicating that nutrient supplementation increased seedling nutrient

Table 3.3. Effct of ectomycorrhizal inoculation and fertilization regimes on seedling growth parameters and N, P, and K concentration of black spruce seedlings after 20 weeks of growth.

Fungal treatment	N level (mg/seedling per season)				
	12.5C	12.5E	25E	50E	Means
			Shoot dry weight (mg)		
Hebeloma	455.0	416.2	500.2	543.5	478.7 A
Laccaria	473.4	398.2	512.9	596.2	495.1 A
Control	453.2	413.0	538.2	583.7	497.0 A
Means	460.5 c	409.1d	517.1 b	574.5 a	
			Root dry weight (mg)		
Hebeloma	133.5	85.3	96.4	96.5	102.9 A
Laccaria	141.1	91.4	91.5	105.7	107.4 A
Control	140.7	82.0	96.0	101.0	103.0 A
Means	138.4 a	86.2 c	94.6 bc	101.1 b	
			Total dry weight (mg)		
Hebeloma	588.5	501.6	596.6	640.0	581.7 A
Laccaria	614.5	489.6	604.4	701.9	602.6 A
Control	594.0	495.0	634.2	684.7	602.0 A
Means	599.0 b	495.4 c	611.7 b	675.5 a	
			Shoot:root ratio		
Hebeloma	3.5	4.9	5.2	5.6	4.8 A
Laccaria	3.4	4.4	5.6	5.6	4.8 A
Control	3.3	5.0	5.7	5.8	5.0 A
Means	3.4 c	4.8 b	5.5 a	5.7 a	
			N concentration (%)		
Hebeloma	1.25	1.82	2.04	2.35	1.9 A
Laccaria	1.12	1.86	2.13	2.25	1.8 A
Control	1.24	1.70	1.76	1.95	1.7 B
Means	1.20 d	1.80 c	2.00 b	2.20 a	
			P concentration (%)		
Hebeloma	0.19	0.27	0.26	0.24	0.24 A
Laccaria	0.18	0.26	0.24	0.23	0.23 B
Control	0.17	0.22	0.21	0.21	0.20 C
Means	0.18 d	0.25 a	0.24 b	0.23 c	
			K concentration (%)		
Hebeloma	1.0	1.2	1.1	1.2	1.1 A
Laccaria	1.1	1.3	1.1	1.2	1.2 A
Control	1.0	1.1	1.0	1.1	1.0 B
Means	1.0 b	1.2 a	1.1 b	1.2 a	

Note: Mean values within columns and rows followed by different letters are significantly different at $p < 0.05$ level (LSD test).
All values are mean of four replicates (n = 15).

uptake. Ectomycorrhiza formation further elevated both N (p = 0.0001), and P (p = 0.0001) uptake in seedlings (Fig. 3.4, Table 3.1), presumably because of enhanced nutrient uptake by ectomycorrhizal root systems with enlarged nutrient-absorbing surface area (Navratil *et al.* 1981). The extramatrical mycelium is considered especially important for increasing the absorbing capacity of ectomycorrhizal roots (Harley 1989) by facilitating rapid and efficient transfer of assimilated N to the host and promoting conversion of N compounds into more readily utilized forms (Abuzinadah and Reid 1988). This conversion may take place mainly through the glutamate dehydrogenase (GDH) pathway (Martin and Botton 1993, Quoreshi *et al.* 1995) that requires less energy than the GS-GOGAT (glutamine synthetase-glutamine synthase) pathway in the higher plant roots. Mycorrhizae also may increase N available to seedlings

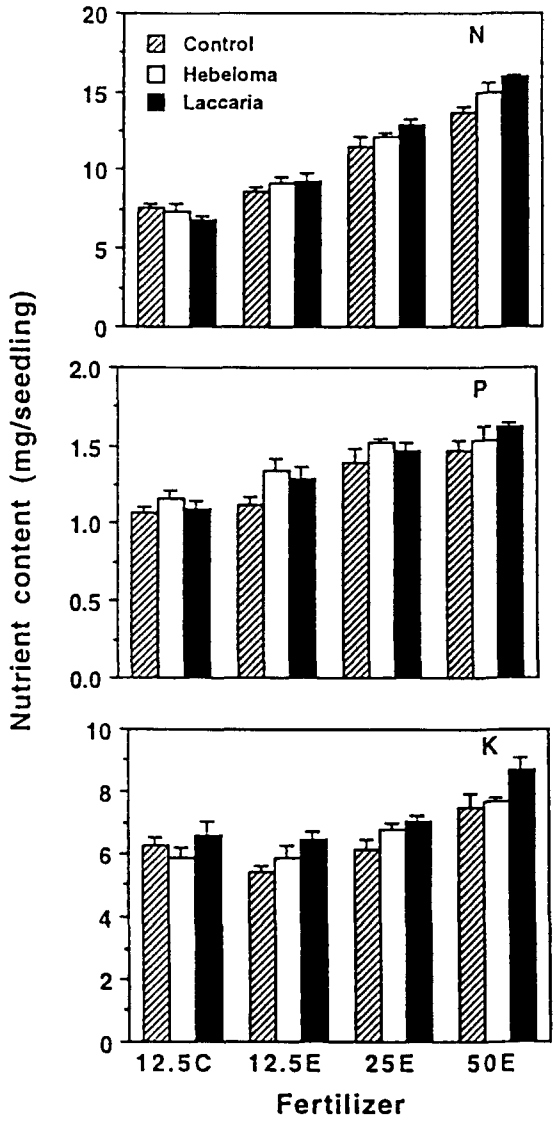


Fig. 3.4. Plant N, P, and K content of seedlings produced under conventional and exponential regimes and three inoculation types. The values are means of four replicates (n = 15) with vertical bars representing standard error of the mean.

by utilizing organic N sources in the growing media (Read *et al.* 1989). Synchronized addition of nutrients with seedling growth under exponential regimes may have further contributed to improved mineral uptake, since addition rates correspond more closely to the desired relative growth rate and nutrient demand which enhanced nutrient uptake efficiency (Timmer 1997). However, under high loading regimes uptake efficiency declined, contributing to nutrient accumulation in the growing media (as noted by higher EC levels) rather than in the seedlings (Fig. 3.3). Not only did loading stimulate plant nutrient uptake, but ectomycorrhizal inoculation also contributed to enhanced plant nutrient uptake. Comparison of inoculated and non-inoculated seedlings revealed that inoculation improved nutrient loading efficiency by increasing N, P and K uptake by 6-17, 5-20 and 4-18%, respectively. The highest tissue nutrient concentration of ectomycorrhizal seedlings was 2.35, 0.27 and 1.3 % of dry mass for N, P and K (Table 3.3), appreciably higher than reported for ectomycorrhizal black spruce seedlings reared in containers in previous studies (Ekwebelam and Reid 1983, Gagnon *et al.* 1987, 1988).

The results demonstrate the advantages of exponential fertilization compared to conventional fertilization for rearing ectomycorrhizal conifer seedlings. Inoculation treatments combining exponential regimes with nutrient loading practices increased element uptake without changing seedling biomass significantly, thus contributing to the buildup of nutrient reserves as luxury consumption for later retranslocation and utilization during field growth (Malik and Timmer 1995, 1996). The fact that ectomycorrhizal inoculations under exponential fertilization regimes can maintain the symbiotic relationship even at high loading levels while enhancing nutrient uptake contradicts recent findings by Gagnon *et al.* (1995) that N status of inoculated seedlings was usually lower than N status of uninoculated seedlings. This study, however, has dealt only with seedlings during the pre-hardening phase of the greenhouse rotation. Post-hardening sampling has revealed declining nutrient levels in both the soil and seedlings after exponential nutrient loading because of leaching and reduced fertilization (Miller and Timmer 1994, 1997), which may further benefit ectomycorrhizal formation during this period.

Nitrogen supply and carbohydrate availability to mycorrhizal fungi are considered the key mechanisms contributing to the mycorrhizal symbiosis (Marx *et al.* 1977). The current results showing that adequate mycorrhizal infection occurred under high nutrient inputs support the view of Nylund and Wallander (1989), and Wallander and Nylund (1991) that mineral nutrient deficiency is not necessarily a precondition for the formation of mycorrhiza. It was originally suggested by Bjorkman (1942) that the host plant allocates less carbohydrate to the fungal partner at high levels of N and P supply owing to the greater demand for C by growing shoots under such conditions, hence

limiting mycorrhiza formation. This result suggests that inhibiting mycorrhizal development noted in previous studies may not be related to carbohydrate deficiency, and concurs with recent findings (by Ingestad *et al.* 1986, Nylund 1988, Nylund and Wallander 1989) that a high nutrient supply does not necessarily restrict the symbiosis when seedlings were reared under a range of relative nutrient addition rates.

CONCLUSIONS

I have found that under experimental greenhouse conditions simulating operational culture, it is feasible to produce acceptable ectomycorrhiza-infected containerized black spruce seedlings by combining early mycelial inoculation with exponential fertilization. Infection rates greater than 70 and 50% of short roots was achieved even at high levels (25 and 50 mg respectively) of N addition. Inoculation was also associated with increased N, P, and K uptake suggesting that the mycorrhizal treatments may contribute effectively to nutrient loading of conifer seedlings. The mode of nutrient delivery rather than the amount of fertilizer was critical for the production of nutrient loaded ectomycorrhizal seedlings. Progressively increasing nutrient exposure under exponential fertilization may favor tolerance and acclimatization of mycorrhizae on seedling to higher nutrient levels. Current nursery practice of applying fertilizer at a constant addition rate can be modified to implement exponentially-based fertilization to produce both nutrient loaded and mycorrhizal infected seedlings. The dry matter productivity of all seedlings equaled or exceeded the provincial standard (500 mg) for containerized black spruce planting stock before the hardening phase of the rotation (Galloway and Squires 1988), demonstrating that exponential culture of mycorrhizal infected seedlings can meet morphological and nutritional standards for seedling quality. Further research is needed to evaluate the benefit of ectomycorrhizal status on survival, growth and nutrition of inoculated seedlings when outplanted in the field.

Chapter 4

GROWTH, NUTRIENT DYNAMICS AND ECTOMYCORRHIZAL DEVELOPMENT OF CONTAINER-GROWN *Picea mariana* SEEDLINGS IN RESPONSE TO EXPONENTIAL NUTRIENT LOADING *

Summary

Containerized black spruce (*Picea mariana* (Mill.) B.S.P.) seedlings fertilized conventionally (12.5 mg N/plant) or exponentially (12.5, 25 or 50 mg N/plant) and inoculated with *Hebeloma crustuliniforme* or *Laccaria bicolor* were periodically monitored for a 20-week greenhouse rotation to assess growth dynamics, steady-state N and P nutrition, and ectomycorrhizal development. Growth and nutrient accumulation increased exponentially for the exponential regimes and more linearly for the conventional regime, although final biomass was similar except for the low dose exponential addition. Shoot:root biomass ratios were relatively stable for most of the growing season characterizing steady-state nutrient supply that benefits seedling outplanting performance and mycorrhizal colonization. Exponential fertilization also stimulated mycorrhiza formation even at high loading (25, 50 mg N) rates that build up nutrient reserves in the seedlings without affecting seedling size. Plant nutrient uptake was more efficient under exponential fertilization and (or) fungal colonization, although efficiency dropped off at high loading levels. Vector nutrient diagnosis revealed marked nutrient dilution under conventional fertilization, but steady-state nutrition under exponential fertilization, which coincided with satisfactory mycorrhizal development on seedlings. Dilution-free nutrient conditions for seedlings may provide stable carbohydrates for symbiosis and may develop enhanced tolerance to high fertilizer inputs.

INTRODUCTION

Regeneration of black spruce with container-grown planting stock is widely practiced in the reforestation of northern Ontario (Arnup *et al.* 1988, Timmer 1997). Although conventional nursery culture may produce planting stock of satisfactory size, seedlings may suffer from inadequate nutrition (as evident from reports of positive growth response to field fertilization (Timmer and Munson 1991, van den Driessche 1991, Imo and Timmer 1997,1999)), lack of ecologically adapted ectomycorrhizal fungi (Marx 1991), and inconsistent natural inoculation from outside spores (Browning and Whitney 1992, Kropp and Langlois 1990). Studies have shown that non-mycorrhizal seedlings may also limit successful regeneration of conifers on both fertile (Marx and Cordell 1988, Browning and Whiteny 1993) and disturbed sites with low inoculum potential (Le Tacon *et al.* 1988). Extra high fertilization, or nutrient loading, in the nursery has enhanced growth and nutrition of newly planted spruce seedlings on a variety of site conditions (Timmer and Munson 1991, Malik and Timmer 1995, 1996; Imo and Timmer 1998, McAlister and Timmer 1998). The response was attributed to the build-up of high nutrient reserves in the seedlings (from 1.0 –1.5 to 2.5-3.0 % N) by luxury consumption during nursery culture for retranslocation and reuse soon after outplanting to meet nutrient demands of new growth (Malik and Timmer 1998). Seedling quality may be further improved by combining both nutrient loading and inoculation of seedlings with site specific ectomycorrhizal fungi that promote subsequent survival and performance in the field (Cordell *et al.* 1987, Browning and Whitney 1992, 1993,

* Reproduced from Canadian Journal of Forest Research, Quoreshi, A.M., and Timmer, V.R. Growth, nutrient dynamics and ectomycorrhizal development of container grown *Picea mariana* seedlings in response to exponential nutrient loading. 30, 191-201, 2000, with permission from CJFR.

Smith and Mohammed 1997).

Mycorrhizal status of current commercial container stock is low because mycorrhizal fungi are usually sensitive to high fertilization regimes applied conventionally in nursery culture (Marx *et al.* 1977, Kropp and Langlois 1990, Väre 1990, Browning and Whitney 1992). Improved colonization is usually achieved by reducing rather than increasing nutrient supply, which produces seedlings with adequate mycorrhizal development but often deficient in nutrients and below acceptable size (Gagnon *et al.* 1987, 1988, Ruehle and Wells 1984). Few studies have examined mycorrhiza formation with respect to progressively increasing fertilizer applications. Gradual exposure to mineral nutrients under exponential fertilization did not inhibit ectomycorrhizal development when seedlings were grown hydroponically under steady-state nutrient conditions (Ingestad *et al.* 1986). This was achieved by applying nutrients incrementally at exponential rates corresponding closely to the relative growth and nutrient uptake rate of seedlings during their exponential growth phase (Ingestad and Lund 1986). Steady-state nutrition in plants was characterized by stable internal nutrient concentration during exponential growth that is relatively free of nutrient stress.

Compared to conventional fertilization, exponential fertilization is considered advantageous for nutritionally preconditioning seedlings for the field because (*i*) dilution-free nutrient accumulation during seedling production (Imo and Timmer 1992, 1997) corresponds well with steady-state nutrient uptake of natural exponentially growing seedlings in the forest (Munson and Bernier 1993), (*ii*) initial low level nutrient additions match field soil-solution nutrient levels more closely (Linder and Rook 1984), and (*iii*) nutrient delivery to container-restricted root systems simulates nutrient flux reached by expanding roots in forest soils with constant rate nutrient availability (Pettersson 1986). Similarly, these conditions are beneficial for mycorrhiza development in the nursery because low starting fertilizer levels enhance inoculum potential and balanced, dilution-free nutrient accumulation favors stable internal carbohydrate availability that sustains symbiotic relationships (Ingestad *et al.* 1986, Nylund and Wallander 1989).

In an earlier study (Chapter 3), I demonstrated that exponential nutrient loading of container-grown black spruce seedlings was compatible with adequate ectomycorrhiza formation without causing serious toxicity in the fungi. I speculatively attributed this response to the gradual exposure of the symbionts to high fertilizer inputs under exponential fertilization while plant nutrient concentration remained stable (Ingestad *et al.* 1986), allowing mycorrhiza to adapt to high nutrient supply. However, I was unable to confirm steady-state nutrient status in the crop because there was a single harvest data. In this Chapter, I investigated these responses in more detail by examining the dynamics of seedling growth and nutrition, ectomycorrhiza formation and growing medium fertility at intervals during the greenhouse-growing season. The specific objectives were to determine whether steady-state nutrition was achieved under this experimental approach, and to examine relationships between exponential nutrient loading, steady-state nutrient conditions, growth medium fertility, and sustained mycorrhizal colonization.

MATERIALS AND METHODS

Cultural conditions and establishment of seedlings

Growth conditions and procedures for seedling production were described in detail in Chapter 3. Container-grown black spruce seedlings were raised for 20 weeks in the greenhouse between June and November of 1995, using steam sterilized, moist horticultural grade sphagnum peat moss and vermiculite in the proportion 10:1 (v/v) with or without ectomycorrhizal inoculum. Seedlings were reared at ambient air temperatures averaging 18-25°C and a relative humidity of 65-85% with supplementary light to an extended 20-h photoperiod at a light intensity of 250 $\mu mol.\ s^{-1}.\ m^{-2}$. Spray nozzles were used to irrigate seedlings when needed to container capacity of the peat growing medium (Timmer and Armstrong 1989) and to avoid moisture saturation and possible fertilizer loss due to leaching. The trays were rotated in position every two weeks to minimize edge effects.

Ectomycorrhizal fungal inoculation

Pure cultures of two species of ectomycorrhizal fungi, *Hebeloma crustuliniforme* (Bull. Ex St-Amans) Quel. and *Laccaria bicolor* (R. Mre.) Orton were used to inoculate the growth media prior to seeding with black spruce. The live vegetative mycelial inoculum was grown aseptically on a vermiculite carrier and obtained in sterilized condition in a special bag and shipped by courier mail from Plant Health Care Inc. Pittsburgh, PA 15238, U.S.A (for details on inoculum preparation, see Appendix 1). The fungal inoculum was thoroughly mixed into the seedling growth media at a rate of 1:10 (by volume) to obtain a homogenous mixture of inoculum and peat medium within each container cavity (Chapter 3). For the non-mycorrhizal (NM) control treatment, instead of a vermiculite inoculum, a similar volume of sterilized vermiculite moistened with sterilized water was used. The 110 cm^3 capacity hard wall containers (Rootrainers®, Spencer-Lemaire Industries Limited, Edmonton, Alberta, Canada) were arranged in trays, each holding 50 cells in rows or "books" of five. Opening the hinged wall of each "book" facilitated inspection of mychorrhizal colonization of the root plug with minimal disturbance of the seedlings.

Fertilization treatments

Two weeks after seed germination, the seedlings received their first weekly fertilization. Seedlings were fertilized for 18 weeks with a commercial water-soluble fertilizer mixture (Plant Products as 20:10:20 N-P_2O_5-K_2O plus micronutrients). Four fertilizer regimes were

applied to the seedlings with increasing amounts of nutrients being supplied either conventionally or exponentially during the culture rotation (Table 4.1). The conventional treatment (12.5C), representing the industry's operational regime (12.5 mg N/seedling/ cavity per growing season) for containerized black spruce seedling production in Ontario (Timmer 1997) was applied at a constant addition rate (0.69 mg N/seedling per week). The exponential fertilization schedules (E) involved three levels of fertilizer application at exponentially increasing rates: 12.5 mg N/seedling per cavity (12.5E) to simulate operational levels; 25 mg N/seedling per cavity (25E) for low nutrient loading; and high nutrient loading at 50 mg N/seedling per cavity (50E) levels. The delivery schedule for exponential fertilization was calculated as previously described (Chapter 3) based on the exponential function described by Ingestad and Lund (1986) and Timmer and Armstrong (1987).

Experimental design and statistical analyses

Treatments were arranged in a complete randomized block design on the four fertilization regimes (F) and the three inoculation (I) treatments. The fertilization regimes consisted of a conventional treatment (12.5C) and three exponential treatments (12.5E, 25E, and 50E) during the 18 weeks growing period. The three inoculation treatments were: (*i*) *Hebeloma crustuliniforme*; (*ii*) *Laccaria bicolor*; and, (*iii*) an uninoculated control (no fungus). Each experimental unit contained two trays consisting of 100 seedlings per treatment for a total of 4800 seedlings (96 trays): 4 blocks x 4 fertilization x 3 inoculation x 100 seedlings. Half of the seedlings were used for monitoring ectomycorrhizal formation, growth, and nutrient status at different harvests and the rest were used for winter hardening and a subsequent outplanting experiment. The trays were grouped in four blocks (replications), containing the 12 treatments that were randomly distributed within the block. All data were tested by

Table 4.1. Weekly application of N and P on black spruce seedlings grown in containers under conventional (12.5C) and three exponential (12.5E, 25E, and 50E) regimes for 20 weeks in the greenhouse.

Fertilization	Nitrogen (mg/seedling per season)				Phosphorus (mg/seedling per season)			
Week	12.5C	12.5E	25E	50E	12.5C	12.5E	25E	50E
1	0.69	0.05	0.06	0.07	0.15	0.01	0.01	0.02
2	0.69	0.07	0.08	0.10	0.15	0.01	0.02	0.02
3	0.69	0.08	0.11	0.13	0.15	0.02	0.02	0.03
4	0.69	0.10	0.14	0.18	0.15	0.02	0.03	0.04
5	0.69	0.13	0.18	0.25	0.15	0.03	0.04	0.05
6	0.69	0.16	0.24	0.33	0.15	0.04	0.05	0.07
7	0.69	0.21	0.31	0.45	0.15	0.05	0.07	0.10
8	0.69	0.26	0.41	0.62	0.15	0.06	0.09	0.14
9	0.69	0.33	0.53	0.84	0.15	0.07	0.12	0.18
10	0.69	0.41	0.69	1.14	0.15	0.09	0.15	0.25
11	0.69	0.52	0.91	1.55	0.15	0.11	0.20	0.34
12	0.69	0.66	1.18	2.10	0.15	0.14	0.26	0.46
13	0.69	0.83	1.55	2.86	0.15	0.18	0.34	0.63
14	0.69	1.04	2.03	3.89	0.15	0.23	0.44	0.85
15	0.69	1.31	2.65	5.28	0.15	0.28	0.58	1.15
16	0.69	1.65	3.47	7.18	0.15	0.36	0.76	1.57
17	0.69	2.08	4.54	9.76	0.15	0.45	0.99	2.13
18	0.69	2.62	5.94	13.27	0.15	0.57	1.30	2.9
Total	**12.5**	**12.5**	**25.0**	**50.0**	**2.7**	**2.7**	**5.5**	**10.9**

Note: Fertilizer was applied two weeks after germination.

analysis of variance (ANOVA) using SAS (SAS® Institute Inc. 1989). The general linear model for the analysis was:

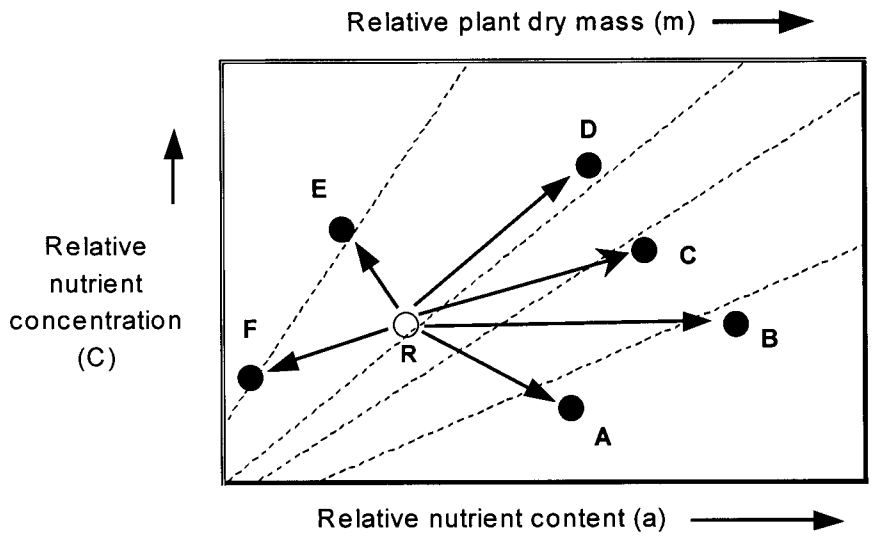
$$Y_{ijk} = \mu + \beta_i + F_j + I_k + (FI)_{jk} + \varepsilon_{ijk}$$

Where levels of factors were, replication i = 4, fertilization j = 4, and inoculation k = 3. Other factors were, Y = dependent variable, μ = constant, β = block, F = fertilization, I = inoculation, FI = fertilization x inoculation interaction and ε = error. The least significant difference (LSD) test was used to determine the significance of between treatment means.

Interactions between the treatment means were further evaluated for significance by predetermined, single degree of freedom orthogonal contrasts. The contrasts selected were (1) fertilization: 12.5C (conventional) versus 12.5E (exponential); (2) fungal inoculation: control versus (*Hebeloma* + *Laccaria*), *Hebeloma* versus *Laccaria*; and (3) fertilizer x fungal inoculation interaction: 12.5C (conventional) versus 12.5E (exponential) x control versus (*Hebeloma* + *Laccaria*), 12.5C (conventional) versus 12.5E (exponential) x *Hebeloma* versus *Laccaria*.

Harvesting and measurements

Seedlings were harvested randomly from each treatment at 8, 12, 16 and 20 weeks after germination. Forty seedlings per treatment were harvested during the first three harvests, whereas 80 seedlings per treatment were harvested at the end of a 20-week growing season. At each harvest, the seedling root system was washed carefully to remove the growing medium. Ten or twenty seedlings per experimental unit were examined using a dissecting microscope (25X) in each harvest to obtain the percentage of ectomycorrhizal development according to the procedure described earlier by Danielson *et al.* (1984) and Chapter 3. Ectomycorrhizal infection was characterized by the presence of fungal networks around short roots as well as laterals that formed a complete to moderately developed external mantle or sheath as reflected in Photo plates 4.1 and 4.2, showing the ectomycorrhizal roots were typically devoid of root hairs and a dense fungal hyphae formed a mantle around the short roots. The uninoculated roots



Vector shift	Change in relative			Interpretation	d(c)/dt	Diagnosis
	m	a	c			
A	+	+	-	Dilution	<0	Growth dilution
B	+	+	o	Sufficiency	=0	Steady-state
C	+	+	+	Deficiency	<1,>0	Deficiency
D	o	+	+	Luxury	=1	Accumulation
E	-	-, +	+	Excess	>1	Toxic accumulation
F	-	-	-	Excess	=0	Antagonism

Fig. 4.1. Interpretations of directional changes with time in dry mass, nutrient concentration, and nutrient uptake during seedling development. The reference point (R) represents an initial status of plant at t0 (week 8), and was normalized to 100. Vector orientation and magnitude characterize parameter relationships [increase (+), decrease (-), or no change (0)] relative to seedlings harvested at subsequent time intervals. Arrow direction also depicts time progression from time t0, t1, t2.... (Modified from Imo and Timmer 1997; Timmer 1991).

showed root hairs and were free of ectomycorrhizae. The presence of Hartig net and fungal mantle were confirmed microscopically from a random sample of short roots described in Chapter 3. The formation of a Hartig net was characterized by the presence of growing mycelia between the epidermal and cortical cells. The remaining 30 or 60 seedlings from each treatment (depending on different harvest dates) were separated into root and shoot component which were then oven-dried at 70°C for 48 hours to determine biomass, shoot:root ratios and for subsequent chemical analysis. Plant nutrient analyses and the electrical conductivity (EC) of growth media were determined by the procedure described in Chapter 3. Only N and P data are presented in this paper since these elements are usually most affected by mycorrhizal colonization (Smith and Read 1997).

Vector diagnosis

Vector diagnosis, a graphical tool for evaluating plant tissue analysis, was used to interpret seedling nutrient status and fertilizer requirements (Timmer 1991, Haase and Rose 1995). The technique has recently been refined to assess steady-state nutrient achievement by incorporating a time variable in the format (Imo and Timmer 1997). The analysis compares relative responses of plant growth and nutrition over time in a single nomogram with dry matter production and nutrient content on the horizontal axes and concentration on the vertical axis (Fig. 4.1). Nutrient concentration (c) is a product ($c = a/m$) of nutrient uptake (a) and biomass accumulation (m) in plants or plant components, hence concentration change with time can be expressed by the function: $d(c)/dt = d(a/m)/dt$ (Imo and Timmer 1997). Relative change in plant samples taken at progressive time intervals can be represented by vectors where the initial sampling (used as reference) is normalized to 100%. Diagnostic interpretations (summarized in the box beneath Fig. 4.1) are based on vector size and orientation (or slope) that reflect the magnitude and direction of nutritional responses induced by treatment.

Thus *steady-state* nutrition is achieved when $d(c)/dt = 0$, as characterized by increased biomass and nutrient content but no change in nutrient concentration (vector shift B), indicating that plant nutrient uptake matched biomass accumulation over time (Imo and Timmer 1997, Ingestad and Lund 1986). Growth *dilution* occurs when $d(c)/dt<0$, as depicted by increased dry mass and nutrient uptake but declining nutrient concentration (shift A). A response to nutrient *deficiency* occurs when $1>d(c)/dt>0$, signified by increased dry mass, nutrient content, and concentration (shift C) reflecting enhanced growth and uptake by the element involved. Vector shifts D, E, and F were absent from the data, thus are not elaborated here. Temporal responses in plant development and nutrition to fertilizer delivery regimes and mycorrhizal treatments were assessed using sequential harvest data collected at 4-week intervals.

RESULTS AND DISCUSSION

Seedling growth dynamics

Fertilization and mycorrhizal treatments did not affect seedling survival, and no visible symptoms of nutrient deficiency or toxicity were evident at any stage of the 20-week growing period. Plant dry mass in all treatments increased progressively for the duration of the experiment (Fig. 4.2) following exponential growth functions (R^2 = 0.96-0.98) for the exponential regimes. Dry matter production was linear for the conventional (R^2 = 0.99) regime reflecting the constant-rate nutrient delivery schedule. Conventionally fertilized seedlings produced more biomass than exponentially fertilized seedlings early in the season presumably because of higher initial nutrient inputs (Table 4.1). However, the exponentially fertilized seedlings caught up in biomass as the season progressed, except for the low-dose treatment (12.5E), which despite slow growth met the provincial biomass standard (500 mg) for containerized black spruce before the hardening phase (Galloway and Squires 1988).

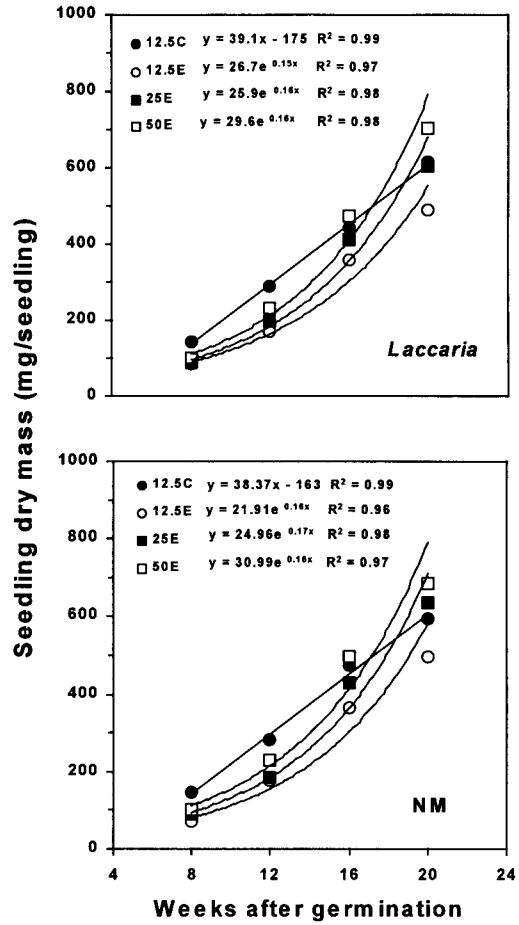


Fig. 4.2. Seasonal dry mass production of black spruce seedlings under conventional (12.5C) or three exponential fertilization regimes (12.5E, 25E, and 50E) and inoculated with (*Laccaria*) or without (NM) ectomycorrhizal fungi. The values are means of four replicates (n = 10 for week 8 and 12, and n = 15 for week 16 and 20).

Table 4.2. ANOVA table (P ≥ F) for plant dry mass (DM), shoot to root ratio (S/R), % ectomycorrhiza (% Ecto), electrical conductivity (EC), N concentration (N%) and N, P, and K content of black spruce seedlings at different harvest during greenhouse culture.

Source of variations	df	P ≥ F							
		DM	S/R	N	P	K	N (%)	% Ecto[a]	EC
Week 8									
Block	3	0.0118	0.0426	0.4262	0.1534	0.1538	0.0006	0.1568	0.0004
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0006	0.0001	0.0001
12.5C vs 12.5E	1	0.0001	0.0003	0.0001	0.0001	0.0001	0.0001	-	-
Fert E linear	1	0.0001	0.7077	0.0001	0.0005	0.0001	0.0810	-	-
Fert E quadratic	1	0.4235	0.7649	0.1181	0.8252	0.3881	0.4684	-	-
Fungus (1)[a]	2	0.0947	0.0221	0.0004	0.0001	0.1048	0.0001	0.2185	0.5982
Fungus C vs (H+L)	1	0.3641	0.0063	0.0002	0.0001	0.2102	0.0001	-	-
Fungus H vs L	1	0.0500	0.7866	0.1170	0.1234	0.0828	0.1890	-	-
Fertilizer x Fungus (3)[a]	6	0.6783	0.0816	0.7447	0.7605	0.7893	0.8249	0.2019	0.9730
12.5C vs 12.5E x C vs (H+L)	1	0.0987	0.0745	0.2411	0.8364	0.3130	0.7750	-	-
12.5C vs 12.5E x H vs L	1	0.9082	0.0238	0.4452	0.5208	0.9947	0.4188	-	-
Error mean square (21)[a]	33	115.72	0.1718	0.0143	0.0012	0.0161	0.0157	60.89	1194
Total (31)[a]	47								
Week 12									
Block	3	0.3314	0.0001	0.0726	0.3909	0.2255	0.0470	0.6659	0.9991
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
12.5C vs 12.5E	1	0.0001	0.0001	0.0001	0.0001	0.0001	0.0007	-	-
Fert E linear	1	0.0001	0.0029	0.0001	0.0001	0.0002	0.0010	-	-
Fert E quadratic	1	0.1425	0.2330	0.1538	0.8286	0.9261	0.1181	-	-
Fungus (1)[a]	2	0.0038	0.0106	0.0156	0.0014	0.0043	0.0001	0.1792	0.0832
Fungus C vs (H+L)	1	0.0022	0.2773	0.0480	0.0050	0.9115	0.0001	-	-
Fungus H vs L	1	0.3282	0.0046	0.0285	0.0113	0.0011	0.1316	-	-
Fertilizer x Fungus (3)[a]	6	0.6491	0.0400	0.4993	0.4246	0.7359	0.5949	0.6871	0.1849
12.5C vs 12.5E x C vs (H+L)	1	0.1390	0.0161	0.7252	0.2812	0.4010	0.3857	-	-
12.5C vs 12.5E x H vs L	1	0.9905	0.0377	0.8133	0.9118	0.6122	0.2928	-	-
Error mean square (21)[a]	33	584.75	0.1506	0.1592	0.0045	0.0923	0.0238	74.84	2051
Total (31)[a]	47								
Week 16									
Block	3	0.0034	0.0034	0.1654	0.0217	0.0447	0.0034	0.7779	0.9064
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
12.5C vs 12.5E	1	0.0001	0.0690	0.7746	0.6379	0.0007	0.0001	-	-
Fert E linear	1	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	-	-
Fert E quadratic	1	0.0602	0.0302	0.0277	0.1197	0.4292	0.3367	-	-
Fungus (1)[a]	2	0.0156	0.0001	0.0025	0.0047	0.5008	0.0001	0.2908	0.4976
Fungus C vs (H+L)	1	0.0061	0.2734	0.0008	0.0012	0.8126	0.0001	-	-
Fungus H vs L	1	0.3544	0.7283	0.3342	0.9327	0.2527	0.0204	-	-
Fertilizer x Fungus (3)[a]	6	0.9626	0.3443	0.7575	0.9964	0.4733	0.3443	0.9349	0.2847
12.5C vs 12.5E x C vs (H+L)	1	0.3092	0.8342	0.6246	0.9817	0.0274	0.8342	-	-
12.5C vs 12.5E x H vs L	1	0.8467	0.0398	0.1322	0.6333	0.7670	0.0400	-	-
Error mean square (21)[a]	33	796.92	0.1283	0.5230	0.0155	0.1561	0.0092	69.22	5735
Total (31)[a]	47								
Week 20									
Block	3	0.0638	0.0130	0.9086	0.1721	0.0410	0.0700	0.0778	0.0266
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
12.5C vs 12.5E	1	0.0001	0.0001	0.0001	0.0181	0.1592	0.0001	-	-
Fert E linear	1	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	-	-
Fert E quadratic	1	0.0004	0.0223	0.0002	0.0025	0.7675	0.1000	-	-
Fungus (1)[a]	2	0.2497	0.4656	0.0025	0.0007	0.3697	0.0001	0.2000	0.1893
Fungus C vs (H+L)	1	0.4221	0.2451	0.0008	0.0003	0.1856	0.0001	-	-
Fungus H vs L	1	0.1445	0.6879	0.4100	0.2487	0.6396	0.4351	-	-
Fertilizer x Fungus (3)[a]	6	0.4987	0.3214	0.0511	0.5385	0.0062	0.0001	0.5350	0.6244
12.5C vs 12.5E x C vs (H+L)	1	0.4823	0.1682	0.1552	0.3032	0.1529	0.0212	-	-
12.5C vs 12.5E x H vs L	1	0.3454	0.3629	0.5153	0.4930	0.7377	0.0754	-	-
Error mean square (21)[a]	33	1567.3	0.2097	0.6930	0.0117	0.3481	0.0088	32.52	16046
Total (31)[a]	47								

Note: Fert E linear and Fert quadratic indicate linear and quadratic relationship for exponential treatments. Fungal treatments C, H, and L refer to non-mycorrhizal control (no fungus), *Hebeloma*, and *Laccaria*, respectively. 12.5C and 12.5E, operational doses of fertilization (mg N per seedling) applied conventionally and exponentially, respectively.

[a] Values in parentheses are the dfs that apply only to the analysis of ectomycorrhizal infection.

Growth partitioning (Table 4.3) was affected by fertilization throughout the season (p = 0.0001, Table 4.2) reflecting the different nutrient flux to roots under the two types of delivery schedules. Conventional fertilization favored shoot growth (23-45%) at the expense of root growth early in the growing season because of the relatively high nutrient inputs in relation to plant demand. Consequently, conventionally fertilized seedlings exhibited higher (p = 0.0001) initial shoot:root biomass ratios than exponentially fertilized seedlings. This trend was reversed later in the season when growth and nutrient demand was larger relative to nutrient inputs. Shoot:root ratios of exponentially fertilized seedlings remained stable until week 16 indicating steady-state supply of nutrients, but increased at the end of the season probably because of high late-season fertilizer applications (Table 4.1). Seedling biomass production was unaffected by inoculation (p = 0.25, Table 4.2) at sampling intervals, indicating that host carbohydrate utilization by fungal symbionts did not reduce plant growth. Seedling growth and nutritional responses were non-significant between *Hebeloma crustuliniforme* and *Laccaria bicolor*, hence only data from the latter are presented in Figs. 4.2-4.4 to minimize duplication.

Nutrient uptake dynamics

Nutrient content in seedlings consistently increased during the season following patterns of biomass production, although uptake rates varied depending on fertilization regimes (Fig. 4.3). A distinct pattern of exponential accumulation of N and P was evident from exponential delivery, confirming that exponentially increasing nutrient additions match exponential growth

Table 4.3. Shoot to root ratio of black spruce seedlings produced under conventional (12.5C) and three levels of exponential fertilization regime (12.5E, 25E, and 50E) and under three inoculation treatments over 20 week period.

Fungal treatments	N level (mg/seedling per season)				
	12.5C	12.5E	25E	50E	Means
			Week 8		
Hebeloma	3.6	3.2	2.8	2.9	3.1 B
Laccaria	3.9	2.5	2.9	3.1	3.1 B
Control	3.8	3.6	3.4	3.1	3.5 A
Means	3.8 a	3.1 b	3.0 b	3.0 b	-
			Week 12		
Hebeloma	4.6	3.2	3.1	3.4	3.6 A
Laccaria	3.6	3.1	2.8	3.2	3.2 B
Control	4.2	2.4	2.8	3.5	3.2 B
Means	4.2 a	2.9 c	2.9 c	3.4 b	-
			Week 16		
Hebeloma	3.6	3.0	4.0	4.5	3.7 A
Laccaria	3.5	3.3	3.8	4.3	3.7 A
Control	3.4	3.4	4.0	4.6	3.8 A
Means	3.5 c	3.2 c	3.9 b	4.5 a	-
			Week 20		
Hebeloma	3.5	4.9	5.2	5.6	4.8 A
Laccaria	3.4	4.4	5.6	5.6	4.8 A
Control	3.3	5.0	5.7	5.8	5.0 A
Means	3.4 c	4.8 b	5.5 a	5.7 a	-

Note: Mean values within columns and rows followed by different letters are significantly different at the p> 0.05 level (LSD test). All values are means of four replicates.

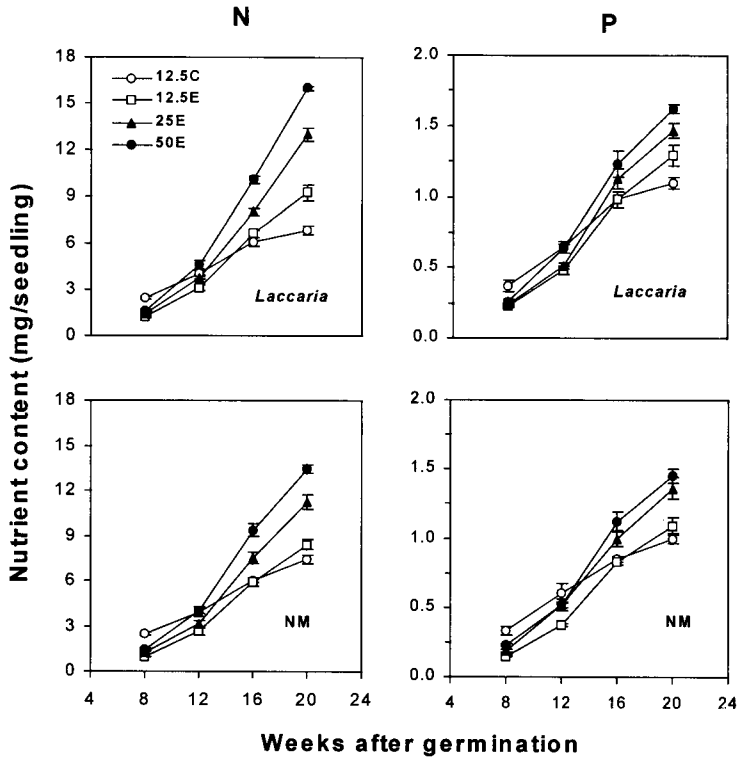


Fig. 4.3. Seasonal N and P uptake of seedlings raised under various fertilization regimes and inoculated with (Laccaria) or without (NM) ectomycorrhizal fungi, during a complete greenhouse rotation. The values are means of four replicates (n = 10 for week 8 and 12, and n = 15 for week 16 and 20). The vertical bars are standard errors of the mean.

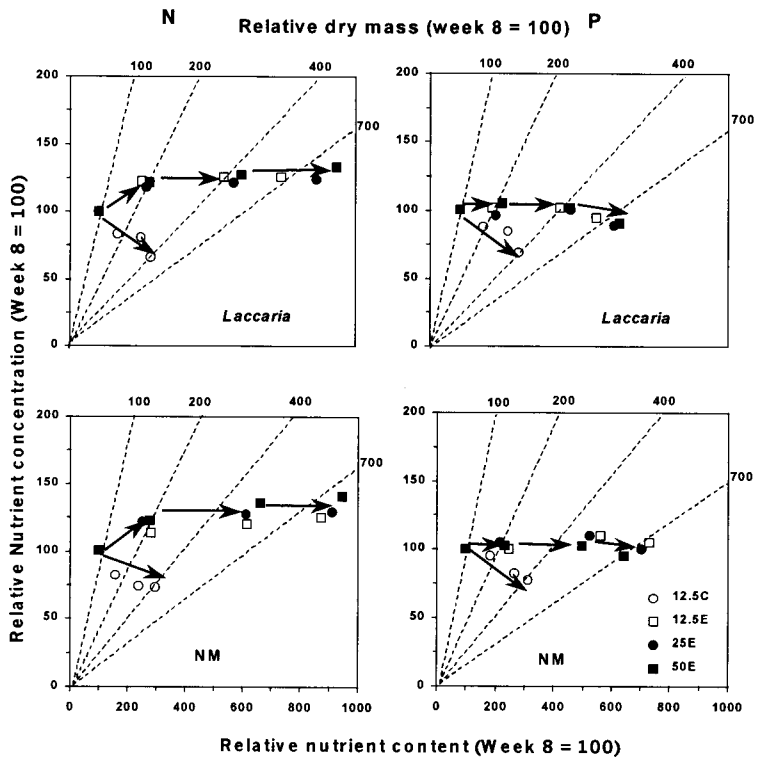


Fig. 4.4. Progression of relative dry mass and nutrient status (N and P) of seedlings reared under conventional (12.5C) and exponential (12.5E, 25E, and 50E) regimes and inoculated with (Laccaria) or without (NM) ectomycorrhizal fungi, sampled periodically from week 8 to 12, 16, and 20 during the growing period. The seedling status at week 8 of each inoculation treatment was normalized to 100.

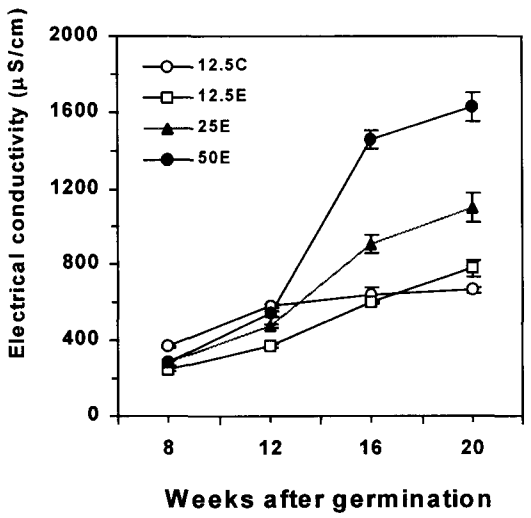


Fig. 4.5. Electrical conductivity of saturated aqueous extracts of peat growing media from containerized black spruce seedlings reared under conventional and three exponential regimes for 20 weeks. The data are from non-inoculated treatments only since there were no differences among the three inoculation types. All values are means of four replicates with vertical bar representing standard errors of the mean.

and nutrient uptake of the plant (Xu and Timmer 1998). Nutrient content in conventional fertilized seedlings fell off between week 16 and 20, suggesting nutrient limitation late in the growing season because of the constant rate addition schedule. The same seasonal dose (12.5 mg N) applied exponentially (12.5E) built up more N (24%, p = 0.0001) and P (10%, p = 0.01) in seedlings, demonstrating superior uptake efficiency under exponential fertilization. The efficiency dropped off at higher loading levels, which may explain correspondingly higher late-season EC levels in the rooting substrate (Fig. 4.5) as nutrients accumulated in the growing media rather than in the plants.

Inoculation increased seedling N (6-27%) and P (4-53%) uptake (Fig. 4.3) throughout the season when compared to non-inoculated trees reflecting improved uptake by ectomycorrhizal root systems and illustrating the practical benefit of fungal inoculation with nursery crops. Compared to non-mycorrhizal treatments, mycorrhizae increased loading efficiency by 16% and 20% for N, 8% and 12% for P at the low (25E) and high (50E) level, respectively.

Vector analysis

The dynamics of growth, N and P composition in seedlings relative to the first harvest (week 8, normalized to 100) was examined in vector nomograms of sequential harvest dates (Fig. 4.4). Similar to Figs. 4.2 and 4.3, the nomograms show a progressive increase in plant biomass, N content and P content (maximum 715, 850 and 650%, respectively) during the rotation, although increases were smaller for the conventional regimes compared to the exponential regimes. Nutrient concentration declined (maximum 27%) progressively for the conventional regime, but remained relatively stable for the exponential regimes, except for N that exhibited an initial increase (maximum 22%). Vector patterns signify a clear dilution of nutrients (shift A, Fig. 4.1) under conventional fertilization, since nutrient concentration declined as growth and nutrient uptake increased.

The exponentially fertilized seedlings exhibited steady-state nutrition for both N and P (horizontal shift B, Fig. 4.1) because nutrient concentration remained relatively constant with time as growth and nutrient uptake increased. The initial gain in N concentration, concomitant with increased dry matter production and nutrient accumulation (shift C, Fig. 4.1), suggested N limitation at that stage in the rotation. Early nutrient deficiency of seedlings during exponential fertilization has been noted before (Timmer *et al.* 1991), and was corrected by using a modified exponential regime in which additions are raised initially at the expense of final additions (Imo and Timmer 1992). Since the modified schedule involved higher starting additions, this delivery model was not adopted in this study to avoid early nutrient toxicity to the mycorrhizae during initial infection.

Ectomycorrhizal development

Periodic microscopic assessment of root systems showed that inoculated seedlings were colonized by ectomycorrhizal fungi (22-96%) by the first harvest (week 8). The uninoculated control seedlings were free of ectomycorrhizae throughout the culture rotation. Infection was not significantly (p = 0.22) different by inoculum type in any fertilizer treatments, suggesting that both *Hebeloma crustuliniforme* and *Laccaria bicolor* were equally effective in forming mycorrhizae with black spruce (Table 4.2). By week 8, inoculated seedlings had 82-89% (p = 0.0001) of the short roots colonized when fertilized exponentially, regardless of the nutrient dose (Fig. 4.6). The infection rate was much lower (22-31%, p = 0.0001) with conventionally fertilized seedlings. Improved infection from exponential fertilization was probably associated with lower amounts of nutrients applied early in the growing season compared to conventional fertilizer additions (Table 4.1). Frequent high level applications of soluble fertilizer can limit mycorrhizal development because of high soluble nutrients in the rooting substrate in the infection process (Ruehle and Wells 1984; Beckjord *et al.* 1985, Kropp and Langlois 1990, Väre 1990).

Since nutrient inputs started low and increased incrementally with exponential regimes (Table 4.1), the mycorrhizae were exposed to progressively higher nutrient levels, hence may gradually develop tolerance to intensive fertilization. Ohenoja (1988) showed that fungal fruiting body production was increased after high N fertilization in the forest, indicating that fungi have the potential to adapt to enhanced N nutrition. Colonization densities were high from the start (82-90%), and remained relatively stable with time when

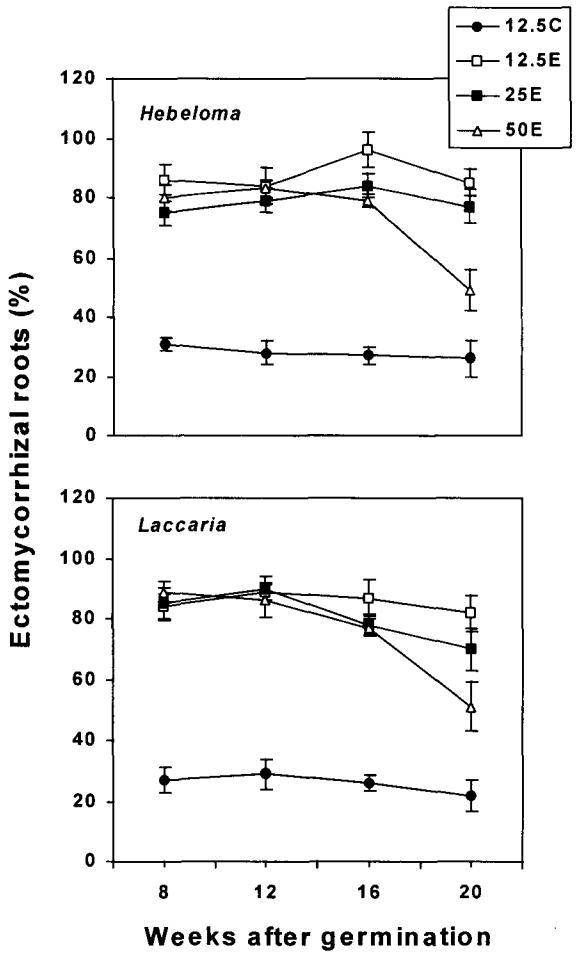


Fig. 4.6. Percentage of ectomycorrhizal short roots of black spruce seedlings inoculated with *Hebeloma crustuliniforme* and *Laccaria bicolor* under conventional (12.5C) and exponential regimes (12.5E, 25E, and 50E). The values are means of four replicate seedlings (n = 10 for week 8 and 12, and n = 15 for week 16 and 20). The vertical bars are standard errors of the mean.

fertilized exponentially, except for the high loading (50E) regime where levels declined at the final harvest (Fig. 4.6). Improved infection rates was associated with elevated N and P concentrations in the seedlings (Table 3.3, Chapter 3), thus contradicting the generalization that seedlings be nutritionally poor for successful infection (Björkman 1949, Wallander and Nylund 1992). The sustained development of ectomycorrhizae coincided with steady-state nutrition of the seedling (Fig. 4.4), reflecting stable conditions in other physiological properties of the plants (Ingestad and Ägren 1995). Presumably, a stable and continuous flow of carbohydrate from the host to the fungi maintained an effective symbiosis. The drop to a 50% infection rate with 50E treatments at the final harvest probably was due to the high fertilizer inputs, although colonization rates were still satisfactory for commercial planting stock (Kropp and Langlois 1990), and were double (p = 0.0001) the level achieved by conventionally (22-26%) fertilized seedlings. The inhibition does show, however, that there is an upper limit to compatible nutrient loading and degree of mychorrhizal colonization.

Growing medium fertility

Nutrient dynamics in the growing media, monitored periodically by EC measurement of saturated extracts of the peat media, exhibited two distinct patterns in the fertilizer delivery schedules (Fig. 4.5), although no significant differences in EC levels were found between the fungal treatments (Table 4.2, p = 0.06). As expected, conventional fertilization induced relatively constant EC concentrations (370-640 μS cm^{-1}) during the growing period leading to nutrient dilution in seedlings (Fig. 4.4), while exponential fertilization induced lower EC levels initially that increased gradually from 284 to 1700 μS cm^{-1} (Fig. 4.5) resulting in essentially steady-state nutrition in trees (Fig. 4.4). Colonization under conventional fertilization (Fig. 4.6) was limited (22%) indicating that initial EC levels (370 μS cm^{-1}) were probably excessive for mycobiont viability (Marx *et al.* 1977). Marked higher infection rates (70-90%) achieved with exponential fertilization were sustained for the entire monitoring period despite exposure to a wide range of EC spanning a five fold increase in concentration (Fig. 4.5). Apparently, the fungi progressively adapted to higher EC levels in the rhizosphere to an upper limit of about 1500 μS cm^{-1}, since inhibition occurred at about 1700 μS cm^{-1} at final harvest (50E treatment). The response patterns suggest two critical thresholds that may serve as useful guidelines for producing mycorrhizal seedlings in containers under steady-state conditions: early fungal inhibition occurring at levels above 284 μS cm^{-1}, and late inhibition occurring at levels above 1500 μS cm^{-1}.

CONCLUSIONS

Restricted development of ectomycorrhizae under intensive fertilization in containerized seedlings may be overcome by adopting exponential fertilization regimes that expose seedlings and fungi incrementally to abundant nutrients at steady-state supply. The approach was compatible with nutrient loading practices that build up internal nutrient reserves of seedlings before planting; in fact, ectomycorrhizal inoculation improved loading efficiency by stimulating tree nutrient uptake (as high as 20% for N, and 12% for P). Vector nutrient diagnosis demonstrated that unlike conventional fertilization, which caused seasonal nutrient dilution in seedlings, exponential fertilization induced steady-state nutrition that exhibited initially slight deficiency for N, but not for P. Steady-state nutritional conditions achieved in this study signify synchronized growth and nutrient uptake of seedlings (Xu and Timmer 1998), which most likely induced stable carbohydrate flow from host to fungus for sustained symbiosis and facilitated tolerance buildup to high fertilizer inputs. Seasonal monitoring of EC measurement of saturated extract of the peat growing media indicated that early- and late-season thresholds for mycorrhizal inhibition were associated with concentrations exceeding 248 and

1500 μS. cm^{-1}, respectively. Further research is required to test the effectiveness of ectomycorrhizal inoculation and steady-state nutrient loading of seedlings for outplanting in terms of survival, growth and nutrition in the field environment.

Chapter 5

EARLY OUTPLANTING PERFORMANCE OF NUTRIENT-LOADED CONTAINERIZED BLACK SPRUCE SEEDLINGS INOCULATED WITH *Laccaria bicolor*: A BIOASSAY STUDY *

Summary

Early growth potential of nutrient-loaded and (or) *Laccaria bicolor* inoculated (*Picea mariana* (Mill.) B.S.P.) seedlings was investigated using pot bioassays retrieved from a low competition Feathermoss site and a high competition Hardwood-Alnus site in the boreal forest. Mycorrhizal seedlings were similar in biomass and shoot:root ratio to non-mycorrhizal seedlings at planting, but significantly higher in nutrient content depending on fertilization regime and loading rate. After transplanting, both nutrient-loaded and inoculated seedlings outperformed conventional seedlings, increasing dry matter production by 20-49% with loading and by 45-92% with combined treatments. Nutrient uptake followed similar trends, increasing N, P, and K uptake by 80-124%, 89-129%, and 72-106%, respectively, with combined treatments compared to conventional seedlings, demonstrating the advantage of both nutrient loading and inoculation in early plantation establishment. Seedling response was greater on the Feathermoss site presumably because of less competition for nutrients and light. Vector diagnosis indicated the response was associated with a primary limitation of N and P that was alleviated by nutrient loading and mycorrhizal inoculation, particularly when treatments were combined. A strong correlation between pre-plant N content and outplant biomass suggests that initial nutritional status is a better criterion for predicting stock quality than traditional morphological parameters of seedlings.

INTRODUCTION

Black spruce is the most important commercial tree species in Ontario because of its preferred pulping characteristics and its abundance on forest land (Arnup 1996). However, there is concern about adequate regeneration of young black spruce due to intense competition from natural vegetation after logging (Jeglum 1983, Malik and Timmer 1995). Poor nutrient preconditioning and insufficient mycorrhizal infection of seedlings planted on competitive and low fertility sites are considered factors that may contribute to regeneration problems (Perry *et al.* 1987, Timmer *et al.* 1991, van den Driessche 1991). Although fertilization after transplanting is one way to increase early outplanting performance, fertilizer applications in the field may often intensify competition from natural vegetation (Burdett *et al.* 1984, Munson and Timmer 1989, Imo and Timmer 1998). Competition control is effective using chemical herbicides, but there are strong public concerns against extensive application of herbicides in forestry (Buse *et al.* 1995), because of possible harmful effects to wildlife and human populations living in or near the forest (Wagner 1993). Hence, there is increasing pressure to find alternative approaches to herbicide use for vegetation control and ensure successful regeneration of black spruce.

One approach demonstrated recently by Malik and Timmer (1995, 1996, 1998) is to improve the competitiveness of planted seedlings by nutrient loading in the nursery. This practice builds up nutrient reserves in seedlings by luxury consumption that boosts subsequent field growth and can suppress neighbouring vegetation (Timmer and Munson 1991, Timmer 1997). Another approach is to enhance outplanting performance by inoculating nursery stock with mycorrhizal fungi that are known to promote uptake of nutrients and water, and increase resistance against some pathogens (Valdés 1986, Cordell *et al.* 1987, Stenstrom and Ek 1990, Villeneuve *et al.* 1991, Smith and Read 1997). However, containerized seedlings produced traditionally in intensive nursery culture usually lack sufficient mycorrhizae (Marx and Barnett 1974, Langlois and Fortin 1982) because of generous fertilizer and biocide used to optimize seedling growth is often inhibitory to mycorrhizal fungi (Marx *et al.* 1977, Browning and Whitney 1992).

Although mycorrhiza formation may be incompatible with high fertilizer use in conventional seedling culture, new techniques integrating exponential fertilization, luxury consumption and fungal inoculation may result in production of both nutrient-loaded and ectomycorrhizally infected planting stock (Chapter 3 and 4). Exponential fertilizer delivery avoids high nutrient additions early in the nursery rotation and progressively builds up nutrients in the rooting media to acclimatize mycorrhizae to higher nutrient inputs. The mycorrhizae also improve loading efficiency by stimulating plant nutrient uptake while maintaining adequate infection levels on their root systems (Chapter 3 and 4)). Although combined nutrient loading and inoculation may be successful in the greenhouse production stage, the benefits for early outplanting performance of seedlings has yet to be demonstrated.

The objective of this study was to evaluate the growth and nutritional responses of nutrient-loaded ectomycorrhizal seedlings after outplanting under controlled conditions. The test seedlings were transplanted on intact blocks of soils (or bioassays) retrieved from two contrasting forest sites. The two sites, a high competition (Hardwood-Alnus) site and a

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low competition (Feathermoss) site, were selected to evaluate competitive responses as well as fertility responses. The bioassay approach has been used previously to assess early seedling performance on boreal sites, and responses are well correlated with short-term response in the field (Munson and Timmer 1989, Malik and Timmer 1996).

MATERIALS AND METHODS

Seedling culture and fungal inoculation

Black spruce seedlings were raised in Rootrainers® plastic containers (110 cm^3 cavities) filled with peat growing media and vermiculite (10:1 by volume) as previously described (Chapter 3). The seeds were inoculated at the time of sowing with a species of ectomycorrhizal fungus, *Laccaria bicolor* (R. Mre.) Orton, grown aseptically on a vermiculite carrier obtained from Plant Health Care Inc, Pitsburg, PA 15238, USA. A similar volume of sterilized vermiculite without the fungus was used for the uninoculated (-M) treatments. The seedlings were grown for 20 weeks in a greenhouse simulating commercial nursery conditions except for an extended photoperiod (20-h at 250 $\mu mol.s^{-1}.m^{-2}$ light intensity). Ambient air temperatures ranged between 18° C and 25° C at a relative humidity from 65 to 85%. Fertilization started two weeks after germination and continued for 18 weeks using commercial water-soluble mixed fertilizer (Plant Products as 20:10:20 N-P_2O_2-K_2O plus micronutrients) applied weekly following four fertilization regimes. The conventional (12.5C) regime delivered a seasonal total of 12.5 mg N per seedling at a constant delivery rate. This treatment represented the industry's operational regime for black spruce production in Ontario. The exponential (12.5E) regime delivered the same total amount of nutrients (12.5 mg N per seedling) at exponentially increasing addition rates during the 18-week fertilization period. A 25- and a 50-mg N per seedling dose rate, also delivered exponentially, represented a low (25E) and high (50E) nutrient loading regimes. Irrigation to container capacity with water and (or) nutrient solution was as needed, hence no leaching occurred. After fertilization (week 21), the seedlings were exposed to lower temperatures (18°C day, 12°C night) and 8-h short days for two weeks to induce terminal bud setting. The seedlings were then re-exposed to normal day length and progressively lower temperatures (16-10°C day, 12-4°C night) as well as reduced watering for hardening. At week 27, the seedlings were transferred to cold storage at -2°C to over-winter for a 5 month period.

Bioassay system

After over-wintering, seedling performance was assessed using a bioassay technique described by Munson and Timmer (1989) that involved transplanting seedlings on intact blocks of soil substrates (bioassays) collected from two contrasting forest sites under license to Abitibi-Price Inc. near Iroquois Falls (48° 28'N and 80° 40'W), Ontario. One site was a low competition Feathermoss site on fresh-moist coarse sandy soil with shallow organic matter, moderately drained and ground cover dominated by *Pleurozium* mosses, and few species of *Ledum* and *Vaccainium angustifolium*, which was classified as Operational Group 4 (Jones *et al.* 1983). The other was a competitive Hardwood-Alnus site (Operational Group 10) on moist fine loamy-clayey soil with thick black organic-mineral mull humus layer, imperfectly drained and characterized by the presence of *Alnus rugosa* and *Rubus idaeus* (The site was clear-cut in 1990 and shearbladed in 1991). Other ground vegetation in the Hardwood-Alnus site were *Viburnum* spp, *Ribes glandulosum*, *Rosa acicularis*, *Mitella nuda*, *Galium triflorum*, and *Viola renifolia* representing a competition prone site. In spring at each site, sixteen intact (36 × 30 cm) blocks of substrate were cut to a depth of 15 cm without disturbing the natural vegetation (Photo plate 5.1), and inserted in a rectangular plastic container (15 × 36 × 30 cm) with drainage holes at the bottom. Thirty-two bioassays from two sites were transported carefully to the greenhouse at the University of Toronto (Photo plate 5.2).

Before planting, a plastic baffle was inserted in the middle of each substrate (pot) to split the bioassay in half to avoid possible below ground root interference between the ectomycorrhizal and non-ectomycorrhizal seedlings. Black spruce seedlings were sorted by size class in each fertility regime to reduce possible confounding effect due to pre-plant size differences within the treatment. Six over-wintered seedlings per treatment were transplanted in the spring into each half of an intact soil substrate (bioassay) at an approximate 9 × 9 cm spacing. Treatments were arranged as a randomized complete block design with four replicates. The seedlings were grown for 18 weeks in the greenhouse and watered when needed according to container capacity (Timmer and Armstrong). The bioassay containers were rotated every two weeks to reduce possible edge effect.

Plant harvesting and chemical analysis

Seedlings were harvested in the fall at the end of the growing season. The root systems were carefully washed to assess the ectomycorrhizal status. Three randomly selected large lateral roots from two seedlings per experimental unit were examined for ectomycorrhizal presence on short roots and lateral branches as described by Gagnon *et al.* (1987) and Browning and Whitney (1992). The average of three laterals was used to calculate weighted means of each treatment. Each seedling of the experimental unit was then measured for height and root collar diameter and then separated into shoots and roots. Shoot and root components were pooled separately for each replication and oven-dried at 70°C for 72 h for dry-mass determination. Oven dried plant samples were ground and wet-digested in a sulphuric acid-hydrogen peroxide mixture using a block digester at 380°C (Lowther 1980) for subsequent chemical analysis. The total N was

determined using a dual channel Technicon II Autoanalzer Industrial Model No. 154-71 W (Eastin 1987), P by the molybdenum blue method (Allen 1974), and K by atomic absorption spectrometry (model Perkin-Elmer 3100). Nutrient concentration of components was multiplied by corresponding biomass to determine specific nutrient content. Growth and nutrient composition of the seedlings were evaluated by vector diagnosis (Timmer 1991, Haase and Rose 1995) to explain nutritional responses and identify limiting nutrients. The technique has been applied previously to ectomychorrhizal studies of black spruce seedlings by Quoreshi and Timmer (1998), as outlined in Chapter 3.

Experimental design and statistical analysis

The pot bioassays were laid out in a factorial randomized complete block design with four replications. The experimental design consisted of seedlings raised under four fertilization regime (F) and two inoculation (I) treatments and planted on two site (S) types. Sixteen treatment combinations (2 sites × 4 fertilization treatments × 2 inoculations) were randomly planted to each block (replication) of eight pots. Each plastic pot (main plot) was divided into two subplots and planted with 12 seedlings. Each subplot comprised six seedlings from inoculated (+ M) or non-inoculated (- M) cultural conditions as shown in Photo plate 5.3). Analysis of variance (ANOVA) was carried out on data for each variable (Table 5.1) using SAS® (SAS Institute Inc. 1989). The general model for analysis was:

$$Y_{ijkl} = \mu + \beta_i + F_j + I_k + S_l + (FI)_{jk} + (FS)_{jl} + (IS)_{kl} + (FIS)_{jkl} + \varepsilon_{ijkl}$$

where levels of factors were, replication i = 4; fertilization j = 4; site l = 2 and inoculation k = 2. Differences among treatment means were separated by least significant difference test (LSD) and significant site differences by confidence intervals ($p < 0.05$).

RESULTS AND DISCUSSION

Seedling status before planting

Pre-plant status of seedlings was similar to those seedlings at the pre-hardening stage (Chapter 3) except

Table 5.1. Pre-plant and outplant ANOVA table ($P \geq F$) for plant dry mass (DM), shoot to root ratio (S/R), root-collar diameter (RC), % ectomycorrhiza (% Ecto), and N, P, and K content of black spruce seedlings.

Source of variations	df	$P \geq F$							
		DM	S/R	N	P	K	RC	% Ecto[b]	%Ecto[c]
Pre-plant									
Fertilizer	3	0.0001	0.0001	0.0001	0.0001	0.0001	-	0.0001	-
Fungus	1	0.8827	0.2210	0.0002	0.0001	0.0019	-	0.0001	-
Fertilizer x Fungus	3	0.0632	0.6821	0.3708	0.6552	0.6219	-	0.0001	-
Error mean square	21	806.86	0.3464	0.7257	0.0071	0.3839	-	25.44	-
Total	31								
Outplant (FM)[a]									
Fertilizer	3	0.0001	0.3254	0.0001	0.0002	0.0001	0.0011	0.0001	0.0031
Fungus	1	0.0001	0.0027	0.0001	0.0006	0.0001	0.0001	0.0001	0.0002
Fertilizer x Fungus	3	0.4762	0.0591	0.3940	0.6309	0.5492	0.3608	0.0001	0.0031
Error mean square (21)[b]	24	0.1478	0.2517	0.0001	0.0000	0.0000	0.0874	22.42	4.06
Total	31								
Outplant (HA)[a]									
Fertilizer	3	0.0001	0.0060	0.0001	0.0022	0.0001	0.0490	0.0001	0.0010
Fungus	1	0.0001	0.3912	0.0001	0.0001	0.0001	0.0032	0.0001	0.0001
Fertilizer x Fungus	3	0.9391	0.2950	0.9183	0.6858	0.0584	0.8841	0.0001	0.0010
Error mean square (21)[b]	24	0.0520	0.1983	0.0000	0.0000	0.0000	0.1573	17.30	2.94
Total	31								
FM vs HA									
Site	1	0.0001	0.9828	0.0023	0.0001	0.0001	0.0063	0.0021	0.8338
Error mean square (52)[b]	62	0.3531	0.3349	0.0001	0.0000	0.0000	0.2342	30.11	2.96
Total	63								

Note: [a] FM and HA represents Feathermoss and Hardwood-Alnus site, respectively. [b] Applies only to the analysis of *Laccaria bicolor* infected seedlings. [c] Applies only to the analysis of indigenous fungal infections.

for increased growth and lower shoot:root ratios that incurred during hardening (Table 5.2). Both fertilization (Table 5.1, $p = 0.0001$ for N, P, and K) and inoculation treatment ($p = 0.0002$ for N, $p = 0.0001$ for P, and $p = 0.0019$ for K) increased nutrient uptake of seedlings during the nursery phase depending on loading rate (Table 5.2). The exponentially fertilized seedlings contained 43-116% more N, 60-87% more P, and 30-68% more K than the conventionally fertilized seedlings. Although fungal inoculation did not affect plant dry mass ($p = 0.88$) and shoot:root biomass ratio ($p = 0.22$), nutrient uptake was increased significantly reflecting higher absorption capacity of nutrients by infected root systems. Nutrient-loaded ectomycorrhizal seedlings contained, respectively, 120-155%, 105-113%, and 67-90% more N, P, and K than conventional non-mycorrhizal seedlings. Seedlings inoculated with *Laccaria bicolor* had 56-82% of their short roots ectomycorrhizal when fertilized exponentially compared to only 20% ectomycorrhizal roots for conventionally fertilized seedlings, exemplifying the compatibility of fungal formation with exponential nutrient delivery regimes. The uninoculated control seedlings remained free of ectomycorrhizae before planting.

Growth characteristics after outplanting

There was no mortality after transplanting into the bioassays; the seedlings developed normally for the 18-week growing season. Both nursery inoculation and exponential fertilization treatments consistently improved growth performance after outplanting (Fig. 5.1), illustrating the advantage of pre-plant exponential fertilization and ectomycorrhizal inoculation in early seedling establishment. At harvest, total dry matter production of inoculated (+M) seedlings was increased by 20-92% and 5-67% on the Feathermoss and Hardwood-Alnus sites, respectively, when compared with uninoculated (- M) conventional seedlings (see Photo plate 5.4). Although smaller than conventional (12.5C) mycorrhizal seedlings at planting (Table 5.2), the exponentially fertilized (12.5E) mycorrhizal seedlings caught up with the latter at the end of the bioassay study (Fig. 5.1), presumably because of the higher infection levels and N, P and K status (Table 5.2) obtained under steady-state nutrition associated

Table 5.2. Pre-plant dry mass (DM), shoot to root ratio (S/R), percent ectomycorrhizal short roots (%ESR), and N, P, K contents of black spruce seedlings inoculated with (+ M) or without (- M) ectomycorrhizal fungi (*Laccaria bicolor* (R. Mre.) Orton) and fertilized with different nutrient regimes.

Parameters	Mycorrhizal treatments	Fertilizer treatments				
		12.5C	12.5E	25E	50E	Mean
DM (mg)	+ M	655	576	692	739	665 A
	- M	607	592	720	748	667 A
	Mean	631 c	584 d	706 b	744 a	-
S/R ratio	+ M	2.7	3.8	4.3	4.3	3.8 A
	- M	2.5	3.7	3.7	4.2	3.5 A
	Mean	2.6 b	3.8 a	4.0 a	4.2 a	-
% ESR	+ M	21	88	77	52	60 A
	- M	0	0	0	0	0 B
	Mean	21 d	88 a	77 b	52 c	-
N content (mg)	+ M	7.1	9.5	13.2	15.3	11.3 A
	- M	6.0	8.6	12.0	12.9	9.8 B
	Mean	6.5 d	9.1 c	12.6 b	14.1 a	-
P content (mg)	+ M	0.96	1.4	1.5	1.6	1.4 A
	- M	0.75	1.2	1.4	1.3	1.2 B
	Mean	0.85 c	1.3 b	1.5 a	1.5 a	-
K content (mg)	+ M	5.2	6.0	6.6	7.6	6.3 A
	- M	4.0	5.2	6.3	6.7	5.5 B
	Mean	4.6 c	5.6 b	6.5 a	7.1 a	-

Note: Mean values within columns and rows followed by different letters are significantly different at the $p < 0.05$ level (LSD test). All values are means of four replicates.

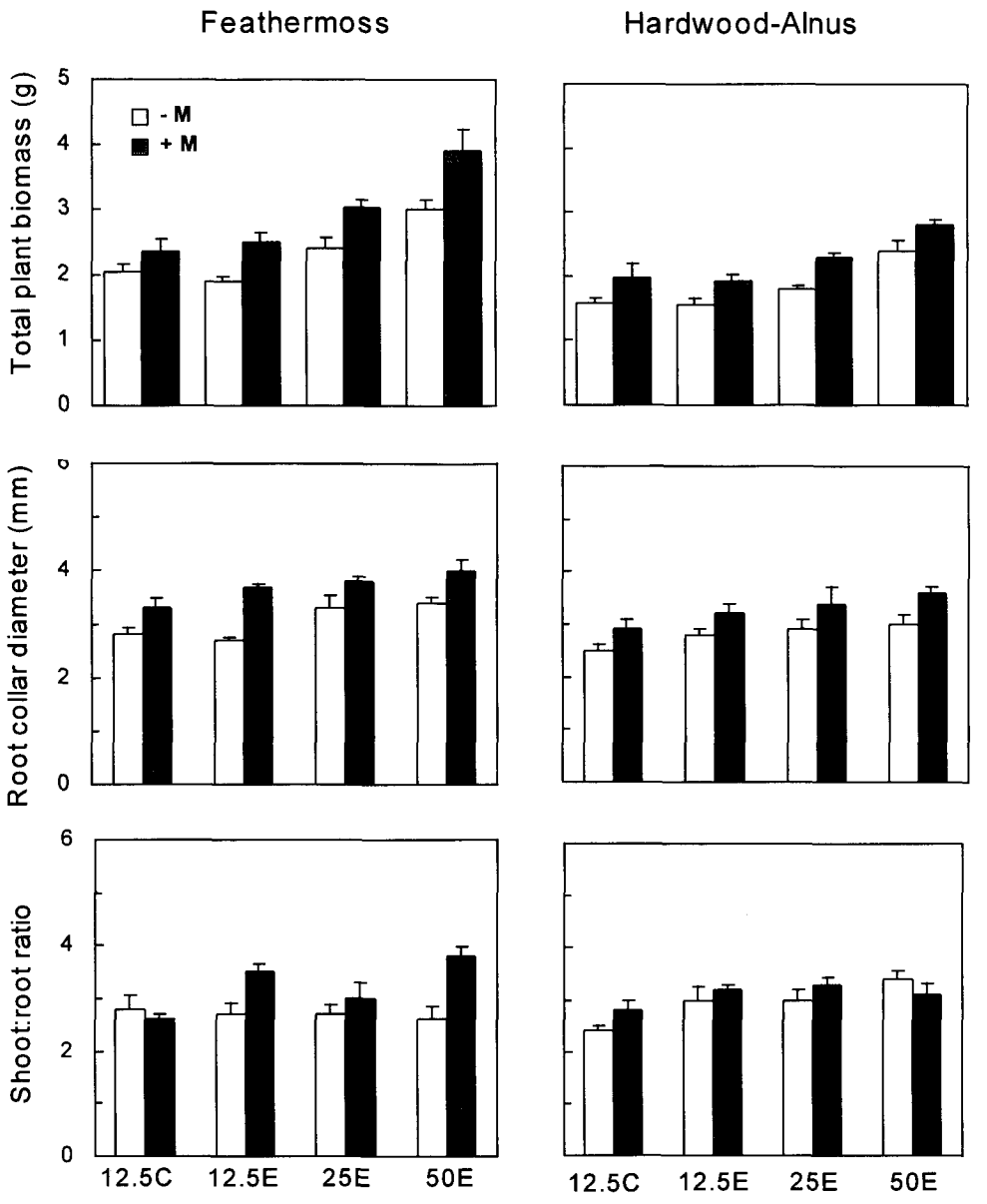

Fig. 5.1. Plant biomass, root collar diameter and shoot:root ratios of conventional (12.5C), exponential (12.5E), and exponential nutrient-loaded (25E and 50E) seedlings with (+ M) or without ectomycorrhizae (- M) planted for one season on intact bioassays retrieved from contrasting forest sites. The values are means of four replicates with vertical bars representing standard error of mean.

with exponential fertilizer delivery (Quoreshi and Timmer 1999). Pre-plant nutrient loading (25E and 50E) also enhanced biomass production of outplanted seedlings, the magnitude increasing with loading rate. On the Feathermoss site low (25E) and high loading (50E) treatments alone increased respective plant biomass by 20 and 49%, and 45 and 92% when combined with mycorrhizal inoculation. Similar trends in dry matter production were also observed on the Hardwood-Alnus site. The superior growth performance obtained by nutrient loading for both mycorrhizal and non-mycorrhizal seedlings reflects efficient utilization of nutrient reserves to the actively growing parts of the plants during early plantation establishment (Malik and Timmer 1998). The response, however, was significantly greater on the Feathermoss site than Hardwood-Alnus site (see ANOVA Table 5.1), probably exemplifying the higher competition for available nutrients and light between natural vegetation and tree seedlings on the second site, as noted by Imo and Timmer (1998, 1999).

Root collar diameter of inoculated seedlings was significantly greater (Table 5.1, p = 0.0001 and p = 0.0032) than those of non-inoculated seedlings on both sites (Fig. 5.1), indicating better stability and survival potential (Thompson 1986) of inoculated seedlings in the outplanting environment. Shoot:root ratio was higher (Table 5.1, p = 0.0027) with inoculated seedlings compared to non-mycorrhizal and conventional seedlings on the Feathermoss site (Fig. 5.1), suggesting that both mycorrhizal association and nutrient loading favored shoot growth more than root growth due to

increased nutrient availability (Ingestad and Ågren 1988). However, this effect was non-significant (p = 0.3912) on the Hardwood-Alnus site, probably due to the intensive competition for soil resources.

Nutrient accumulation after planting

Pre-plant nutrient status was higher for ectomycorrhizal (+ M) than non-mycorrhizal (-M) seedlings presumably because fungal inoculation improved nutrient uptake and loading efficiency during seedling production (Table 5.2, see also Chapter 3 and 4). This response persisted after planting since the main effect of fungal inoculation on plant nutrient uptake was positive and significant (Table 5.1, p = 0.0001 for N, p = 0.0006 for P, and p = 0.0001 for K), increasing N (20-86%), P (21-62%), and K (29-92%) content depending on loading levels on both study sites (Fig. 5.2). Thus not only did *Laccaria bicolor* inoculation stimulate nutrient uptake during nursery culture, but also after transplanting where the effect was more pronounced.

The nutrient loading response also persisted after planting. Before outplanting, the loaded seedlings contained 89-117% more N, 56-87% more P, and 27-68% more K than the non-loaded conventional seedlings. One season after transplanting, the main effect of nutrient loading was 20-50% more N, 43-74% more P, and 29-43% more K in the seedlings (Table 5.1, p = 0.0001 for N, p = 0.0002 for P, and p = 0.0001 for K). Nutrient uptake was further enhanced (80-124% for

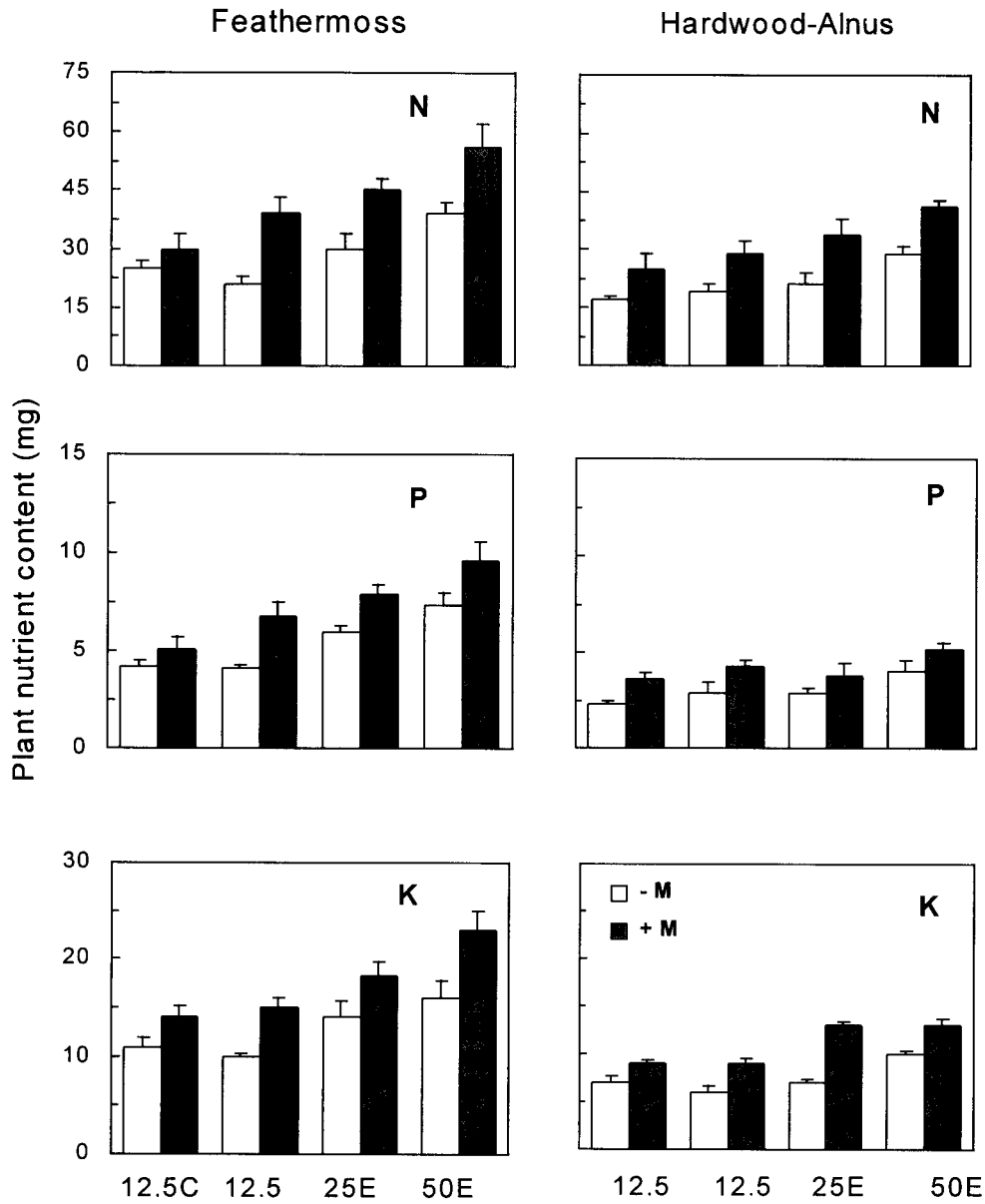

Fig. 5.2. Plant N, P, and K content of conventional (12.5C), exponential (12.5E), and exponential nutrient-loaded (25E and 50E) seedlings with (+ M) or without ectomycorrhizae (- M) planted for one season on intact bioassays retrieved from contrasting forest sites. The values are means of four replicates with vertical bars representing standard error of mean.

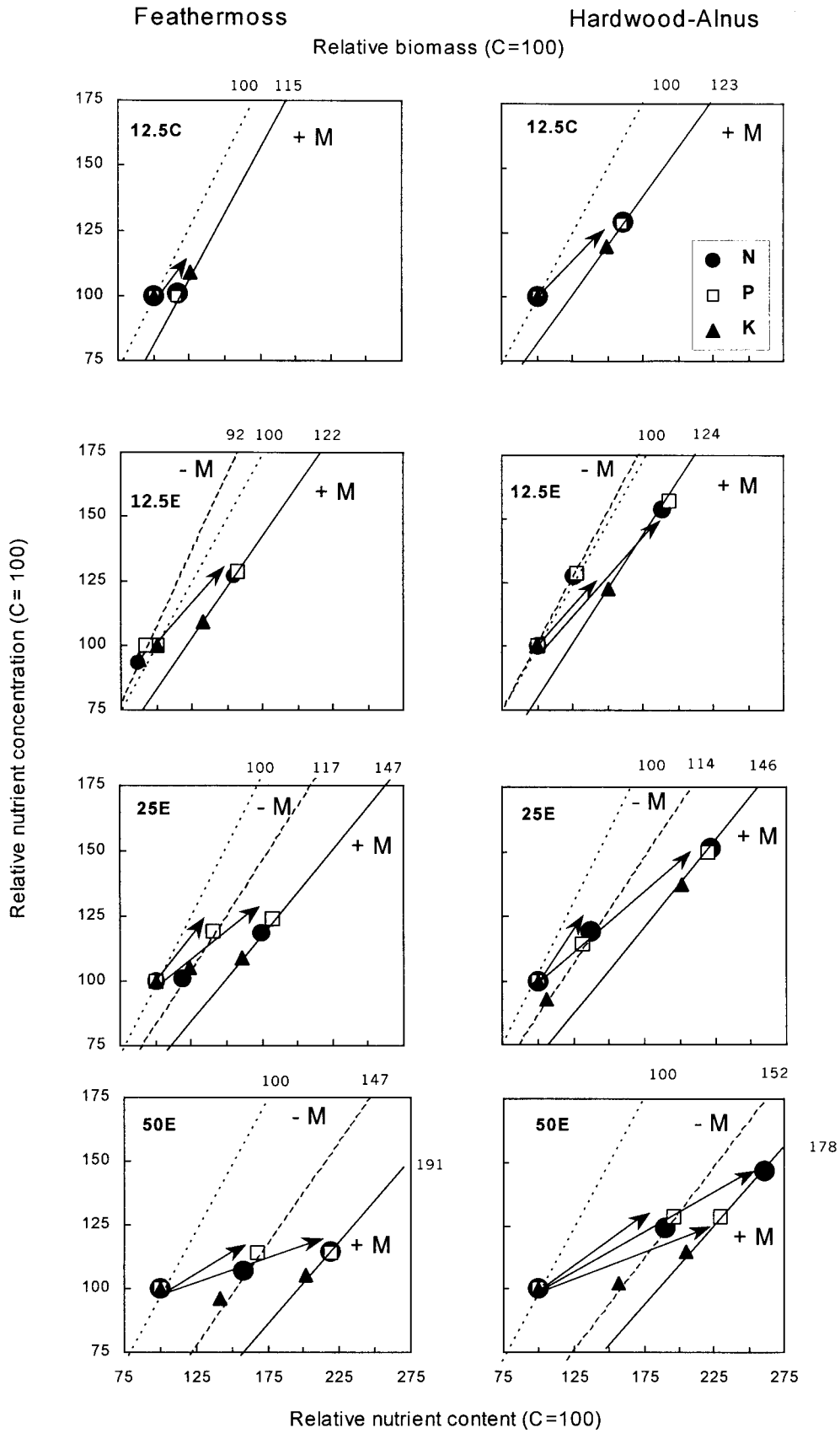

Fig. 5.3. Vector nomogram of relative difference in nutrient content, concentration and plant biomass of conventional (12.5C), exponential (12.5E), and exponential nutrient-loaded (25E and 50E) seedlings with (+ M) or without ectomycorrhizae (- M) grown for one growing season in potted, intact blocks retrieved from two contrasting forest sites. The seedling status of non-mycorrhizal (-M) conventional control was normalized to 100 (C=100) for comparison. Only vectors associated with major responses are shown.

N, 89-129% for P, and 72-106% for K) with both nutrient loading and inoculation when compared to conventional treatments; the response was additive since fertilizer-fungus interactions were non significant (Table 5.1). The higher plant nutrient status suggests more efficient utilization of internal nutrient reserves built up by loading, and greater acquisition of soil nutrients due to higher absorption capacity and uptake efficiency of ectomycorrhizal root systems (Smith and Read 1997). The main effects of site type on seedling nutrition were significantly higher (Table 5.1) on the Feathermoss site than Hardwood-Alnus site, indicating probably greater competition with non-crop vegetation for available nutrients and moisture on weed-prone Hardwood-Alnus site and also demonstrating that the preconditioning responses are site specific in the boreal region (Imo and Timmer 1998).

Vector diagnosis

The vector nomograms (Fig. 5.3) compare the relative responsiveness of seedlings using the non-inoculated conventionally fertilized seedlings as the control. This treatment was normalized to 100. Only major vectors are drawn in Fig. 5.3 to reduce clutter. The nomograms show a distinct pattern of upward, right pointing vectors (Shift C, see Imo and Timmer 1998) that increased in magnitude with nutrient loading and mycorrhizal inoculation (+ M), and were associated mostly with N and P. Except for the non-mycorrhizal (- M) 12.5E regime that lack significant responses, the major vectors depicted increased biomass, nutrient concentration and nutrient content (Shift C) signifying deficiency responses mainly to N and P, which enhanced growth and nutrient uptake after outplanting. Thus, exponential nutrient loading increased biomass as much as 47 and 52%, and nutrient uptake as much as 82 and 95% compared to the conventional seedlings for the Feathermoss and Hardwood-Alnus sites, respectively. Together with mycorrhizal inoculation, respective dry mass production was increased as high as 91 and 78%, and nutrient uptake as high as 130 and 160% for the Feathermoss and Hardwood-Alnus sites, illustrating the benefits of an integrated treatment approach.

Potassium uptake was also stimulated by treatments (20-60%), but not as much as N and P, suggesting that the latter two nutrients were the most limiting at this early stage of seedling establishment. In general, mycorrhizae have been shown to benefit seedlings mostly with improved absorption of N and P compared to K, although the uptake ability of seedlings may vary greatly according to fungal species and nutrient availability (Harley and Smith 1983). Vector magnitude increased consistently with mycorrhizal treatment (+ M), and also with loading rate, reflecting the responsiveness of seedlings to these preconditioning treatments.

Correlation of pre-plant seedling status with subsequent outplanting performance

Correlations between dry matter production after planting and pre-plant N content (r = 0.72), or biomass

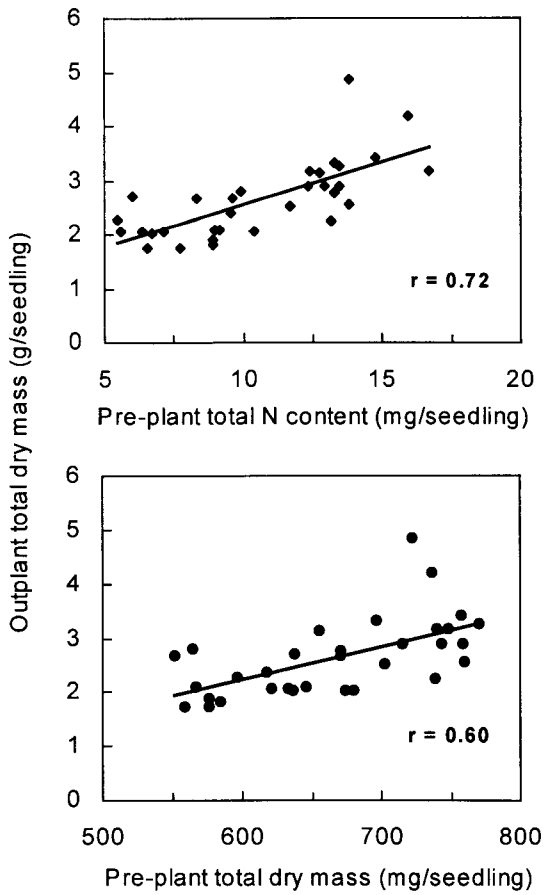

Fig. 5.4. Linear relationships between outplant dry mass and pre-plant seedling status (N content and biomass production) of black spruce. Data from both conventional and exponentially fertilized seedlings are included, each point represents a pooled sample of six seedlings on the Feathermoss site.

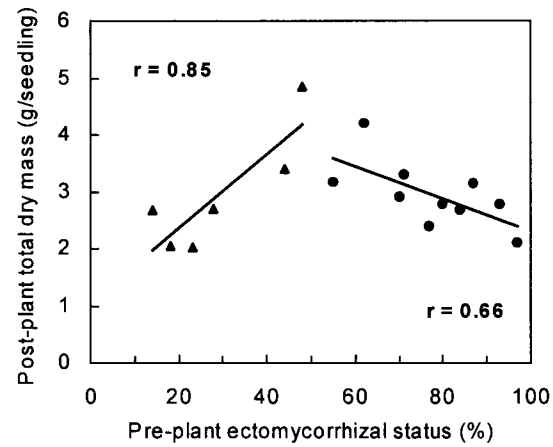

Fig. 5.5. Relationships between post-plant dry mass production and pre-plant seedling ectomycorrhizal status of black spruce. Data from both conventional and exponentially fertilized seedlings are included, each point represents a pooled sample of six seedlings from the Feathermoss site.

(r = 0.60) of seedlings suggest that initial N status is a better predictor of seedling quality for field performance than traditional morphological size criteria (Fig. 5.4). Apparently early growth of newly planted seedlings is highly dependent on the amount of nutrients built up as internal reserves during nursery culture (van den Driessche 1985, Malik and Timmer 1995).

A 50% or greater infection level of mycorrhizae on root systems of planting stock is suggested for optimal benefits of artificial inoculation after outplanting (Marx and Cordell 1988, Kropp and Langlois 1990). I have tested this threshold by examining the relationship between initial mycorrhizal infection rates and post-plant seedling biomass production (Fig. 5.5). Maximum tree growth peaked at about 50% infection of short roots, which confirmed the validity of this threshold. Below this level (triangular symbols) seedling growth was probably limited (r = 0.85) by low infection, above this level (circular symbols) growth was likely limited (r = 0.66) by inadequate nutrition because most of these plants were not nutrient-loaded. Apparently, both adequate root colonization and plant nutrient reserves are required for maximum productivity of seedlings after planting. My result suggested that a pre-plant infection level at 50-60% is adequate for maximum outplanting performance. Higher pre-plant infection levels, usually associated with increasing carbon drain from the host (Smith and Read 1997), may not be necessary for optimum seedling performance in the field.

Ectomycorrhizal status after outplanting

Post-plant infection levels of mycorrhizae on seedling root systems are also critical factors determining seedling performance and survival in the field (Trofymow and van den Driessche 1991). Root examination of the seedlings 18 weeks after transplanting revealed continued presence (15-62%) of inoculant ectomycorrhizal fungi on both site types (Table 5.3) based on morphological characteristics and differences in color and texture. The unidentified wild type fungus was characterized by dark brown hyphae found loosely on the uninoculated roots compared to well-defined, light brown to honey colored manltes with a purple tint associated with the inoculated seedlings. The fungus was also isolated from the roots and grown on pure culture and identified as *Laccaria* by their purple tint.

Natural root colonization of outplanted seedlings by indigenous ectomycorrhizal fungi was low (5-7%), and was confined to uninoculated seedlings (Table 5.3). Introduced fungi have shown a strong dominance over natural inoculum, thus preventing colonization of indigenous fungi immediately after outplanting (Browning and Whitney 1992, Buschena *et al.* 1992). Since ectomycorrhizae formation depends on a source of active inocula and other environmental variables (Amaranthus and Perry 1987), the slow colonization of

Table 5.3. Ectomycorrhizal colonization observed on the root system of inoculated (+ M) and non-inoculated (- M) black spruce seedlings after one season at the outplanting environment.

Fertilizer treatments	Mean of percent mycorrhization (%)	
	Inoculated (+ M)	Non-inoculated (- M)*
	Feathermoss	
12.5C	18 (± 2.0)	7 (± 3.0)
12.5E	62 (± 4.5)	0
25E	54 (± 5.2)	6 (± 2.0)
50E	38 (± 4.1)	0
	Hardwood-Alnus	
12.5C	15 (± 2.0)	0
12.5E	47 (± 4.4)	7 (±2.0)
25E	42 (± 3.6)	0
50E	33 (± 3.4)	5 (± 2.0)

Note: All values are average of four replicates. Data in parentheses are the standard error for the mean. * Indicates presence of indigenous fungal infection onlv.

new roots by indigenous fungi noted here suggests that the planted sites were low in natural inoculum potential (McAfee and Fortin 1989). Hence introduced *Laccaria bicolor* may be well adapted to the study sites for at least one growing season and may persist for initial seedling establishment, although the inoculated fungi will gradually be replaced by the resident ectomycorrhizal fungi over time (McAfee and Fortin 1986, Villeneuve *et al.* 1991, Buschena *et al.* 1992).

CONCLUSIONS

The results demonstrate that exponential nutrient loading combined with mycorrhizal inoculation during seedling production can significantly improve growth and nutrition of black spruce in the outplanting environment. Nutrient loading during nursery culture enhanced dry matter production and nutrient uptake of planted seedlings on both site types, suggesting efficient nutrient utilization of internal reserves and increased uptake from the soil after outplanting. These responses were further improved when integrated with ectomycorrhizal inoculation because of increased acquisition of soil nutrients by the mycorrhizal root systems. The results suggest that high pre-plant nutrient reserves and mycorrhizal colonization acquired during greenhouse culture continue to influence plant growth and nutrition following outplanting. However, loading and inoculation treatment responses varied with site type, indicating that responses are site specific. Mycorrhizal nutrient loading may be particularly suitable for weed prone sites to reduce competition for nutrients. *Laccaria bicolor* seems to be a fairly tenacious species for the two sites tested, since the fungus sustained symbiotic relationship with seedling roots at least one season after outplanting. Although natural colonization will occur eventually in the field, pre-plant inoculation and nutrient loading practices provide an alternative biological tool to improve early reforestation success. Further experiments are required to confirm the results in actual field conditions rather than short-term bioassay techniques used here.

Chapter 6

MYCORRHIZAL NUTRIENT LOADING: A SYNTHESIS

INTRODUCTION

In forest regeneration, successful establishment of trees depends on several factors. Among these factors, initial nutrient status and the capacity for early resource capture by planted seedlings are the most important. In previous chapters, I have shown that black spruce container seedlings with higher internal nutrient reserves and active ectomycorrhizal root systems acquired in the nursery exhibited significant increased growth and nutrition in a simulated outplanting environment. Improved plant nutrient uptake was achieved in the nursery through exponential nutrient loading and mycorrhizal inoculation, inducing steady-state luxury consumption of nutrients. Although mycorrhizae are sensitive to high nutrient availability (Marx *et al.* 1977, Harley and Smith 1983), exponential fertilizer delivery at high levels allowed adequate ectomycorrhizal formation and nutrient accumulation without detrimentally affecting tree biomass (Chapter 3 and 4). This new technique is termed "mycorrhizal nutrient loading" for operational purposes.

The objective of this chapter is to review and integrate the experimental findings of previous chapters (Chapter 3, 4, and 5) into simplified conceptual models to illustrate the critical processes involved in mycorrhizal nutrient loading. As noted earlier, the build up of nutrient reserves, the maintenance of adequate ectomycorrhizal development in seedlings, and the utilization of nutrient reserves for growth and acquisition of nutrients after outplanting are key factors that contribute to the superior regeneration of mycorrhizal nutrient-loaded seedlings (see Fig. 6.1, 6.3, 6.4, and 6.8).

MODEL STRUCTURE

A model is an abstraction or simplification of reality, which represents the form and (or) function of processes in a system (Kimmins 1987). Models can depict the dynamic nature of mechanisms operating in a system, as well as the interactions between components. In my review of pertinent literature, I did not find a comprehensive, conceptual model of plant-mycorrhizal nutrient interactions, hence I developed my own model. The interactions of critical processes operating under three different fertilization treatments are presented in a series of flow diagrams (see Fig 6.1, 6.3 and 6.4). The basic structure of each diagram is similar. The flow is from top to bottom with the upper portion, or the "Pre-plant" stage, separated from the bottom portion or "Outplant" stage by a thick shaded line.

Seedling development begins with seed germination (top rectangle with rounded corners), followed by one of three fertilization schemes (2nd level of rectangle with rounded corners) applied as: 1) Conventional Fertilization (constant addition rate, Fig. 6.1), 2) Exponential Fertilization (progressive addition rate, Fig 6.3), and 3) Exponential Loading (progressive addition rate at loading dose, Fig. 6.4). The fertilization regimes are characterized by their effect on: initial nutrient supply (left ovals), initial EC levels (centre ovals) and the occurrence of synchronized or non-synchronized growth and nutrient uptake (right ovals). How these initial nutritional factors (ovals), controlled by fertilizer delivery and dose levels, affect critical seedling developmental processes (rectangles) is shown through connections (arrows) between ovals and rectangles, as well as between rectangles, and possible interactions by double headed arrows. The large, broken dashed line rectangle on the left side of each diagram encloses the processes that are part of the mycorrhizal system. The dotted rectangles with thick dashed arrows represent processes only under excessive mycorrhizal development occurring under exponential fertilization only. Each condition either alone or in combination

affects seedling nutrient uptake, internal nutritional status, and formation of ectomycorrhizae through processes (rectangles) depicted in the models.

The major goal of my thesis was to test new fertilization techniques, such as exponential fertilization and nutrient loading combined with mycorrhizal inoculation, during greenhouse culture to enhance seedling quality. This approach was expected to result in significantly improved outplanting performance of planted seedlings. While the model representations are simplistic, they highlight critical controls of seedling growth and development, and mycorrhizal formation affected by the different fertilization regimes. The functioning of key biological and chemical mechanisms driving the response will be discussed next emphasizing the fundamental processes involved.

NUTRIENT ACCUMULATION IN SEEDLINGS IN THE NURSERY

Conventional fertilization model

When seedlings were reared conventionally (Fig. 6.1), fertilizers were delivered periodically (weekly) at constant rates during the growing season (Fig. 3.1, Chapter 3, Table 4.1, Chapter 4), resulting in a relatively high initial nutrient supply (oval 1), causing

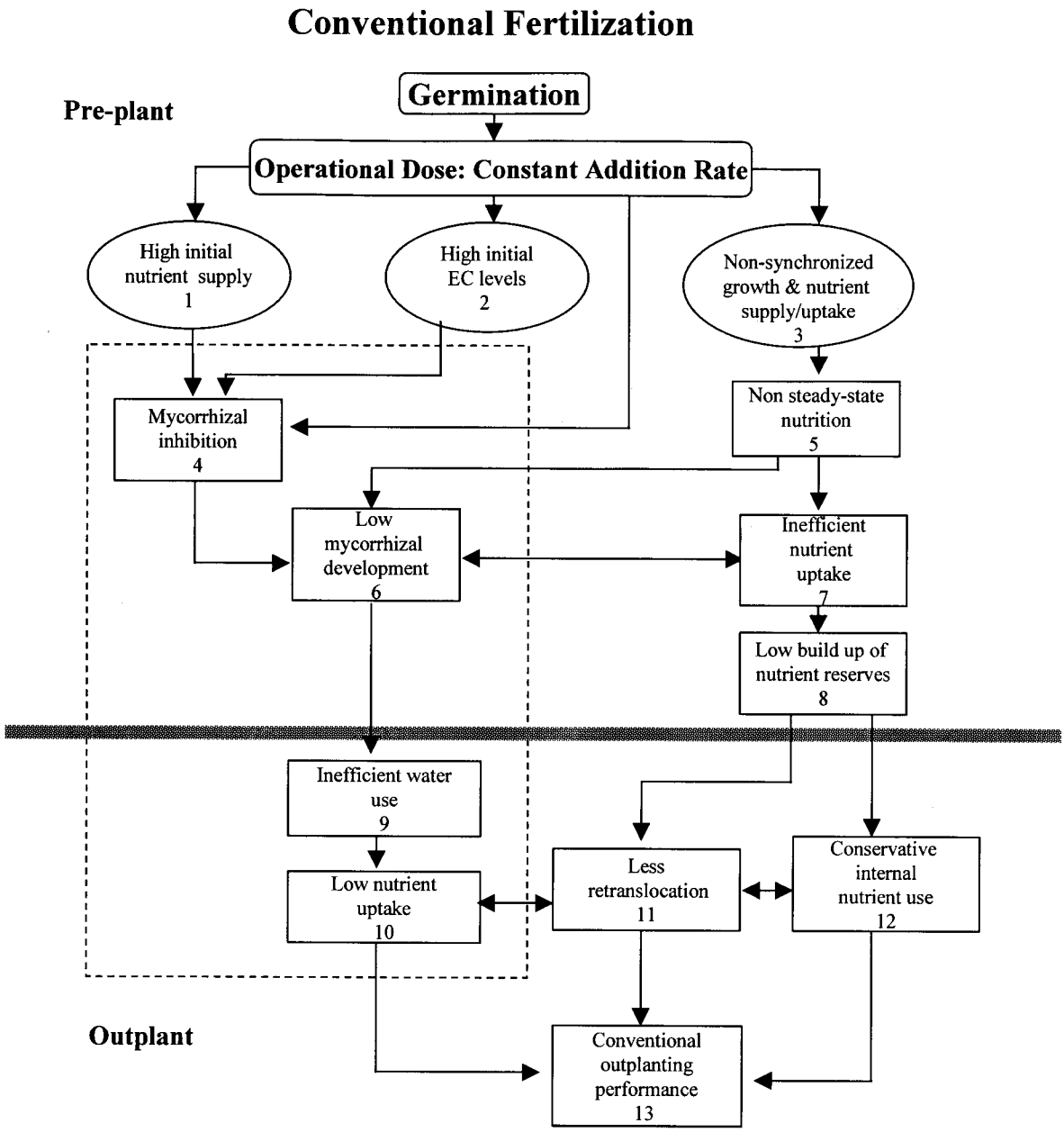

Fig. 6.1. Schematic representation showing critical conditions and processes involved in ectomycorrhizal formation, nutrient uptake, and nutrient storage in seedlings during nursery culture and early outplanting performance. The ovals represent the conditions that characterize the conventional fertilization regime. Rectangles represent the suggested processes involved in mycorrhizal inhibition, low nutrient reserves build up, and substandard outplanting performance. Dashed rectangle represents mycorrhizal system.

high initial EC levels (oval 2) in the rooting substrate that leads to non-synchronized growth and nutrient uptake (oval 3). The addition rate did not keep up with the exponential growth and nutrient demand of the seedlings, as shown in Fig. 6.2, where N and P assimilation under the conventional (12.5C) fertilization was proportionately lower than dry matter production of the seedlings as the season progressed. Consequently, internal nutrient concentration decreased rapidly with time because of growth dilution that is typical of non steady-state nutrition (rectangle 5) in seedlings. The dilution effect is depicted schematically by vector diagnosis (Fig. 4.4, Chapter 4) as shift A (Fig. 4.1, Chapter 4), significantly decreasing nutrient concentration with increasing nutrient uptake and growth with time. Hence growth and nutrient uptake were non-synchronized, leading to inefficient nutrient uptake (rectangle 7), and low build up of internal nutrient reserves (rectangle 8).

Exponential fertilization model

In contrast to conventional fertilization, exponential fertilization with an equivalent seasonal dose (12.5E), resulted in a progressive increase in nutrient supply, commencing with low fertilizer inputs and EC levels (rectangle 14 and 15), that induced synchronized growth and nutrient accumulation (rectangle 16) as reflected in Fig. 6.3. Consequently, exponential dry mass production, and N and P uptake were well matched for the entire growing season (Fig. 6.2). Synchronized growth and nutrient uptake resulted in stable internal nutrient concentrations typical of steady-state nutrition (rectangle 19, Fig. 4.4, Chapter 4) and efficient nutrient uptake (rectangle 21) that led to higher build up of nutrient reserves (Fig. 3.4, Chapter 3 and Fig. 4.3, Chapter 4) compared to conventional fertilization. Steady-state nutrition was characterized by vector diagnosis (Fig 4.4, Chapter 4), as shift B illustrating conditions of stable nutrient concentration with increasing nutrient uptake and growth in the plant (Fig. 4.1, Chapter 4). Therefore, under this regime, seedling growth and nutrient uptake were synchronized inducing steady-state nutrition and efficient nutrient accumulation, that resulted in higher internal nutrient reserves (rectangle 22) compared to conventional seedlings. Exponential fertilization (12.5E) was also much more efficient than conventional fertilization (12.5C) as shown for N and P uptake (Fig. 6.5), demonstrating an important advantage for seedling stock production.

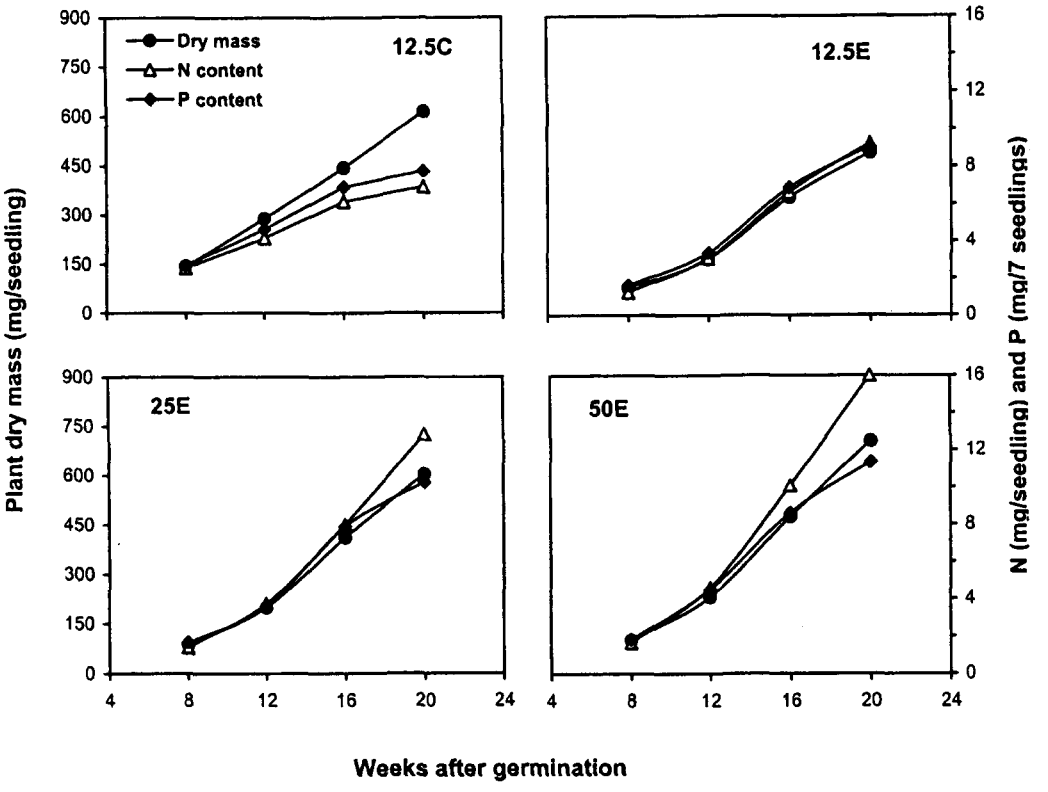

Fig. 6.2. Progression in dry mass production and N, P uptake of black spruce seedling at operational dose conventional (12.5C), operational dose exponential (12.5E), low dose exponential loading (25E), and high dose loading (50E) regimes during the growing season. N and P accumulation either matched the growth increments or luxury consumption of nutrients depending on loading dose under exponential regimes, but not in conventional fertility regime.

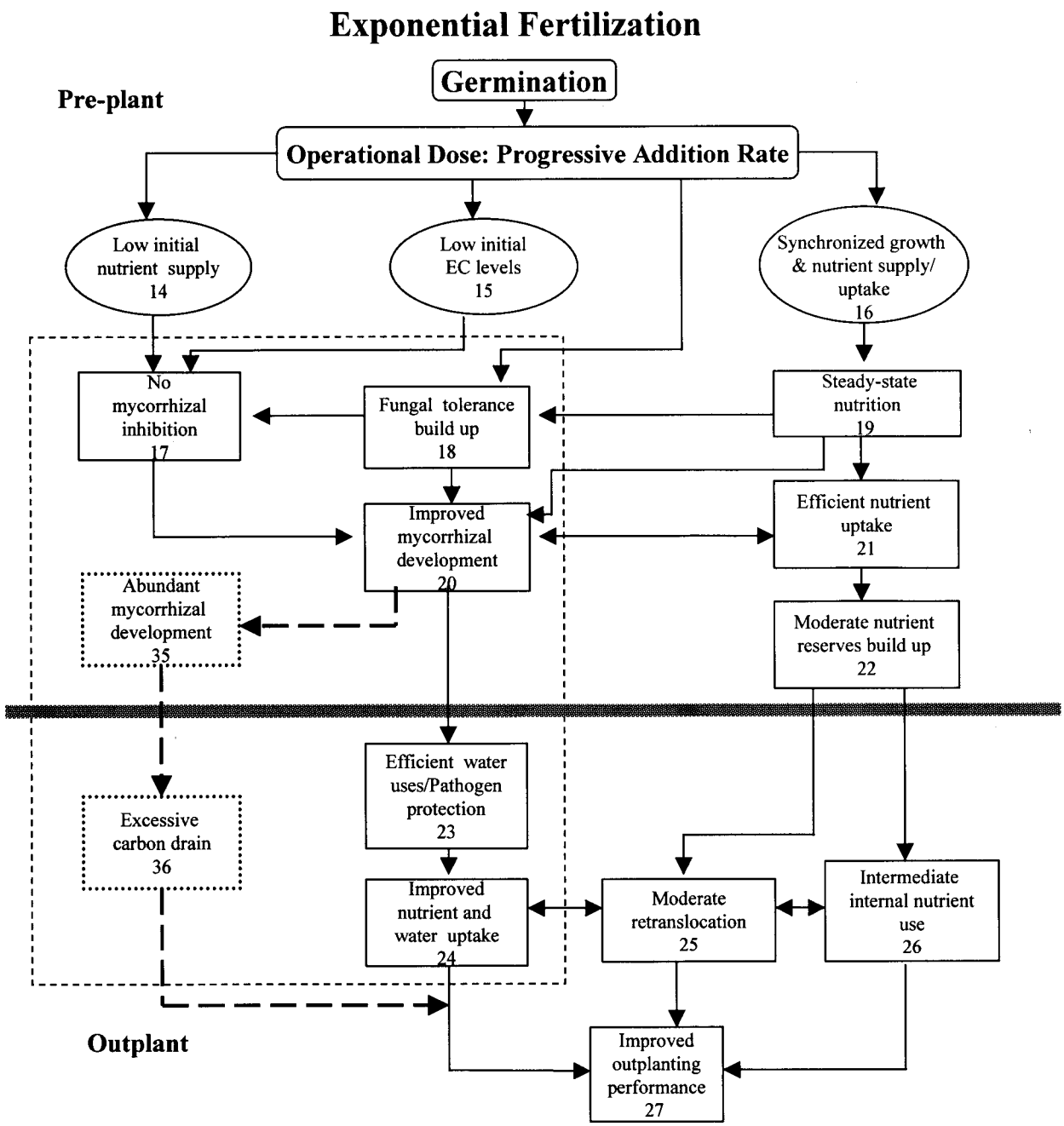

Fig. 6.3. Schematic representation showing critical conditions and possible processes involved in ectomycorrhizal formation, steady-state nutrition, and nutrient storage in seedling during nursery culture and early outplanting performance. The ovals represent the conditions that characterize the operational dose exponential fertilization regime. The rectangles represent the suggested processes leads to improved mycorrhizae formation, moderate nutrient reserve build up, and improved outplanting performance. Dotted rectangles with thick dashed arrows represent special circumstances in the case of excessive mycorrhizal formation. Interactions between the processes are shown as double-headed arrows. Dashed rectangle represents mycorrhizal system.

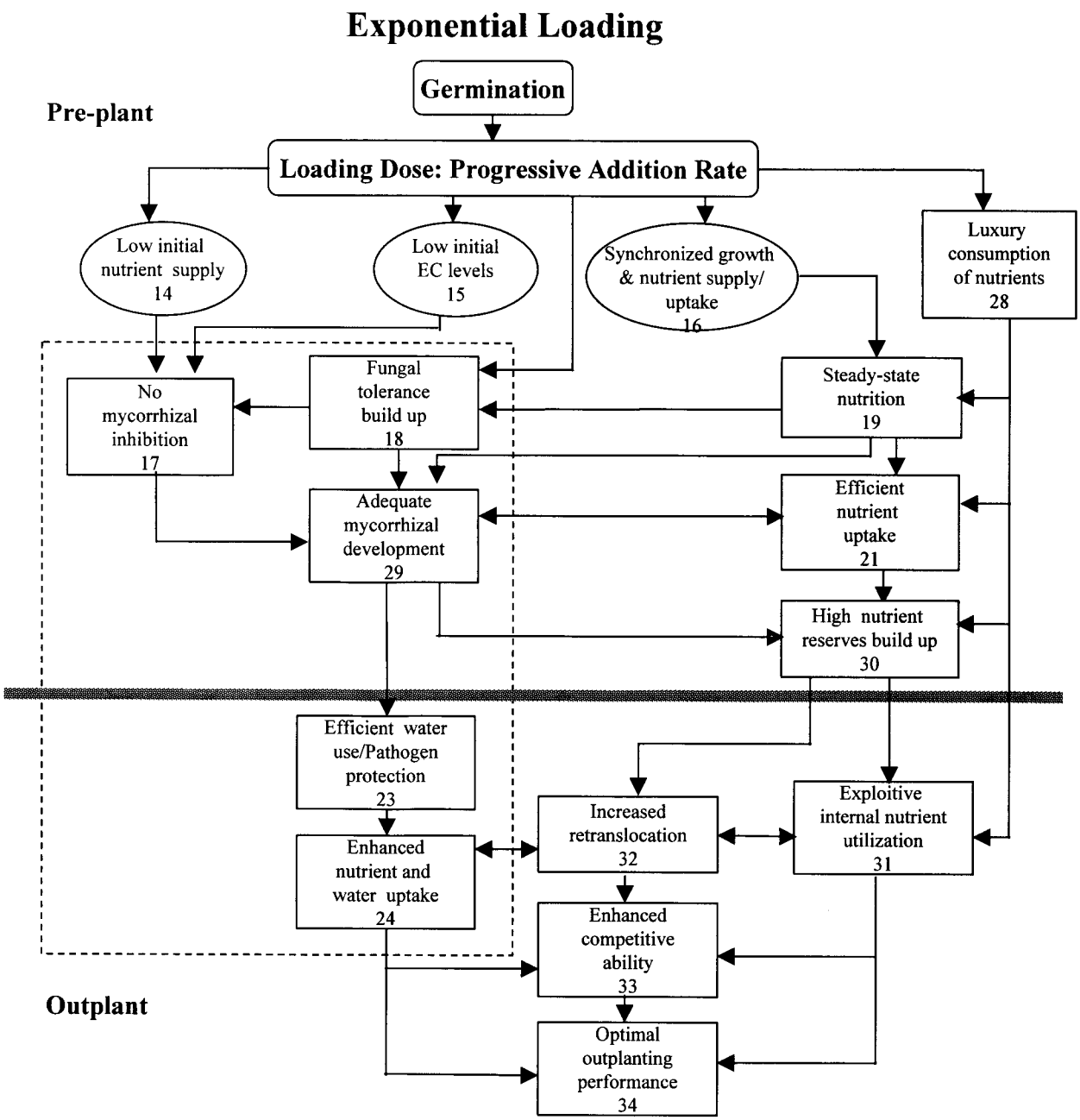

Fig. 6.4. Schematic representation showing critical conditions and processes involved in ectomycorrhizal formation, luxury nutrient uptake, steady-state nutrition, and nutrient storage in seedlings during nursery culture and early outplanting performance. The ovals represent the factors that characterize the loading dose exponential fertilization regime. The rectangles represent the suggested processes that lead to improved mycorrhizae formation, high nutrient reserve build up, and enhanced outplanting performance. Interactions between the processes are shown as double-headed arrows. Dashed rectangle represents mycorrhizal system.

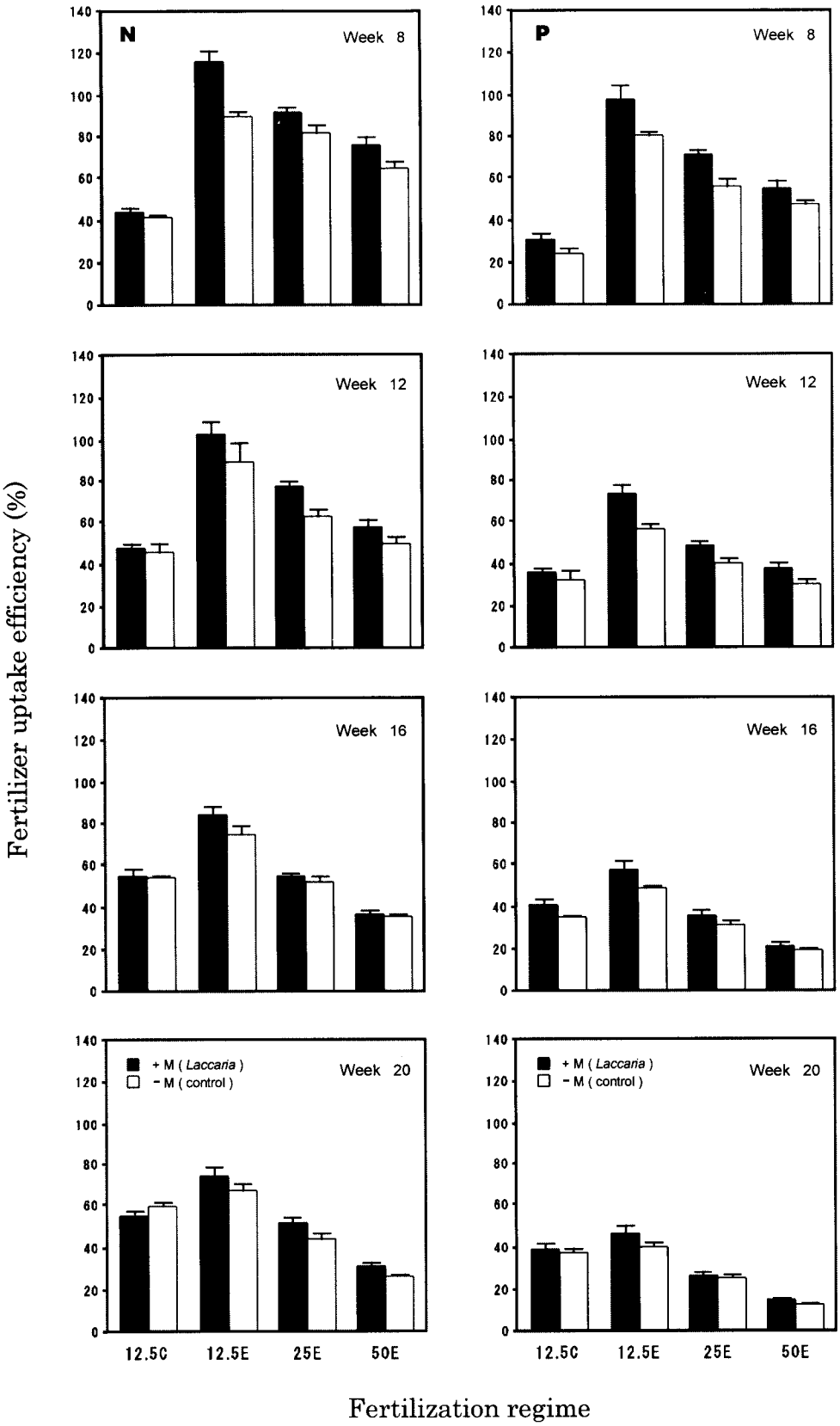

Fig. 6.5. Nitrogen and phosphorus uptake efficiency (NUE and PUE) of mycorrhizal (+ M) inoculated with *Laccaria bicolor* and non-mycorrhizal (- M) control (no fungus) black spruce seedling at conventional (12.5C), exponential (12.5E), low exponential loading (25E), and high exponential loading (50E) regimes during the growing season. Fertilizer uptake efficiency can be defined by the amount of nutrients (N and P) taken up in relation to total nutrients applied during a given time. Vertical bars represent standard error of the mean (SEM).

Exponential loading model

Exponential loading (Fig 6.4) at increased dose levels (25E and 50E) involved incrementally raising fertilizer inputs (Fig. 3.1, Chapter 3, Table 4.1, Chapter 4) that gradually builds up soluble salts in the rooting media (Fig. 4.5, Chapter 4), allowing both the trees and mycorrhizal fungi to adapt progressively to high nutrient additions. Although nutrient uptake rate exceeded growth rate inducing luxury consumption (rectangle 28) of nutrients, assimilation was proportional, accumulating higher nutrient reserves at steady-state conditions. Thus synchronized growth and luxury uptake induced stable internal nutrient concentration at higher levels than the conventional (12.5C) or exponential (12.5E) regimes. This was achieved at steady-state nutrition (rectangle 19, Fig. 4.4, Chapter 4) and at luxury consumption (rectangle 28). The application schedule promoted efficient nutrient uptake (rectangle 21) and higher nutrient accumulation (rectangle 30) in seedlings (Fig. 3.4, Chapter 3 and Fig. 4.3, Chapter 4). Steady-state achievement was validated by vector diagnosis as shift B (Fig. 4.1, Chapter 4) vectors in nomograms for N and P (Fig. 4.4, Chapter 4). Thus under exponential nutrient loading, seedling growth and luxury nutrient uptake were synchronized to ensure steady-state accumulation of extra high nutrient reserves in seedlings (rectangle 30) for utilization later for new growth after outplanting.

ECTOMYCORRHIZAL DEVELOPMENT IN SEEDLINGS IN THE NURSERY

Conventional fertilization model

Ectomycorrhizal development under conventional fertilization regime was inhibited (Chapter 3 and 4), resulting in lower infection rates (22-26%, p = 0.0001) compared to exponential fertilization regimes, indicating nutrient supply during seedling culture can control (Fig. 3.2, Table 3.1, Chapter 3 and Table 4.2, Chapter 4) mycorrhizal symbiosis in the nursery. Apparently, fertilizers supplied weekly at constant rates during seedling culture result in a relatively high initial nutrient supply to trees (oval 1), causing high initial EC levels in growing media (oval 2) that are toxic to mycorrhizae initially. Substrate fertility, type of container, growing media, inoculum storage, and frequency of watering can all influence the effectiveness of the inoculum for the successful production of mycorrhizal seedlings (Marx and Barnett 1974, Dixon *et al.* 1979, Ruehle *et al.* 1981). But, in this case, mycorrhizal inhibition (rectangle 4) was associated with reduced inoculum potential when exposing young vegetative inoculums early in the inoculation process to relatively high levels of nutrient exposure (Fig. 3.1, Chapter 3), resulting in low mycorrhizal formation on root systems (rectangle 6, Fig. 3.2, Chapter 3 and Fig. 4.6, Chapter 4) as illustrated in Fig. 6.1.

Exponential fertilization model

Exponential fertilization significantly increased (p = 0.0001, Table 3.1, Chapter 3) ectomycorrhizal

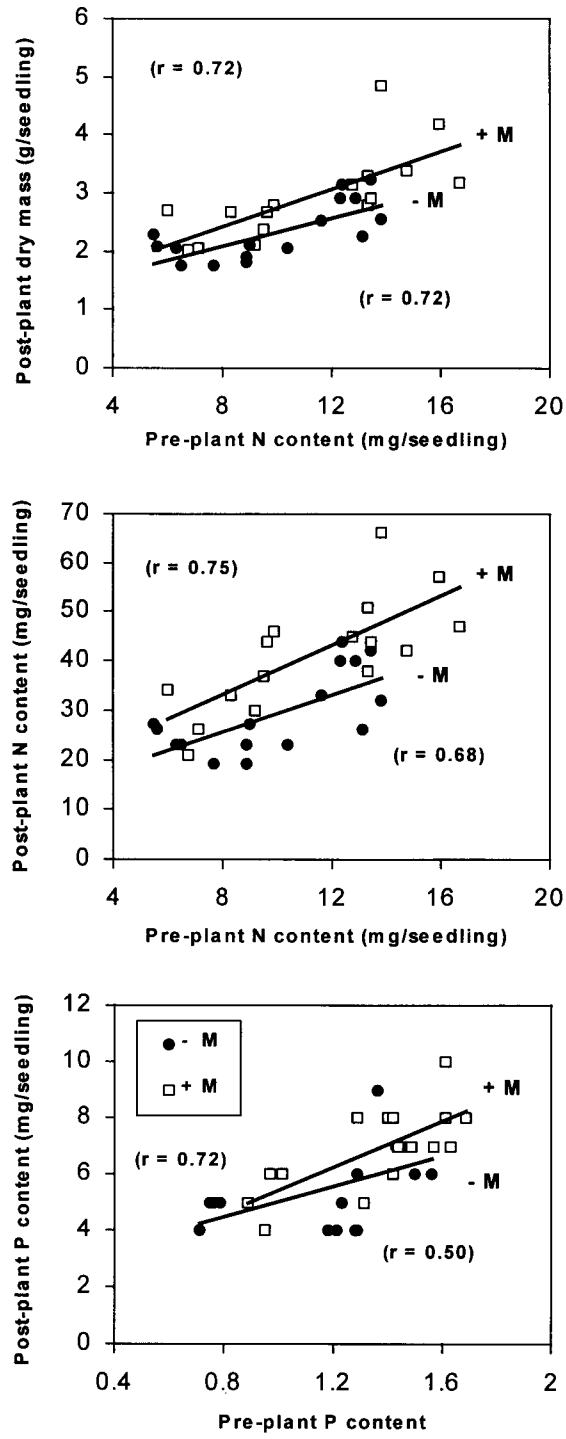

Fig. 6.6. Relationship between outplant N and P content and Pre-plant N and P content of black spruce reared under conventional and exponential fertilization regimes with (+ M) or without (- M) mycorrhizal infection. Each point represents a pooled sample of six seedlings outplanted on Feathermoss site.

formation compared to conventional fertilization because of relatively lower amounts of fertilizer applied initially (oval 14), thus avoiding low initial growing media EC levels (oval 15) that minimized early nutrient toxicity to fungal inoculum and favored the infection process. Exponential fertilization (Fig. 6.3) involved progressive increases in nutrient enrichment (see Fig. 3.1, 3.3, Chapter 3, and Fig. 4.5, Table 4.1, Chapter 4), promoting synchronized growth and nutrient uptake (rectangle 16) at steady-state nutrition (rectangle 19) in seedlings, and facilitated fungal symbionts to adapt and build up tolerance to relatively higher nutritional conditions incrementally (rectangle 18). Thus, the improved ectomycorrhizal development (rectangle 20, Fig. 3.2, Chapter 3 and Fig. 4.6, Chapter 4) occurred under exponential fertilization was attributed to enhance inoculum viability and gradual development of fungal tolerance to high nutrient exposure at steady-state nutrition.

Exponential loading model

Ectomycorrhizal infection rates obtained under the exponential loading regime (50E) were twice those of conventionally (12.5C) fertilized seedlings (Fig. 3.2, Chapter 3 and Fig. 4.6, Chapter 4) exemplified the compatibility of exponential nutrient loading practices with mycorrhizal development in the nursery despite high (50 mg N/plant) fertilizer inputs. Adequate ectomycorrhizal infection (rectangle 29) was associated with lower initial nutrient supply (oval 14) and lower EC levels (oval 15), and progressive increases in nutrient delivery (Table 4.1, Chapter 4). This schedule facilitated the fungi to acclimatize and build up tolerance (rectangle 18) to higher nutrient levels. Mycorrhizae formation achieved under exponential regime stimulated nutrient uptake (rectangle 21) as reflected in Fig. 6.6. Seedling N and P uptake efficiency were consistently higher in mycorrhizal seedling (+ M) than non-mycorrhizal control (- M), reflecting the superior nutrient uptake ability of mycorrhizal seedlings. Thus both fertilizer addition and mycorrhizal infection increased plant nutrient uptake, demonstrating effect of both chemical and biological mediated nutrient loading mechanisms

UTILIZATION OF NUTRIENT RESERVES AFTER OUTPLANTING

Conventional fertilization model

Improved competitiveness of newly planted seedlings has been attributed previously to the size of the internal reserve pool (Malik and Timmer 1995, 1996, 1998, Xu and Timmer 1998, 1999). Conventionally reared seedlings acquired less nutrient reserves than exponentially fertilized seedlings (Table 5.2, Chapter 5) and exhibited lower outplanting performance when compared to nutrient-loaded ectomycorrhizal seedlings (Fig. 5.1, 5.2, Chapter 5). Apparently, conventional seedlings spend more energy acquiring below ground resources, resulting in a negative effect in seedling shoot growth. Seedlings with low nutrient reserves followed a conservative internal nutrient use strategy (rectangle 12), where nutrients are primarily assimilated into functional proteins and enzymes that are likely less mobile than are available amino acids. Consequently, retranslocation in plants was restricted (Titus and Kang 1982, Malik and Timmer 1998), and plants needed to conserve their nutrient reserves after planting, yielding moderate new growth, but ensuring nutrient supplies for future development (Malik and Timmer 1998). Furthermore, conventionally reared seedlings had low mycorrhizal development (rectangle 6) that negatively affected water use efficiency (rectangle 9) (Auge *et al.* 1986), and restricted use of available resources yielding conventional, sub optimal outplanting performance of seedlings (rectangle 13).

Exponential fertilization model

The improved outplanting performance (rectangle 27) of exponentially reared seedlings when compared to conventional seedlings was attributed to moderate pre-plant internal reserves (Fig. 3.4, Chapter 3, rectangle 22) and their efficient utilization, greater nutrient uptake due to higher mycorrhizal infection, greater protection from root-rotting pathogens (Fig. 5.2, rectangle 24), and preferred retranslocation of nutrient reserves (rectangle 25) during early plantation establishment. Higher pre-plant nutrient reserves and increased colonization rates induced efficient utilization of internal reserves and resource acquisition (rectangle 23 and 24) from soil than in conventionally reared seedlings as shown in the bioassay study (Fig. 5.2, Chapter 5). Internal nutrient use of exponential seedlings was recognized as an intermediate nutrient use strategy (rectangle 26), leading to moderate retranslocation (rectangle 25) of nutrient reserves compared to conventional seedlings based on the magnitude of pre-plant internal nutrient pool.

Exponential loading model

Exponentially loaded seedlings (Fig. 6.4) accumulated higher internal reserves (rectangle 30) than conventionally and exponentially fertilized seedlings (Table 5.2, Chapter 5) and yielded optimum outplanting performance (rectangle 34). The superior growth and nutrient uptake of planted seedlings (Fig. 5.1, 5.2, Chapter 5) obtained by nutrient loading was associated with higher internal nutrient pools built up in the nursery (rectangle 30) and more efficient utilization of reserves by accelerated retranslocation (rectangle 31 and 32) of nutrients. Much of the variation in response can be attributed to the magnitude of the nutrient status of seedlings at planting. Seedlings with greater reserves were probably less stressed and used less energy to assimilate external nutrients immediately after planting.

The extra response in plant biomass and N, P status after transplanting (Fig. 5.1, 5.2, Chapter 5) confirmed that mycorrhizal nutrient loading stimulated growth and nutrient uptake of seedlings compared to non-mycorrhizal conventional seedlings (with low nutrient reserves). The superior performance after planting was presumably due to higher nutrient reserves (Fig. 3.4,

Chapter 3 and Fig. 4.3, Chapter 4.3) and mycorrhizal infection (Fig. 4.6, Chapter 4) at planting that increased retranslocation of internal reserves (rectangle 31, 32), improving nutrient and water uptake (rectangle 23, 24), suppress root-rotting pathogens, and enhancing competitive ability (rectangle 33) that led to optimal outplanting response (rectangle 34). The importance of pre-plant nutrient reserves for early plantation establishment was reflected in Fig. 6.6. Higher growth and nutrient uptake (N and P) after transplanting was closely correlated to pre-plant N and P pools in seedlings. Furthermore, in each case seedling response after transplanting was surpassed with- rather than without- mycorrhizal inoculation, exemplifying the merits of combing nutrient loading with mycorrhizal association.

In mycorrhizal root systems, assimilated inorganic N is stored in the form of simple amino acids before it converts to more complex organic molecules (Smith and Read 1997). These free pools of amino acids are considered to be the main forms of recycled nitrogen within plants (Margolis and Brand 1990). Furthermore, with luxury consumption (rectangle 27) the internal nutrients are probably stored .in the more readily available forms found in the retranslocatable nutrient pool. These internal nutrient reserves are thought to be simple amino acids, less structurally bound than reserves in conventional seedlings, and hence more easily available when required for growth (Malik and Timmer 1998). Unlike conventional seedlings, loaded seedlings adapt an exploitive internal nutrient utilization (rectangle 31) strategy after outplanting resulting in rapid initial growth at the expense of accumulated reserves. Consequently, nutrient-loaded seedlings show faster and increased retranslocation, leading to an enhanced competitive ability (rectangle 33) among seedlings as demonstrated by Imo and Timmer 1999, Malik and Timmer 1996.

Carbohydrate relations and mycorrhizal loading

Discussion of aspects of carbohydrate dynamics between the symbionts after mycorrhizal nutrient loading is needed to relate responses to the symbiotic association. In most mycorrhizal systems, symbiosis is characterized as the transfer of organic C from the plants to the fungus for their growth and development and nutrients from the fungus to the plants. A condition not common to all three models occurs under exponential fertilization regime (12.5E) when pre-plant mycorrhizal infection of seedling root systems was far above the threshold 50% infection rate of short roots (see Fig. 5.4, Chapter 5). In this particular case, the extremely high mycorrhizal development (rectangle 35) would result in excessive carbon drain from the host (rectangle 36) which may detrimentally affect outplanting performance (Fig. 6.7), suggesting that

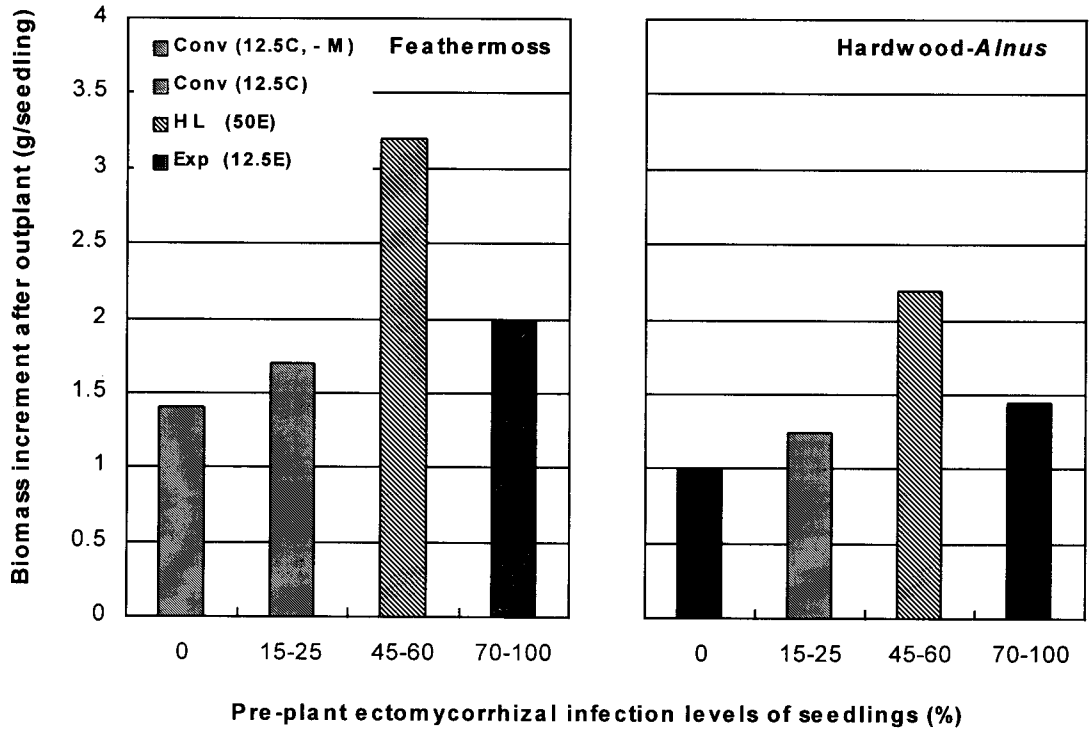

Fig. 6.7. Post-plant biomass increment in relation to the pre-plant ectomycorrhizal infection of conventional [Conv (12.5C, - M) or [Conv (12.5C), high loaded [HL (50E)], and exponential [Exp (12.5E)] black spruce seedlings transplanted on intact bioassay retrieved from two different forest sites. The biomass increment data represents the pooled values from the corresponding infection classes. Pre-plant N content in non-inoculated conventional on (12.5C, - M), inoculated conventional (12.5C), exponential [Exp (12.5E)], and high loaded [HL (50E)] were 6.0, 7.1, 9.5, 15.3 mg/plant, respectively.

abundant pre-plant infection levels may not be essential for optimal seedling response in the field.

The mechanism by which mycorrhizal symbiosis is regulated has been the subject of controversy for a long time (Nylund 1988). The interactions between N and P supply, carbohydrate availability, and ectomycorrhizal formation have been studied extensively and are considered to be crucial factors for the plant-fungal symbiosis (Nylund 1988). The inhibitory effect of higher N on mycorrhizae has been interpreted as an indirect effect of reducing sugar concentration in the roots (Wallander and Nylund 1991). However, other reports contradict the view that, at high nutrient levels, sugar concentrations decrease (Rudawska 1986, Wallander and Nylund 1991). It is generally agreed that, at high N supply, the host allocates less photosynthate to the mycobiont as a result of a greater demand for carbon by the growing shoots (Bjorkman 1942). According to the carbohydrate theory, mycorrhiza can develop only when the host plant contains surplus levels of soluble carbohydrates (Bjorkman 1942). Contrary to Bjorkman's (1942) view that deficiency of nutrients is a precondition for formation of mycorrhiza, experimental evidence (Chapter 3 and 4) demonstrated that adequate mycorrhizal development occurred (Fig. 3.2, Chapter 3 and Fig. 4.6, Chapter 4) even at high loading dose under exponential regimes. These results suggest that synchronized growth and nutrient uptake (Fig. 6.2) under exponential nutrient loading technique resulted in steady-state nutrient status in plants (Fig. 4.4, Chapter 4), which in turn induced stable carbohydrate relations between the symbionts.

Ectomycorrhizal infection can change the carbohydrate relations of trees through a number of interrelated plant processes including net photosynthesis, biomass production, biomass partitioning, and mineral nutrient uptake. Since mycorrhizae constitute a considerable carbon sink to plants, it is possible to change carbon allocation from shoot to root by stimulating net photosynthesis (Read *et al.* 1983). Other views advocate that improved N and P nutrition of seedlings due to mycorrhizal formation plays a substantial role in modifying plant carbon assimilation and allocation thus increasing net photosynthetic rate (Bowen 1973, Rousseau and Reid 1990). However, results of seedling growth, nutritional status, and ectomycorrhizal formation in this study (Chapter 3 and 4), suggest that successful mycorrhizal colonization can be achieved even at high levels of nutrient supply (loading dose), if applied exponentially. Formation of mycorrhizae under conditions where growth was not restricted by nutrient supply has been reported earlier by Ingestad and coworkers (1986). I suggest that under exponential fertilization and steady-state nutrition, a stable supply of carbohydrate from the host to fungus must occur once the fungal infection is established, which is necessary for maintaining the symbiosis. Hence, a stable carbohydrate pool in seedlings and a steady flow of carbohydrate from host to fungus is much more important than nutritionally stressed seedling with greater root carbohydrate reserves. Further research is required to support these suggestions and to quantify the rate of photosynthesis and carbohydrate pool flux that takes place during seedling production.

CONCLUSIONS

The generous supply of nutrients at constant addition rates used by commercial nurseries to produce planting stock potentially conflicts with the compatible nutritional levels required for adequate ectomycorrhiza formation. The traditional view that poor nutritional condition is the prerequisite for development of mycorrhizal symbiosis in plants was found to be untenable based on my experimental findings (Chapter 3, 4, and 5). In fact, results showed that mode of fertilizer delivery and stable internal nutrient concentration may be far more critical than the total amount of fertilizer applied for the production of mycorrhizal seedling. Superior mycorrhizal association was obtained by adopting exponential fertilization regimes that exposed symbionts incrementally to higher nutrients at steady-state supply, resulting in synchronized growth and nutrient uptake. This exponential fertilization approach was also found compatible with nutrient loading practices that were intimately associated with build up of internal nutrient reserves and subsequently enhanced outplanting performance on bioassays.

The fundamental aspects of seedling response to various nutritional regimes were not recognized as a whole in the past research. Therefore, a conceptual representation of the relationships between the mycorrhizae and build up of seedling nutrient reserves was developed to break down the inherent complexity of the critical processes of mycorrhizal nutrient loading and functioning after planting. Despite the complexity, an integrated modeling approach (Fig. 6.1, 6.3, and 6.4) identified the critical conditions and processes that affected mycorhizal development, mycorrhizal nutrient loading and resource utilization after planting. Furthermore, to simply the models I have combined three models into a single model (Fig. 6.8). These key processes and their essential differences between the fertilization regimes are depicted in simplified system diagrams. Understanding these critical processes (Fig. 6.1, 6.3, 6.4 and 6.8) would effectively integrate this novel approach (mycorrhizal nutrient loading) into current operational forestry. However, further research is needed to determine the link between mycorrhizae and water uptake efficiency and retranslocation processes involved within the system to confirm their role and to validate the models.

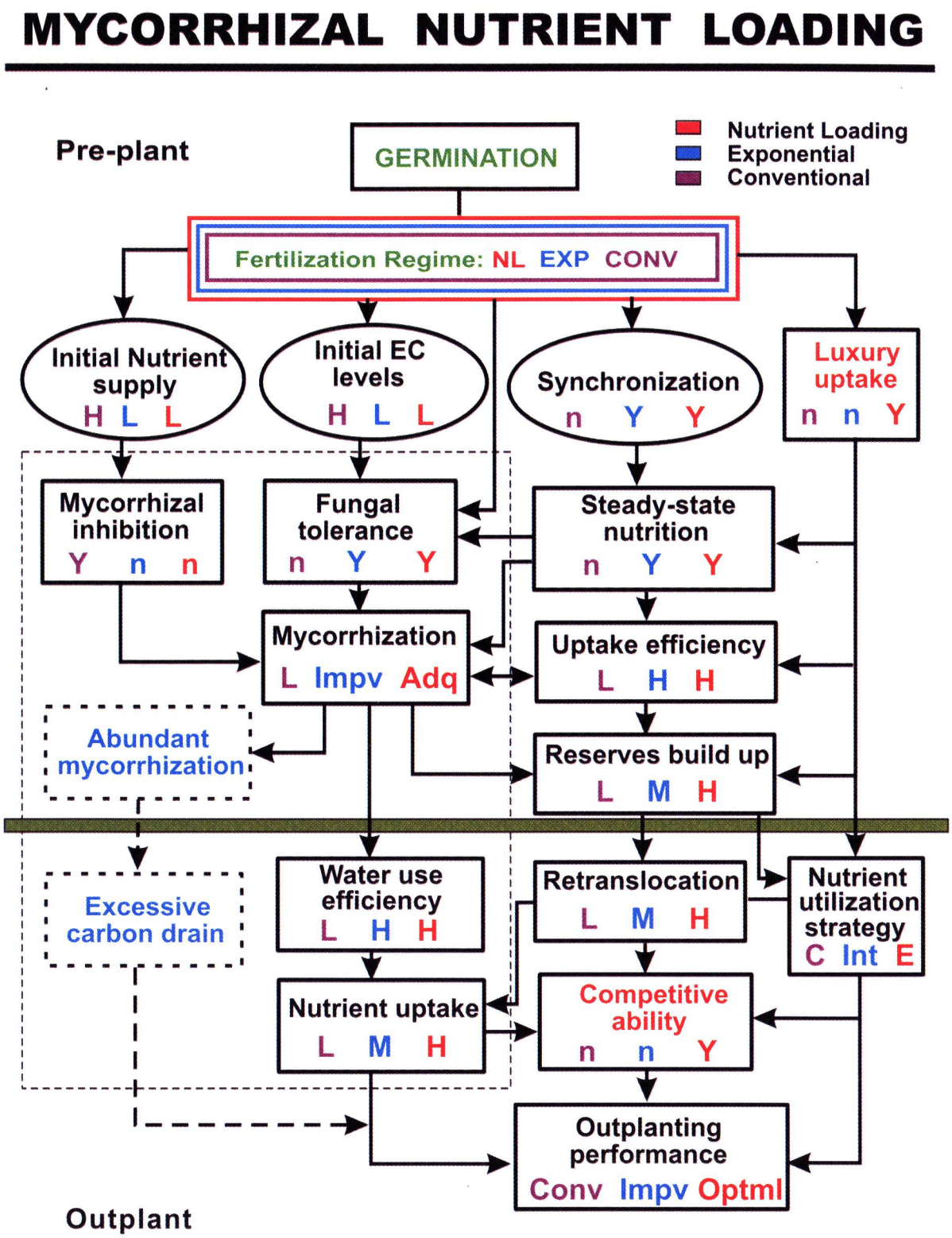

Fig. 6.8 An integrated schematic representation of three (Fig. 6.1, 6.3 and 6.4) models called mycorrhizal nutrient loading showing critical conditions and processes involved in ectomycorrhizal formation, luxury nutrient uptake, steady-state nutrition , and nutrient storage in seedlings during nursery culture and early outplanting performance. The interactions of critical process are separated by colors: red, blue and purple. The top portion is pre-plant stage, bottom portion is outplant phase separated by thick green line. Capital H and L stand for high and low, and small y and n indicate yes and no, respectively.

Chapter 7 : Summary

GENERAL CONCLUSIONS

Although specific conclusions for each experiment are presented in chapters 3, 4, and 5, this chapter further summarizes the results and provides a brief outline of the implications for commercial nursery and field application. Based on present experimental evidence and discussions from the literature, it is possible to make the following general conclusions on the role of nursery fertilization and seedling nutrition in mycorrhizal development:

(1) Although mycorrhizal development is often incompatible with high nutrient availability, this study demonstrated the successful production of mycorrhizal black spruce seedlings in containers at high nutrient inputs if the nutrients are applied exponentially during the growing season. This is contrary to classical theory (Bjorkman 1942) and invalidates the postulate that deficiency of mineral nutrients is a precondition for formation of mycorrhiza.

(2) The carbohydrate theory proposed by Bjorkman (1942) does not seem to be applicable to this study. The nutritional effect on mycorrhiza may not be simply the effect of high nutrient availability on carbohydrate reduction in seedlings. The results suggest that a larger carbohydrate pool may not be important since a continuous supply of carbohydrate from host to fungus is essential to maintain sustained symbiosis. A stable carbohydrate supply may be maintained when seedlings are grown under steady-state nutrient culture and a constant relative growth rate (Ingestad and Lund 1986).

(3) Study results indicate that the mode of nutrient delivery rather than the total amount of fertilizer applied is crucial for the production of mycorrhizal seedlings. Therefore, it is suggested that the conventional fertilization regime practiced in operational nurseries should be modified to an exponentially-based fertilization regime. This will facilitate integration of mycorrhizal inoculation and nutrient loading for efficient internal nutrient build up and biological conditioning of plants.

(4) The mycorrhizal nutrient loading technique shows significant potential in enhancing seedling nutrition during nursery culture and seems particularly effective in building up reserves by increasing fertilizer uptake efficiency via luxury uptake, and may provide an opportunity for greater outplanting performance.

(5) Nutrient loading combined with ectomycorrhizal inoculation provides nutritional advantages to preconditioning nursery seedlings, and protects against early nutritional stress encountered during outplanting. This could play an important role in the formulation of intensive reforestation programs.

(6) Contrary to the traditional view, adequate ectomycorrhizal formation in inoculated seedlings due to progressively increasing nutrient exposure under an exponential regime may benefit mycelia, allowing fungi to acclimatize to higher nutrient levels. This method may enable fungi to build tolerance to high nutrient levels. Exponential fertilization regimes induced steady-state nutrient status in the seedlings, which may benefit sustained symbiosis. The exponential fertilization technique is recommended for ectomycorrhizal nutrient loading for nursery stock production.

(7) Examination of the nutrient dynamics of the growing media during seedling development suggests that initial soluble salt concentration early in the inoculation process is a crucial factor affecting colonization success despite the build up in EC levels later in the season. Therefore, the need for using low dose fertilizer applications initially and then increasing levels progressively during seedling development is recommended for integrating mycorrhizal technology in seedling culture.

(8) New guideline values or critical levels were determined for monitoring production of mycorrhizal seedlings. Early- and late-season thresholds for mycorrhizal inhibition were associated with growing media soluble salt levels exceeding 248 and 1500 $\mu S\ cm^{-1}$, respectively. Graphical vector diagnosis was effective in detecting steady-state nutrition, luxury consumption, element dilution, and nutrient deficiency. Maximum nutrient loading occurred at steady-state concentration of 2.4% (dry weight) tissue N. Pre-plant tree N content proved to be useful predictor of initial outplanting performance of seedling stock. A 50-60% mycobiont infection rate of root systems was confirmed as satisfactory for mycorrhizal nutrient loaded seedlings.

(9) The outplanting results confirm the dependence of seedlings on pre-plant internal nutrient reserves and ectomycorrhizal root systems to support early establishment of black spruce seedlings. The superior response of mycorrhizal-loaded seedlings at both high and low competitive sites also confirms the importance of mycorrhizal nutrient loading in reducing plantation problems at routine reforestation sites.

(10) The present approach significantly improved the quality of containerized black spruce seedlings in nurseries in terms of enhanced nutrition, and increased growth and nutrient uptake on simulated reforestation sites. Therefore, the use of an ecologically adapted fungal symbiont as a biological tool, combined with new fertilization technique for reclamation and reforestation efforts in Canada appears highly promising.

(11) Conceptual models were effective in characterizing the growing conditions attained under intensive fertilization regimes, identifying the key mechanisms involved, and demonstrating interactions between soil fertility, seedling growth,

nutrient uptake and mycorrhizal infection processes both in the nursery, and when outplanted.

Suggestions for future research

The general goal of this research was to test the compatibility of exponential fertilization and nutrient loading with mycorrhizal inoculation under steady-state nutrient culture in the nursery, and to investigate the growth and nutrition of these seedlings in the field. However, there remains an enormous scope for investigating both the fundamental and practical aspects of symbiotic relationships to tree nutrition. My results raise some physiological and applied prospects for this approach. This leaves room for future investigation for further advancement of the technique:

(1) Although satisfactory mycorrhiza formation in the nursery was obtained, my research was not able to characterize many of the physiological events (e.g., changes in chemical compositions, proteins, enzymes, hormonal effects, etc.) that may occur at the cellular level during the inoculation process. This realm requires further investigation. Understanding these processes may improve the potential use of ectomycorrhizal inoculation in commercial nurseries for successful reforestation.

(2) Since nursery managers are reluctant to use nutrient loading and inoculation techniques for commercial seedlings, it is apparent that large-scale field trials of these seedlings, followed by an economic evaluation, are essential in order to show their benefits before they will be adopted universally.

(3) Future research is needed in order to characterize the chemical composition of internal reserves, and to ascertain whether the formation and composition of these reserves is altered by the mycorrhizal development and (or) the fertilization regime. Answers to these questions will provide further understanding of the symbiotic relationships and retranslocation processes. This will facilitate precise recommendations for loading schedules, using the biological component and (or) higher fertilizer application for improved reforestation.

(4) Carbon flow from host to fungus is an essential process for mycorrhizal symbiosis and the carbon is required for fundamental growth processes, such as the assimilation of N into organic molecules. This raises the question whether or not efficiencies in loading and symbiosis can be further enhanced by carbon loading during seedling culture. Furthermore, it is essential to directly test the levels of soluble carbohydrate in roots under both steady-state and non-steady-state conditions, with or without inoculation, to reveal exact carbohydrate relationships. Carbohydrate concentrations may vary widely with plant physiological status and cultural conditions (Ericsson 1980).

(5) The models that I have presented addressed conditions and processes that may be involved in mycorrhizal formation under intensive nutrient culture with containerized black spruce seedlings. The model also illustrates how enhanced field performance can be obtained, but needs further validation with several other host/mycobiont associations since mycorrhizal symbiosis and its effectiveness varies greatly according to species and site concerned.

(6) The model may need adjustments to further improvement of seedling development depending on the grower's objective. The model can also serve as a useful tool for other seedling preconditioning practices, such as carbon loading, which have great potential to improve planting stock quality.

(7) This study recommends testing the findings of this research approach with other conifer species of northern biosphere in order to validate the current approach. Since this research showed significant improvement in the quality of containerized black spruce seedlings in nurseries as well as increased growth and nutrient uptake on simulated reforestation sites. This approach may be useful to adapt in reforestation programs in northern Japan. In this part of Japan, where Sakhalin spruce (*Picea glehnii* Masters) that is quite similar to black spruce in Canada planted mostly on infertile serpentine or volcanic ash soils and burned forest areas that often exhibited poor growth. Another important species in Hokkaido is Japanese Larch also used for various plantation programs need to be practiced with this research approach. This study supports the concept that a wide array of ecotypes should be tested before extensive nursery inoculations. Recently, we had isolated fungal strains from a plantation of *Picea mariana* in Teshio Experimental Forests, Hokkaido, Japan. The potential of these ecologically adapted to improve survival and growth of *Picea mariana* and other conifer species should be determined.

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APPENDIX

Production of ectomycorrhizal fungal inoculum for this study

The pure vegetative inoculum of ectomycorrhizal fungi mixed with a growing substrate used in this study to inoculate the containerized black spruce seedlings was obtained from a commercial inoculum producer (Plant Health Care Inc., Pittsburg, PA 15283, U.S.A.). A standard procedure as described by Marx and Kenney (1982) and Marx et al. (1982) was adapted with some modifications to produce the live vegetative inoculum. The first step was to find a pure culture of selected fungi. This procedure involved growing mycelium of selected fungi under sterilized condition using a modified Melin-Norkrans (MMN) liquid medium containing glucose (Marx and Bryan 1975) with pH adjusted to 5.5 for 3 weeks. The fungal culture was then homogenized in a Waring blender for 15 s to achieve a homogenized mixture of mycelial peat moss moistened with same modified MMN liquid medium, and the inoculum fungus was allowed to develop aseptically in autoclavable plastic bags for 2-3 months at room temperature. The vermiculite substrate, when permeated by the fungal inoculum, was removed from the bags, then leached with distilled water to remove unassimilated nutrients. The inoculum was then dried to a moisture content 15-20 % based on oven dry weight. Dried inoculum was subsequently packed in sterile breathable transparent bags and stored at 5 ℃.

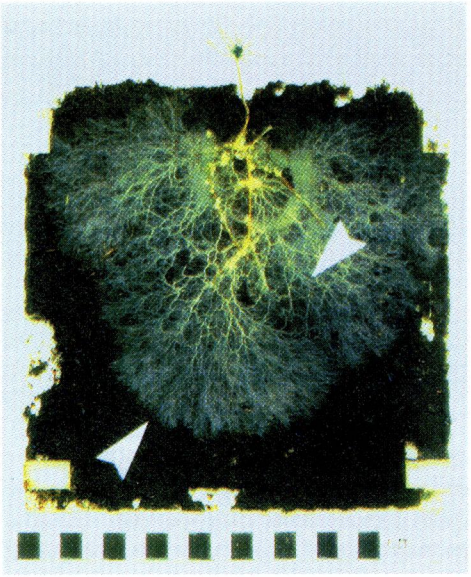

Photo plate 2.1. Example of greater absorptive area of *Pinus* seedlings infected with the ectomycorrhizal fungus *Suillus bovinus* and grown in transparent observation chamber containing non-sterile forest soil. The effectiveness of the exploitation of the environment by the mycelial fan is clear. The mycelium is an early stage of development but differentiation between the advancing hyphal front (lower arrow) and mycelial strands (upper arrow) is evident. (Adapted from Read 1991.)

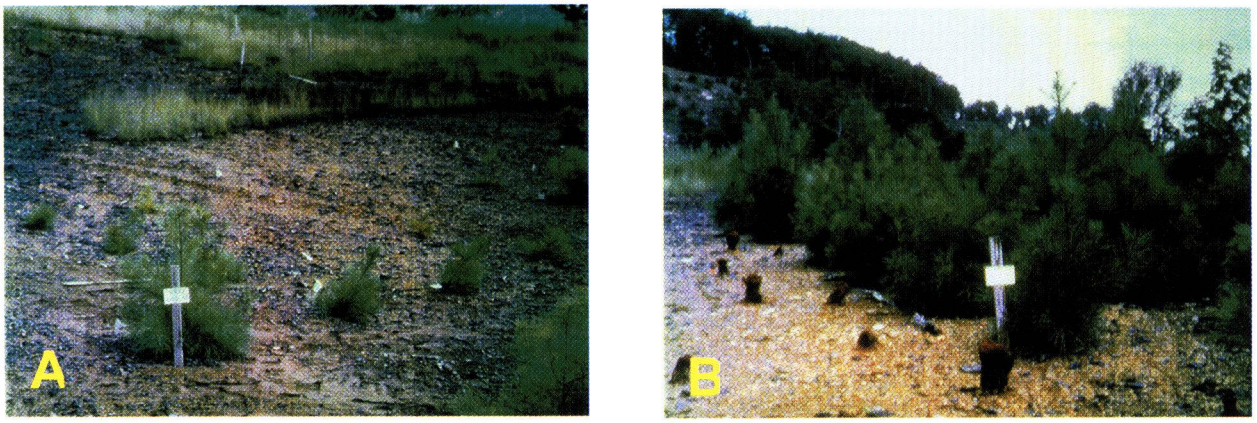

Photo plate 2.2. The importance of ectomycorrhizae to artificial regeneration has been observed in field studies on acid coal spoils. Loblolly pine grown two years on an acid coal spoil soil with (A) naturally occurring ectomycorrhizae or (B) *Pisolithus tinctorius* ectomycorrhizae at planting. Pre-plant inoculation with ectomycorrhizae shows dramatically greater survival and growth than naturally occurring ectomycorrhizae. (Adapted from Marx 1991.)

Photo plate 3.1. Black spruce seedlings were grown in Spencer-Lemaire Rootrainers® plastic containers (110 cm^3 capacity) and arranged in trays with 50 cavities in rows or books of five, filled with peat moss and vermiculite (10:1 by volume) with or without ectomycorrhizal inoculum.

Photo plate 3.2. Greenhouse set up of containerized seedling culture arranged in trays on the high bench. Four fertilizations (12.5C, 12.5E, 25E, and 50E) and three inoculations (*Hebeloma crustuliniforme*, *Laccaria bicolor*, and uninoculated control with no fungus) treatments were arranged in a complete randomized block design. Sodium vapor lamps provided for supplementary light to ensure an extended photoperiod.

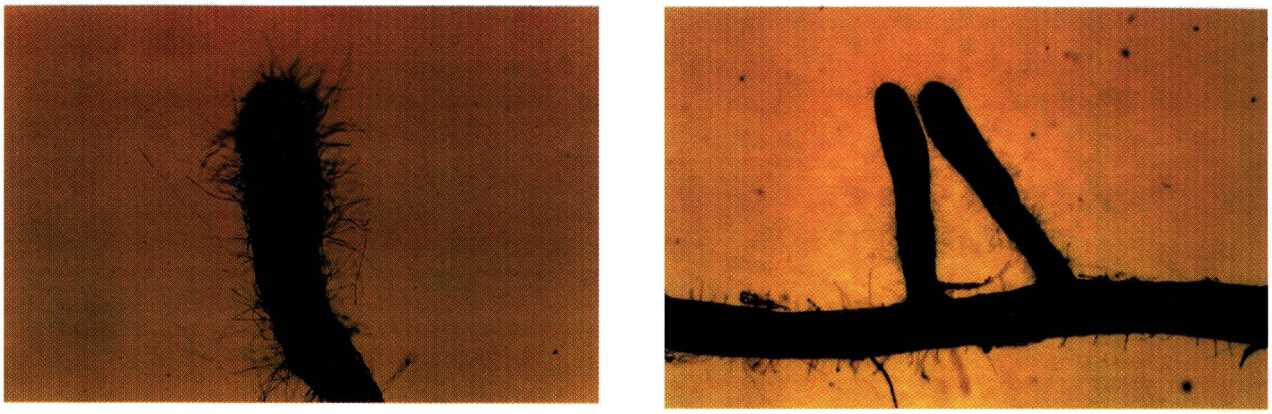

Photo plate 4.1. Microscopic assessment of root systems (+ M) showed ectomycorrhizal short roots formed after inoculation with *Laccaria bicolor*. Short roots were densely covered with fungal hyphae and developed an external mantle or sheath (Magnification, X40). Typically, root hairs almost disappeared. Original color and appearance of the root samples were somewhat distorted due to prolonged staining before photographing.

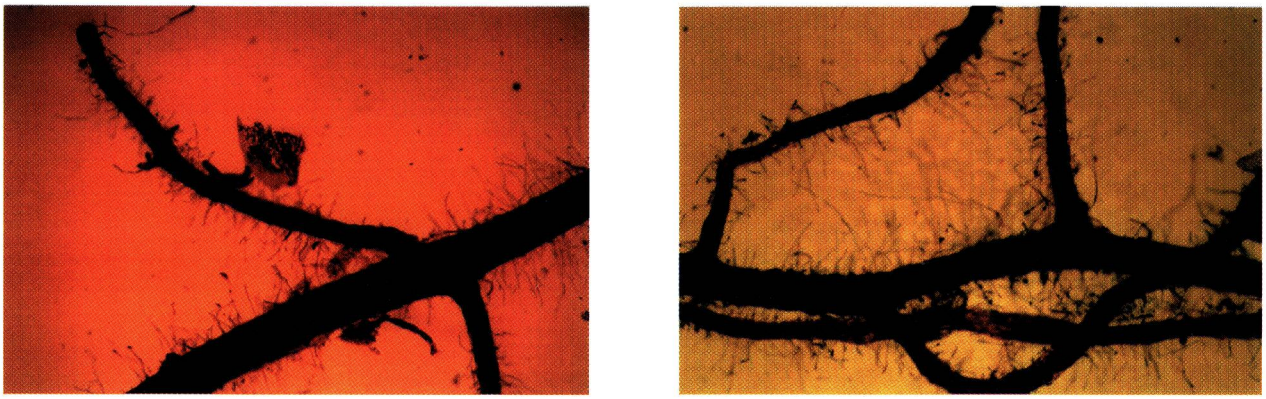

Photo plate 4.2. Microscopic assessment of non-mycorrhizal root systems (- M) of black spruce showed presence of abundant root hairs, absence of short roots and free of ectomycorrhizae (Magnification, X 40). Original color and appearance of the root samples were somewhat distorted due to prolonged staining before photographing.

Photo plate 5.1. Intact (36 x 30 x 15 cm) blocks of substrate (bioassay) were collected from forest sites without disturbing the natural vegetation and then inserted into a plastic container (15 x36 x30 cm). The block is placed on white inverted plastic pot for display. Seedlings were transplanted into intact soil substrate and grown for 18 weeks in the greenhouse.

Photo plate 5.2. Greenhouse set up of bioassay retrieved from two contrasting forest sites: Hardwood-*Alnus* (rear) dominated mainly by *Alnus* and *Rubus* species, representing a weed prone competitive site, and Feathermoss site (front) showing ground cover dominated by *Pleurozium* mosses, representing a low competitive site. Intact bioassays were transported to the greenhouse at the Faculty of Forestry and arranged in a complete randomized block design with four replicates.

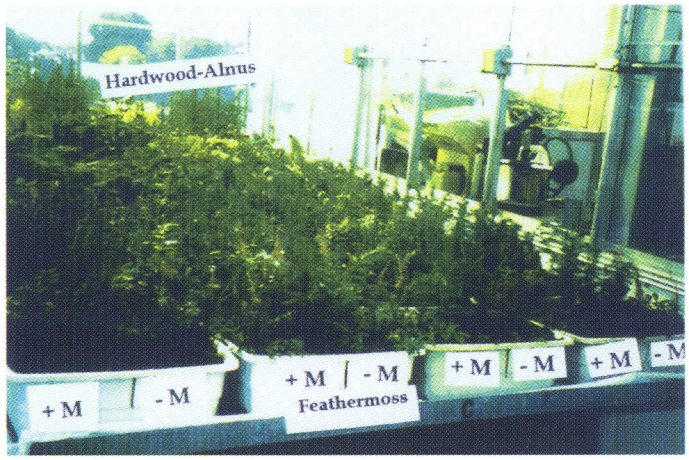

Photo plate 5.3. Transplanted seedlings growing on pot bioassay under greenhouse conditions. Each plastic container was divided into two sub plots and planted with either mycorrhizal (+ M) or non-mycorrhizal seedlings (- M). Abundant vegetation almost covered the planted seedlings on Hardwood-*Alnus* site compared to Feathermoss site, reflecting high competition for available resources.

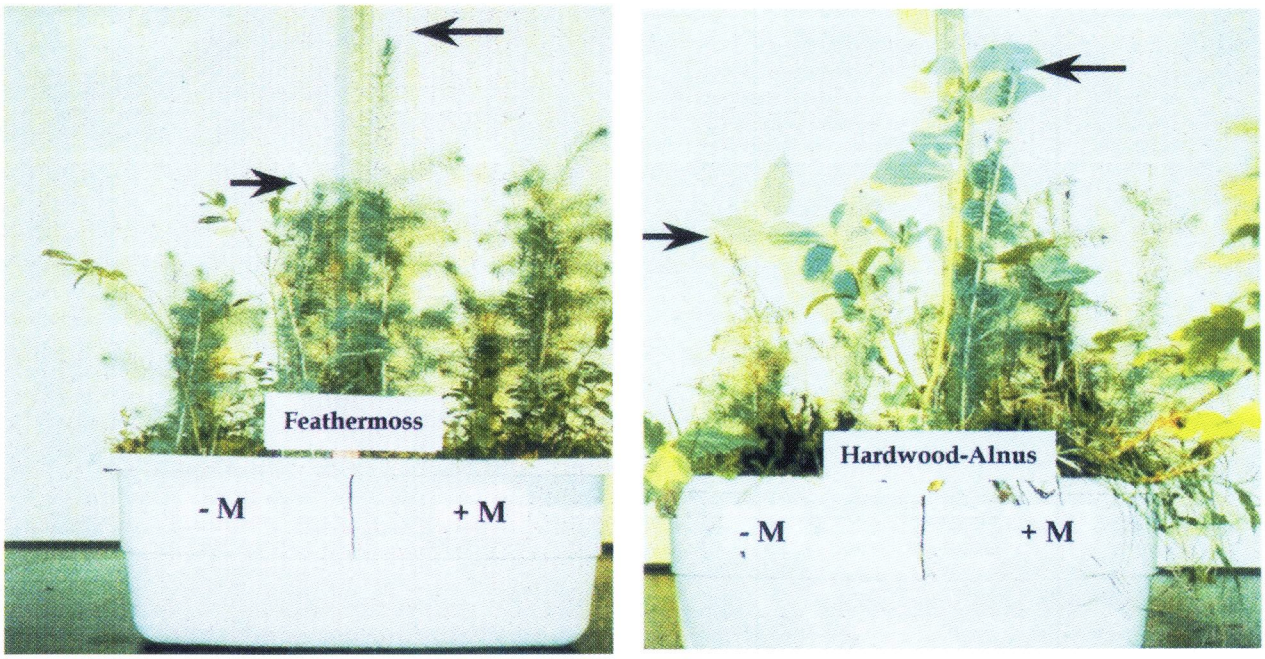

Photo plate 5.4. Outplanting performance of mycorrhizal-loaded (+ M) and non-mycorrhizal (- M) seedlings planted on Feathermoss (low weed competition) and Hardwood-*Alnus* (high weed competition) sites. The mycorrhizal-loaded seedlings outperformed the non-mycorrhizal seedlings on both sites. Arrows indicate height of tallest plant from treatments.