STUDIES OF WESTERN HEMLOCK NUTRITION

by

JOSEPH BARRY WHITE

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Department of **Forestry**

The University of British Columbia
Vancouver, Canada

Date **Dec. 19/2000**
ABSTRACT

Western hemlock (*Tsuga heterophylla*) is an important commercial tree species in coastal British Columbia. A series of studies was carried out to further our understanding of western hemlock nutrition, which may ultimately provide for an operational fertilization program involving this species.

Eight immature western hemlock stands were fertilized with additions of N, two levels of P (100 and 500 kg/ha) and a blend treatment. All stands responded to N additions to varying degrees but did not show evidence of a response to P or blend additions. Arginine concentrations increased following N only additions but decreased when P or the blend fertilizer was also applied. Concentrations of organic and inorganic P were determined. The organic fraction peaked at approximately 0.12% and further increases in total P were allocated to the inorganic fraction.

$^{32}$P uptake was measured in excised fine roots from eight stands that had been previously fertilized. Uptake varied significantly with treatment but was not related to growth response. Uptake rates were related to total P and inorganic P in current-year foliage. The latter suggested that tree perception of P deficiency is foliar based.

A 5-year-old western hemlock plantation was fertilized to investigate the effect of improved nutrition on the photosynthetic apparatus. The additions of N and P, the latter at a rate of 300 kg/ha, improved height growth. The effects of nutrient additions were examined by analyses of photosynthetic rates, carbon isotope discrimination, chlorophyll concentrations, and chlorophyll fluorescence. Improved growth, and the concomitant changes in the various photosynthetic parameters, was mirrored by changes in chloroplast ultrastructure.

An 18-year-old stand comprised of Douglas-fir and western hemlock was fertilized with N and two levels of P. The distribution of P among various P metabolites using perchloric acid extracts was attempted using NMR. An *in vitro* investigation was attempted to fractionate total P, at a cellular level, into vacuolar and cytoplasmic fractions.

The physiological parameters measured in this thesis were more sensitive indicators of N and P status, but a longer growth response period is required before their value in predicting the long-term response of this species to fertilization can be concluded.
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J. B. White
DEDICATION

This thesis is dedicated to the loving memory of my dear uncle, Patrick White, who taught me at a young age the love of the woods and all that that encompasses, the love of music and science, but most importantly, the love and respect for family.
CHAPTER I
INTRODUCTION

Western hemlock (*Tsuga heterophylla*) is an important commercial tree species in coastal British Columbia. Western hemlock occupies an area of approximately 45,000 km$^2$, represented 32 percent of the coastal annual harvest in 1997/98 (Ministry of Forests, 1999), and is the dominant species in several of the coastal Timber Supply Areas facing timber shortages in the near future. Its range extends from the Kenai Peninsula in Alaska south to central California (Pakee, 1990). It also occurs on the windward side of the Rocky Mountains in the Interior of British Columbia and along the eastern slopes of the Cascade range in northern Oregon and Washington. Western hemlock is the principal species of the coastal Western Hemlock (CWH) biogeoclimatic zone. The latter is characterized by a cool mesothermal climate with mean annual precipitation of 2228 mm which is mainly in the form of rainfall (Pojar *et al.*, 1991).

Many of the management units within coastal British Columbia in which western hemlock is the primary species have unbalanced age class distributions characteristic of many Canadian forests. The forest industry in these areas is facing reductions in future wood supply. Increasing the growth of young stands of western hemlock through fertilization, and thereby accelerating their operability, remains a viable option for addressing the problem of future wood supply provided a significant number of stands within a given forest are fertilized. Indeed, while a silviculturist can accelerate individual tree growth through thinning, fertilization is the only silvicultural option available that actually increases the growth rate of a stand.

Prerequisites for operational fertilization

An operational fertilization program has three essential requirements. Firstly, silviculturists must be able to diagnosis the nutritional status of a stand. This involves identifying the nutrient, or nutrients, that are the primary factors limiting the productivity of a stand. Secondly, silviculturists must be able to accurately predict the growth response of the stand when the nutrient deficiency is removed by fertilization (Jokela, 1988), with
particular reference to the form in which the nutrient is applied and its rate of application. The final prerequisite, which is often overlooked in the management of public forest lands, is the impact of increased growth at the stand level, taken as a reduction in rotation length, on wood supply scheduling and timber management goals at the forest level.

In recognition of the importance of the above prerequisites, operational fertilization programs in North America have been preceded by what has often been decades of intensive research to identify the nutritional requirements of the species under management and to learn of innovative ways to predict response. The successful fertilization of Douglas-fir (*Pseudotsuga menziesii*) in British Columbia and the Pacific Northwest, and the preceding research into the nutritional requirements of this species (*e.g.*, Radwan *et al.*, 1991; Weetman *et al.*, 1989; Stegemoeller *et al.*, 1989; Miller *et al.*, 1989; Radwan *et al.*, 1984; Gill and Lavender, 1983; Radwan and Shumway, 1983; Radwan and DeBell, 1980) is an excellent case in point. Chappel *et al.* (1991) reported that approximately 50,000 to 55,000 ha of Douglas-fir stands were fertilized during the 1980’s. Similar research into lodgepole pine (*Pinus contorta* Doug.) is in progress for the interior of British Columbia to assist silviculturists in the incorporation of fertilization into their prescriptions for these stands (Brockley, 1996). Perhaps the best examples of comprehensive fertilization research and subsequent operational fertilization can be seen in the United States. The research conducted since 1967 by the Forest Nutrition Cooperative at North Carolina State University towards increasing forestland productivity has culminated in approximately 400,000 hectares of southern pine being operationally fertilized in 1997 alone (Dr. Lee Allen, pers. comm.). Similarly, research into tree nutrition carried out by the Cooperative Research in Forest Fertilization (CRIFF) research group affiliated with Florida State University, resulted in approximately 500,000 ha of southern pine fertilized in 1998 in that state (Dr. Jokela, pers. comm.).

**History of hemlock trials**

The first comprehensive studies into the nutrition of western hemlock and its potential response to fertilization were initiated in 1969 by the Regional Forest Nutrition
Group, an industry funded nutrition cooperative which works in cooperation with the University of Washington. This initial trial, “Phase I”, involved the N fertilization of 26 installations but these stands were not thinned (RFNRP, 1980). Only a single, young stand in the Cascade Region showed evidence of response (RFNRP, 1978). This preliminary study was followed by what has been termed “Phase II” in 1971. This study was undertaken to assess the effect of N fertilization at two rates of application, 200 and 400 kg/ha. Unlike the installations that had been used in the Phase I study, each of the current eight installations had been previously thinned. The eight-year measurements had indicated that response to fertilization was variable with only a single coastal installation showing evidence of a response to 200 kg/ha treatment (RFNRP, 1982). The 6-year basal area response to N-fertilization in young (< 25 years old), precommercially thinned western hemlock stands was also undertaken by the Regional Forest Nutrition Research Project group and termed “Phase IV”. The experiment consisted of a total of four installations, two on the coast of Washington State and two in the Cascade Region. At each installation, N was applied at the rate of 200 kg/ha in the form of urea. At one installation, the experiment also included the application of P in the form of dicalcium phosphate at the rate of 200 kg/ha. The six-year measurement of basal area indicated that only one of the installations responded to N additions and the response to P additions was inconclusive (Stegemoeller, 1989).

Research into the nutrition of western hemlock was also carried out in the province of British Columbia. The largest of these was the Experimental Project 703 which was a fertilization and thinning study which attempted to investigate the response of Douglas-fir and western hemlock stands to fertilization and thinning, alone, and in combination. The study consisted of four levels of N (0, 225, 450, 675 and 900 kg/ha) where nitrogen was applied in the form of urea. The thinning treatment consisted of the removal of 20%, 35% and 50% of the initial basal area. The treatments were replicated within a total of 85 installations located within the Vancouver Forest Region, of which 24 installations were pure western hemlock, or where hemlock was the dominant species (Omule, 1990). The experiment was somewhat problematic in so far as the experiment lacked the collection and analysis of yearly foliage samples. In addition, the treatments were not replicated
within all installations, and in fact, some installations lacked control plots. It is difficult to conclude much from this experiment but investigators have reported that 50% of the stands had responded to N additions at the 3-year measurement period, which had fallen to 38% at the 6-year measurement period. There was no additional response to N additions greater than 225 kg/ha and generally, younger stands (< 40 years of age) were more responsive than older stands (Godfrey, 1985).

**Research questions raised by other researchers**

The research trials noted above, in both the province of British Columbia and the Pacific Northwest, could be described as “extensive” due to the large number of research plots and installations. The above research trials demonstrated that many western hemlock stands do respond to N additions, that young stands respond more often than older stands, but that this response is very erratic in that many stands do not respond to fertilization.

The research approach adopted by each of the above research organizations, however, did not allow the investigators to conclude as to why a particular stand failed to respond to fertilization. Researchers still had little knowledge as to the nutritional requirements of this species, and equally little knowledge as to how to identify the nutritional status of a western hemlock stand, or how to predict its response to nutrient additions. These remaining questions lead to a more intensive approach to studying hemlock nutrition with a variety of questions being proposed to explain the erratic response.

**Do western hemlock stands vary in their response to different forms of N?**

Dangerfield and Brix (1979) reported that the response of a 24-year-old stand of Douglas-fir was approximately 21% greater when the N fertilizer was applied in the form of ammonium nitrate rather than urea at the rate of 200 kg/ha. The higher foliar N concentrations following the addition of ammonium nitrate indicated that the observed
growth differences were most probably due to an increase in N uptake with the ammonium nitrate fertilizer. The source of nitrogen used in each of the Regional Forest Nutrition Research Projects as well as the EP 703 trial was urea, which lead Radwan et al. (1984) to question whether another form of N, such as ammonium nitrate, may provide a more consistent response. They conducted a fertilization experiment that tested the growth response of three young hemlock stands to five different forms of N. Three of the installations that had been studied in the “Phase II” study noted above were used which ranged in age from 17 to 28-years of age. The data indicated that the poor growth response in non-responsive stands could not be improved with the addition of N in a form other than urea. Radwan and DeBell (1989) later tested whether a slow release form of urea, or one combined with a nitrification inhibitor may have beneficial effects in terms of growth response over that of urea alone at a single installation. No difference in height growth response was reported between the three forms of urea, but basal area and individual tree volume was highest when urea was applied as slow release fertilizer. The latter consisted of urea coated with sulfate so the additional effect of the S addition may, in part, explain the enhanced growth response to this treatment. The authors speculated that the slow release form of urea may also have been beneficial to the fine roots by reducing the likelihood of an accumulation of NH₄-N, or by releasing N at a rate that was in better synchrony with the supply of other nutrients. Radwan et al. (1991) also reported that there did not appear to be any difference in response of western hemlock to either ammonium nitrate or urea sources of nitrogen.

Do western hemlock stands fail to respond to N additions because their growth response is limited by secondary nutrients such as P?

Numerous researchers had questioned whether the response of western hemlock to N fertilization was limited in some stands due to deficiencies in other elements. For example, Gill and Lavender (1983a) studied the effect of urea fertilization of hemlock stands on foliar nutrient concentrations. Foliage was collected from three stands in the coastal region of the state of Washington and three stands in the Cascade Region. They
reported that the concentrations of many of the macro- and micro-nutrients were significantly decreased during the first growing season following urea additions, and furthermore, that the reduction in concentrations was greatest in the stands that were located in the coastal region of the state. They suggested that their findings supported the conclusion that N fertilization of hemlock stands in the coastal region may have induced secondary deficiencies and that for the growth of these stands to be significantly improved, elements other than N would have to be applied.

Heilman and Ekaun (1980) reported that Douglas-fir seedlings grown in a greenhouse experiment, using soil collected from the field, showed evidence of P deficiency. The authors noted that these soils came from an area that supported native stands of western hemlock, which raised the question as to whether low P supplies within the mineral horizon may be limiting the productivity of hemlock. Accordingly, they replicated their earlier greenhouse study but used seedlings of western hemlock instead of Douglas-fir. The two soils were left untreated (control) or amended with the addition of P at the rate of 300 kg/ha. The P addition resulted in a dramatic increase in seedling height and biomass over a nine-month growing period. Anderson et al. (1982) carried out a similar greenhouse experiment involving P additions, this time at the rate of 448 kg/ha, and reported similar results.

Radwan et al. (1991) investigated the role of P nutrition in limiting the response of both Douglas-fir and western hemlock to N fertilization. The study involved the fertilization of a 34-year-old Douglas-fir stand and a 25-year-old hemlock stand, each located within the state of Washington. The treatments consisted of two levels of N (0 and 224 kg/ha) where the latter was applied in the form of urea and four levels of P (0, 100, 300, 500 kg/ha), applied in the form of triple-super-phosphate, which were applied to each stand using a randomized complete block design. It is worth noting that the highest P application was only effective at increasing the total P concentration in current-year foliage from a control level of 0.13% to 0.20%. None of the fertilizer additions resulted in a significant increase in height or basal area increment of either species. This lack of response was surprising since the foliar analysis of control foliage indicated N and P concentrations were in the range generally considered deficient for this species. These
same investigators also fertilized a 34-year-old western hemlock stand and applied similar treatments except that N was supplied in the form of ammonium nitrate and the heaviest P application rate was excluded. As noted in the first experiment above, neither the basal area increment, nor height growth, was significantly affected by N or P additions. Foliar nutrient concentrations were not determined for this investigation.

In a similar study, Carter et al. (undated) also studied the effect of adding nutrients in addition to N in an effort to understand the variation in the response of hemlock stands. The study involved a total of 44 stands, ranging in age of from 15 to 40 years, located throughout coastal British Columbia. Each of the stands was fertilized and the six-year basal area increment was measured. The treatments included N, applied in the form of urea at the rate of 225 kg/ha, alone and in combination with a blend addition. The blend included the addition of P at the rate of 100 kg/ha in the form of triple-super phosphate, 60 kg of K as potassium-sulfate, 40 kg of Mg in the form of magnesium-sulfate, 100 kg/ha of S applied as sulfate, 10 kg of Cu applied in the form of copper sulfate, 20 kg of Zn applied in the form of zinc sulfate, and 2.5 kg of B. Five of the 44 stands responded positively to N only additions, and 11 stands responded to additions of N and the blend. Perhaps the most important finding from the investigation was that no discernable relationship between foliar nutrient concentrations and the three-year basal area increment was found.

Can first-year needle weight responses to fertilization, vector analysis, or DRIS aid in the diagnosis of the nutritional status of western hemlock stands or the prediction of long-term growth response?

Foliar vector analysis is a graphical technique that relates plant growth as represented by increased needle weights during the first growing season for determinant species, nutrient content and nutrient concentrations (Haase and Rose, 1995; Timmer and Armstrong, 1983). The original concept for vector analysis is generally attributed to Krauss (1965) and Heinsdorf (1967). Vector analysis takes advantage of the fact that during the first growing season following fertilization the number of needles in a
determinate species cannot be increased and increased growth is reflected in increased needle size. The response of these pre-determined needles to fertilization during the first growing season is used as a predictor of future growth response of the stand. The value of vector analysis is that it can diagnosis nutrient deficiencies and predict a stand’s responsiveness after a single growing season, thereby dramatically reducing the time and cost of screening trials.

Timmer and Morrow (1983) applied vector analysis to diagnose the nutritional status of a 28-year-old jack pine (*Pinus banksiana* Lamb.) stand and to predict its response to additions of N, P and K. They reported that the growth response of needles in the first year (100 needles/mg) was strongly correlated with 6-year radial growth measurements, confirming the value of this technique in screening trials. The application of vector analysis confirmed that the stand was deficient in N, but not limited by P or K, which was in agreement with the long-term growth measurement. Timmer and Ray (1988) and White and Krause (2000) reported similar results with black spruce (*Picea mariana* [Mill.] B.S.P.). Vector analysis has also proven valuable in diagnosing nutrient deficiencies in seedling stock (Timmer and Armstrong, 1987).

Despite the above successes, Carter *et al.* (undated) reported that the first-year needle weight response, measured as the average weight of 100 needles, was not correlated with long-term basal area increment. For example, five installations which had a significant increase in the first-year-needle weights, did not show evidence of a long-term growth response as measured by the six-year basal area. This finding indicates that estimation of first-year needle response cannot be used in screening trials involving western hemlock and also that the vector analysis technique holds little promise in assisting in the interpretation of foliar nutrient data from hemlock investigations.

The findings by Carter *et al.* (undated) are, however, contrary to those presented by Weetman *et al.* (1989) who measured first-year needle response as a predictor of response of western hemlock growing on a recent cutover on Vancouver Island. As hemlock is an indeterminate species, Weetman *et al.* (1989) substituted mean needle weight of preformed needles with mean needle weight of the main lateral shoot and with a mean needle weight of a sub-sample of 1000 needles. Using this approach they identified
deficiencies in N and P which were latter confirmed with an increase in the three-year height growth response following N and P additions. It should be cautioned, however, that the stand used by Weetman et al. (1989) was severely nutrient deficient. A further cause for caution in applying this technique to hemlock nutrition lies in the fact that western hemlock is indeterminate, which violates the basic assumption behind the application of vector analysis, and its growth response, in terms of needle weight and number or length of shoots across a range of nutritional levels, has not been studied. In any regard, the recent findings by Carter et al. (undated) noted above, indicate that vector analysis has little utility in assisting in the study of western hemlock nutrition.

An alternative approach to the assessment of nutrient status using conventional foliar nutrient concentrations in foliage is the use of Beaufils nutrient indices, commonly referred to as the diagnosis and recommendation integrated system or DRIS. This technique was developed, as the name indicates, by Beaufils, a South African agricultural scientist (Black, 1992). Essentially, this approach considers the concept of nutrient balance to be more meaningful than mere nutrient concentrations. Nutrient ratios are constructed using data collected randomly from high yielding populations (Walworth and Sumner, 1987).

The DRIS approach has had limited application within the field of tree nutrition. Noted exceptions include Leech and Kim (1981; 1986) working with poplar (Populus deltoides Marsh.), Romanya and Vallejo (1996) working with radiata pine (Pinus radiata D. Don), and Moreno et al. (1998) working with fig trees (Ficus carica L. cv. Pellejo Duro). The DRIS approach has also been applied in the commercial Christmas tree industry (Arnold et al., 1992; Rathfon and Burger, 1991a, 1991b; Kopp and Burger, 1990; Hockman et al., 1989).

In their comprehensive review of approaches to nutrient analyses, Weetman and Wells (1990) highlighted that the primary problem with DRIS is that there are seldom sufficiently large data sets for nutrient indices from stands exhibiting high yields or optimal performance. For example, in their application of the DRIS approach to the study of nutritional status of potato (Solanum tuberosum L.), MacKay et al. (1987) relied on a data set which exceeded 2300 individual data sets. In addition, as noted by Powers
(undated), most data sets of foliar nutrient concentrations for mature stands, as opposed to greenhouse investigations, have been focused on nutrient-poor sites. In light of these limitations, there would appear to be little utility in applying this approach to the study of western hemlock nutrition at this point in time.

Is the lack of response of individual western hemlock stands to nutrient additions caused by an adverse effect of fertilizers on fine root growth and function, and mycorrhizae?

Gill and Lavender (1983b) hypothesized that western hemlock stands fail to respond to urea fertilization because fertilization results in an increase in the mortality of fine roots and a reduction in mycorrhizal associations. They studied the spatial distribution and morphological characteristics of fine roots of six natural young-growth stands in the state of Washington and investigated how urea fertilization affected these characteristics in each stand. They concluded that urea fertilization at the rate of 224 kg/ha appeared to result in a significant reduction in the number of mycorrhizal roots. Three of the stands showed evidence of a small, though significant, increase in the number of dead roots in the litter layer and two stands showed evidence of a similar increase in dead roots in the humus layer. Concomitant with this increase in dead roots, there was evidence that in each of the latter horizons the number of mycorrhizal root tips decreased. Interestingly, the authors documented that the response to fertilization varied with mycorrhizal types and the frequency of several types actually increased following fertilization. The authors speculated that a reduction of mycorrhizae would result in a reduction in P uptake and might explain the lack of a growth response to urea fertilization in certain stands.

However, Kernaghan et al. (1992) investigated the effect of urea fertilizer on the ectomycorrhizal community within the forest floor of a 20-year-old hemlock stand growing in Coastal British Columbia. The experiment included the application of urea at the rate of 224 kg/ha, the rate used by Gill and Lavender (1983), and 448 kg/ha. Despite using large sample sizes than Gill and Lavender (1983), no changes in the ectomycorrhizal
community could be directly attributed to urea fertilization, at either rate of urea application.

Each of the above studies investigated the effect of fertilization on the status of fine roots over a fairly short period of time. In an earlier study, Alexander and Fairly (1983) investigated the effect of N fertilization of the fine root production and turnover and the mycorrhizal population in a 35-year-old Sitka spruce (*Picea sitchensis*) plantation in the United Kingdom. Nitrogen was applied in the form of ammonium at the rate of 300 kg/ha. Fertilization resulted in a decrease in the fine root and mycorrhizae by approximately 30% and 15% respectively. More importantly though, they reported that fertilization resulted in both a reduction in fine root mortality and an increase in fine root longevity, both of which may have significant effects on carbon allocation.

Unfortunately, the hypothesis that the response of western hemlock stands to urea fertilization is limited by fine root mortality and/or loss of mycorrhizae has not been critically tested. The findings of Alexander and Fairly (1983) indicate that the effect of fertilization must take into account the long-term effects of C allocation. In addition, a study that attempts to link the loss of fine roots or a decline in mycorrhizal populations with tree growth should include a test of root function, such as the capacity for nutrient uptake.

**Can soil chemical properties be used to identify western hemlock stands that are prone to nutrient deficiencies and aid in the prediction of long-term fertilization response?**

Soil testing has been an accurate and indispensable approach to defining both the fertility and productivity of agriculture soils (Peck and Soltanpour, 1990). Indeed, identifying nutrient deficient soils on the basis of chemical properties (e.g. Liu and Bates, 1990) has been a cornerstone to agricultural soil practices. Researchers in forest nutrition have also attempted to rely on soil chemical properties to understand the nature of nutrient disorders and deficiencies as well as predicting long-term response to nutrient additions. For example, Ballard and Prichett (1974) reported that soil testing for P availability in
mineral soil could be used to develop fertilization prescriptions to evaluate the potential response of slash pine (*Pinus elliottii* var. *elliottii* Engelm.) stands to P fertilization in the state of Florida. White and Leaf (1965) demonstrated that K availability in the solum of sandy soils in the state of New York was strongly correlated with tree growth and was a valuable approach to identifying K deficiency in red pine (*Pinus resinosa* Ait).

Subsequent research by Shepard and Mitchell (1990) confirmed these earlier conclusions. Fisher and Garbett (1980) and Kushla and Fisher (1980) reported that grouping soils on the basis of drainage, depth of rooting, and nature of the B horizon, aided in the prediction of the growth response of pine stands to N and P fertilization in the lower coastal plain of the United States. Comerford and Fisher (1982) investigated the role of two different P extractants in identifying soils with low P and in predicting long-term fertilization response. Interestingly, they reported that soil-extracted P from the surface soil was a poor predictor of long-term response. In contrast, soil sampling to a depth of 60 cm was found to be a reliable approach to assessing short-term response. Lea and Ballard (1982) reported that soil analysis for N availability was not a reliable predictor of the growth response of loblolly pine (*Pinus taeda* L.) stands in North Carolina to N fertilization.

Radwan and Shumway (1983) examined the relationship between soil N, S and P and the response of western hemlock stands to N fertilization. The study involved 16 stands, which were located throughout the state of Washington, ranged from between 10 and 40 years in age, and varied strongly in productivity. Previous response to N fertilization at the rate of 224 kg/ha also varied strongly amongst the stands. Soil parameters measured included total N, mineralizable N and S, extractable P and sulfate S for both the forest floor and 0–15 cm of mineral soil. Extractable P in the forest floor was the variable reported to be most strongly correlated with previous response (r = 0.77) indicating that positive responses to N fertilization were most likely to occur when concentrations of extractable P were high, an indication that P nutrition may have been a secondary limiting factor to hemlock response in these stands. There was no evidence in this experiment, using a soil chemistry approach to assessing nutritional status, to suggest that S was a factor limiting response.
In the study by Carter et al. (undated), noted earlier, soil chemical parameters were measured in 44 western hemlock stands to determine if variation in the response of hemlock stands to nutrient additions could be explained by variation in soil chemical variables. They measured total C and N, mineralizable N, extractable P, K, Mg, Ca, S) for both forest floor and mineral soil (0-30 cm) but detected no discernible relationship between the soil variables assessed and the six-year basal area increment. Kayahara et al. (1995) also reported that neither total P in the forest floor, nor extractable P in mineral soil was correlated with site index of western hemlock in a study of 101 hemlock stands in coastal British Columbia. Notwithstanding the results of Radwan and Shumway (1983), these results suggest that soil chemical variables do not appear to accurately predict response of western hemlock.

Highlighted Problem

A careful re-examination of the results of Carter et al. (undated) reveals that the foliar nutrient concentrations in current-year foliage, the conventional approach to defining nutrient status of conifers, do not appear to be useful in assessing the nutrient status of a western hemlock stand, nor in predicting the stand’s response to fertilization. For example, many of the stands that the investigators identified as being responsive to fertilization had nutrient concentrations much higher than many of the stands that failed to respond to either N or the blend additions. The lack of such a relationship is perplexing as the relationship between foliar nutrient concentrations, nutrient status, and response to fertilization, has been the cornerstone of tree nutrition. Consequently, silviculturists in this province have been unable to justify fertilization of hemlock stands because of their inability to discern between potentially responsive and non-responsive stands.

Much of the research into forest fertilization, including that of hemlock nutrition, has involved the documentation of volume response and foliar concentrations following fertilization. This approach fails to address the fundamental physiological response mechanisms to differing nutrient supplies. For example, conventional studies of tree nutrition have relied on foliar analysis where the mineral content is related to the dry mass
of the sample. For many of the applications of foliar analysis, this common approach has proven to be adequate (e.g. Hopmans and Chappel, 1994). However, such an approach fails to consider the physiological functions of mineral elements and consequently offers little utility in solving many of the complex problems currently facing scientists in the field of tree nutrition. A further problem of previous hemlock research is that the treatments tested have often been restricted to nitrogen. As a consequence, we currently have no clear understanding of the nutritional requirements for this species, or the relationship between foliage production, foliar efficiency, photosynthetic rates and nutrient supply (Brix, undated).

There are many different approaches to the study of both plant and tree nutrition. The most simple and undoubtedly the most widely used in tree nutrition is the determination of the relationship between total nutrient concentrations within a plant part (foliage) and yield. This relationship can be expanded to include a determination of critical concentrations following Mitscherlich’s principle. As noted above, researchers have long recognized the shortcomings in this approach which lead to the development of alternative approaches such as vector analysis, nutrient ratios, and DRIS. These approaches share a fundamental drawback in that they utilize total nutrient concentrations in a specific plant part which may have questionable physiological meaning. Total nutrient concentrations, arguably, relay little information on how the plant or tree is utilizing the element in question. Simultaneous to developing new ways of expressing total nutrient concentrations as noted above, other researchers have expressed a desire to determine more physiologically based estimates of nutrient status. These new approaches to assessing nutritional status of plants and trees are (1) the use of biological indicators of nutrient status, (2) use of concentrations of nutrient fractions, as opposed to total nutrient concentrations, and (3) use of sub-cellular compartmental analysis.

Can biological indicators of nutrient status aid in the study of western hemlock nutrition?
In his well known review of the approaches to understanding the nutritional status of plants, Black (1992) noted that when a specific nutrient becomes deficient, certain organic substances that the specific nutrient plays a role in controlling, often become unusually high or low. Consequently, testing for the concentrations of these organic substances may complement studies into the nutritional status of the element in question, or may even prove to be a more sensitive indicator.

Perhaps one of the better known applications of this approach is the assessment of K status through the determination of foliar putrescine concentrations. For example, Sarjala and Kaunisto (1992) studied the relationship between foliar K and putrescine concentrations in Scots pine (Pinus sylvestris L.) trees growing on drained peatlands in western Finland. They reported that the concentration of putrescine increased exponentially with decreasing K concentrations, and furthermore, that the accumulation of putrescine was triggered at K concentrations lower than previously thought to represent the presence of K deficiency. The relationship between foliar K and putrescine concentrations in Scots pine was further reported by Jokela et al. (1997). Smith et al. (1982) had earlier reported that putrescine concentrations in foliage of lucerne (Medicago Sativa L.) were a more consistent indicator of K status than the critical level approach. Most recently, Zaidan et al. (1999) reported that putrescine concentrations in the foliage of two banana (Musa sp. L.) cultivars were affected not only by external K supplies but also by the ratio of ammonium to nitrate in the culture medium. Klein et al. (1979) had earlier raised concern over the relationship between foliar putrescine concentrations and nitrogen sources while working with pea (Pisum sativum L.) plants. These results caution that production and accumulation of putrescine may not be the exclusive result of K deficiency. Murty et al. (1971) had reported the putrescine concentrations in the foliage of black current (Ribes nigrum L.) were no better at indicating the onset of K deficiency than visual symptoms.

Researchers have also explored the use of the activity, or lack thereof, of certain plant enzymes as indicators of nutrient deficiency. As noted by Black (1992), the basic concept is that when a nutrient that is involved in a specific enzyme reaction is deficient, then the activity of that particular enzyme may be altered. For example, Nason et al.
(1951) reported that the concentrations of Zn, Cu, Mn, Fe, Mo and B resulted in significant changes in the activities of polyphenol oxidase, ascorbic acid oxidase, peroxidase, lactic dehydrogenase, glycolic dehydrogenase, and reduced diphosphopyridine nucleotide (DPNH) diaphorase. The following year, Brown and Hendricks (1952) studied the effect of Cu and Fe deficiencies on enzymatic activities in a range of plant species. They reported that ascorbic acid oxidase activity was strongly reduced by low Cu supply and correlated with a reduction in growth. The enzyme catalase was reduced by reductions in Fe supply. Despite this early work, the potential application of enzymatic activities in identifying nutrient deficiencies does not appear to have received much attention. Tests for diagnosing Cu and Fe deficiency have been proposed by Delhaize et al. (1982) in subterranean clover (Trifolium subterraneum L.) and Bar-Akiva et al. (1978) in tomato (Lycopersicon esculentum Mill.) and corn (Zea mays L.) leaves respectively. More recently, Kolari and Sarjala (1995) reported that acid phosphatase activity in current-year foliage of Scots pine needles is a sensitive indicator of changes in foliar P concentrations. The lack of documented cases of micronutrients limiting productivity of commercial tree species may well explain the lack of research into the use of the activity of certain enzymes as indicators of nutrient status, though the recent findings by Kolari and Sarjala (1995) indicate that an enzyme approach to assessing P status may have promise.

Researchers in the field of plant science have long sought to identify more sensitive and physiologically meaningful estimates of N status than mere N contents in foliage. This has lead researchers to investigate whether the concentrations of free amino acids may be more informative of N status and internal utilization. For example, Tromp (1970) and Oland (1959) proposed that free amino acids, and in particular the amino acid arginine, serve as important storage forms of nitrogen in apple trees (Pyrus malus L.). Baxter (1965) recommended the determination of free amino-nitrogen fraction in fruit trees using the ninhydrin method as a simple and effective test to assess nitrogen status. Lorenz (1975) reported that the concentrations of free amino acids in shoots and roots of tomato plants were reliable indicators of the nitrogen source in the rooting medium.

van den Driessche and Webber (1975) investigated whether soluble N compounds were more useful than total foliar N contents in indicating the N status of 3-year-old
Douglas-fir trees. They reported that while total N concentration in one-year-old foliage increased by 40% with increasing N supply, the concentrations of soluble N increased by as much as nineteen times. van den Driessche and Webber (1979) later reported similar results. Kim et al. (1987) attempted to determine if the concentrations of free amino acids in foliage of jack pine and black spruce seedlings could be used as indicators of nitrogen status. They were able to show that the amino acid fraction was sensitive to changes in the amount of N supplied to the seedlings. However, they did not provide the results of foliar analysis so the changes in the amino acid pool could not be related to changes in the total N pool which would be necessary to ascertain changes in internal N utilization or allocation, as well as to determine if the amino acid pool was a superior method of assessing N status.

Notwithstanding the above research, the field of forest nutrition has been somewhat slower to utilize this method of determining nitrogen utilization than the field of plant science. This may be in part due to the significant variation in amino acid concentrations, the fact that they can be related to stress rather than purely nutrition, and the complexity and cost of the laboratory procedures.

Certainly one of the most fascinating aspects of tree and plant nutrition has to be the study of the many physiological response mechanisms that plants and trees have developed to acquire nutrients under varying conditions of nutrient supply. Indeed, Clark (1984) reported on the variation in nutrient acquisition strategies with different genotypes. Numerous researchers have taken advantage of the ability of $^{32}$P, a radioactive form of P, to trace P as it moves from source to sink. For example, Mejstrrik and Krause (1973) continue to be recognized for their work with radiata pine roots in which they reported on the ability of different mycorrhizae to acquire different sources of available P. Maliondo (1988) also undertook similar studies of the variation in P acquisition amongst black spruce families using labeled P. Cumming and Eckert (1986) used labeled P in greenhouse trials to investigate the effect of Al in the growing medium on subsequent P uptake and internal translocation.

Harrison and Stevens (1979) developed a root bioassay technique for ascertaining P status of trees using uptake of $^{32}$P in excised fine roots. Their approach has been
subsequently modified to include studies which have used $^{15}$N and $^{85}$Rb uptake in excised fine roots as estimators of N and K status. For example, Jones et al. (1991) questioned whether excised fine roots of N-deficient trees would have greater rates of N uptake than similar trees grown with adequate N supply. Their study included a series of greenhouse trials using 36-week-old seedlings of Sitka spruce and Scots pine grown under a range of N supplies. The authors reported that the rate of N uptake in the fine roots increased exponentially with N supply in the growing medium. They also reported a strong relationship between total N content of the seedlings and uptake. Their seedling trials were complemented with a series of field experiments in which N uptake in excised roots was related to previous rates of N fertilizer additions. Jones et al. (1992) reported similar findings for common bentgrass (*Agrostis capillaris* L.). Jones et al. (1987) also investigated the potential for using measurements of uptake of $^{86}$Rb in excised roots as an indicator of K status. The authors carried out greenhouse and field trials and, as in their studies with N uptake in excised roots, reported that uptake of $^{86}$Rb was reduced with increasing K supply in the growing medium and with total K content in the plants. The field study was inconclusive.

Can nutrient fractionation be used in studies of western hemlock nutrition to better understand nutrient utilization and requirement?

Total P has been fractionated into inorganic and organic components with the former proving to be a more sensitive indicator of changing P supplies (Marschner, 1990) and has been used to explain ecological adaptation of species to low nutrient supplies (Chapin and Kedrowski, 1983). Sun et al. (1992) and Saarela and Sippola (1990) reported that the inorganic fraction of P was a more sensitive indicator of P supply.

Researchers in tree nutrition have used the sulfate fraction rather than total sulfur as an index of sulfur status (Finch et al. 1997; Black, 1993; Haq and Carlson, 1993; Lambert, 1986; Turner et al., 1977; Kelly and Lambert, 1972). Indeed, Turner et al. (1977) reported that the foliar sulfate concentrations in foliage of Douglas-fir was a better predictor of fertilization response than foliar N concentrations. Haq and Carlson (1993)
reported that the foliar concentration of sulfate was a better predictor of S status than total S, organic-S or the N:S ratio for French prune trees (*Prunus cerasifera x P. munsoniana*).

With the exception of using the sulfate fraction as a reliable indicator of sulfur status in conifers, there has been relatively little research into expanding a fractionated approach to other nutrients by researchers in the field of tree nutrition. An exception is the work by Gadziola (1991) who separated total Zn into various metabolically active components in foliage of western hemlock.

**Can cellular compartmental analysis aid in the interpretation of foliar nutrient concentrations?**

An alternative approach, and important development to furthering an understanding of tree and plant nutrition, has been the study of nutrient concentrations in cellular compartments, rather than relying on total nutrient concentrations per unit of dry matter. There are two primary approaches to subcellular compartmental analysis that have gained recent prominence in the study of plant and tree nutrition.

Glass and associates have utilized the efflux approach to compartmental analysis to assess the uptake of various nutrients and their distribution amongst several root compartments. For example, Wang *et al.* (1993) studied the uptake and distribution of $^{13}$NH$_4$ in rice (*Oryza sativa L. cv*) roots. They reported that after exposure to an external NH$_4$ supply, 41% of the NH$_4$ was in the cytoplasmic fraction, 20% in the vacuolar fraction with a remaining 20% accounted by efflux. This approach to compartmental analysis has been extended to the study of ammonium and nitrate uptake in rice (*Oryza sativa L.*) (Kronzucker *et al.*, 1999), trembling aspen (*Populus tremuloides* Michx.) lodgepole pine (Min *et al.*, 1999), onion (*Allium cepa L.*) (MacKlon *et al.*, 1990), barley (*Hordeum vulgare* L. cv. Midas) (Lee and Clarkson, 1985) and sunflower (*Helianthus annuus* L.) (Jeschke and Jambor, 1981).

Memon *et al.* (1985) used subcellular compartmental analysis to study potential differences in genetic variation in barley with respect to potassium utilization. An analysis
of K concentrations in the cytoplasmic and vacuolar compartments led these authors to hypothesize that high K use efficiencies was correlated with lower K concentrations in the cytoplasmic and vacuolar compartments. In this case, K use efficiency was related to the minimum concentration of K in the cytoplasmic fraction that was necessary to maintain the biophysical and biochemical processes required for maximum biomass production. The conclusion that nutrient use efficiency may be related to cellular distribution would not be possible with the conventional analysis of K concentrations as a percentage of dry matter.

An alternative approach to studying subcellular distribution of nutrients is the use of nuclear magnetic resonance (NMR). This technology has seemingly attracted little attention by researchers within the field of tree nutrition but has been widely applied by investigators in the field of plant sciences during the past two decades. The advantage of NMR technology is that it allows for a non-destructive means of observing the distribution of inorganic elements within different plant compartments. For example, Roberts and Pang (1992) documented the concentration of ammonium in the vacuolar and cytoplasmic compartments in maize root tips across a range of external ammonium supplies. Nuclear magnetic resonance has also been applied by researchers to understand aspects of P nutrition (e.g. Lee and Ratcliffe, 1993).

Future Research Directions

The three alternative approaches to the study of nutrition noted above have had limited application in the field of tree nutrition, and have yet to be applied to the study of the nutrition of western hemlock. Indeed, it could be argued that much of the focus of research activities within the field of tree nutrition during the past three decades has focused on questions related more to the operational management of the forest resource than understanding tree biology. More physiologically based research in tree nutrition has occurred during the past decade following various growth disturbances, and their accompanying economic loss, that have occurred both in North America (Shortle and Smith, 1988; Schlegel et al., 1992; McCanny et al., 1995; Johnson et al., 1994; Bernier and Brazeau, 1988) and Europe (Oren et al., 1988; Zimmermann et al., 1988; Lange et
al., 1987; Krzak et al., 1988; Meiues et al., 1986; Godbold et al., 1988; Zoetlel and Huettl, 1986; and Estivalet et al., 1990).

It is clear from the above synopsis that the conventional approach to the study of western hemlock nutrition, applied over the past three decades, has not been successful in furthering our understanding of the nutrition of this species. In this regard, it is interesting to note the potentially controversial comments of Ingestad (1991) with respect to the science of tree nutrition and the growth of forest trees. He stated that there are, surprisingly, no general theories about plant nutrition, and furthermore, that this field of science has a weak scientific basis. He argued that this was the result of applied research that had been restricted to “endless repetition of existing methods”. At the expense of merely repeating previous approaches proven to be unsuccessful, and consistent with the recommendation of Brix (undated), future research into hemlock nutrition should explore alternative, physiologically based approaches to nutrition research.

**Thesis Questions**

1) Is the response of western hemlock stands to N fertilization limited by deficiencies of other elements such as P?

2) Can physiological indicators be identified and used to accurately ascertain the nutritional status of western hemlock and hence, be used to predict the response of individual hemlock stands to fertilization?

The objectives of this thesis are to address these two questions. This research effort should be viewed as a “key first step” of a long-term research effort aimed at understanding hemlock nutrition to the point where operational fertilization programs might become economically feasible.
This thesis is divided into chapters on the basis of individual field experiments. Chapter II presents the results of an investigation into the response of eight immature western hemlock stands on northern Vancouver Island and the Sunshine Coast to various nutrient additions. Response variables included conventional foliar analysis and three-year basal area increment. A further objective of this experiment was to determine the effect of nutrient additions on the concentrations of free amino acids and the inorganic and organic components of total P in current-year foliage.

Chapter III reports the findings of a root bioassay study to determine if P uptake in fine roots aided in the diagnosis of nutrient status and response of western hemlock stands. Uptake was determined in excised roots collected from each of the eight installations noted in Chapter II. Uptake was strongly affected by previous treatment but was not related to the three-year basal area increment. Mechanisms related to the perception of P deficiency or stress, the transduction of this message, and the mechanisms associated with the regulation of the uptake apparatus are discussed.

The results from a preliminary study by White et al. (1999) indicated that N and P additions resulted in significant and dramatic changes in carbon isotope discrimination in current-year foliage of western hemlock. These results indicated that an improvement in nutrient status following fertilization had a significant effect on the photosynthetic apparatus in western hemlock and that leaf efficiency may have played a significant role in a subsequent growth response. A study was carried out to investigate how nutrient additions affect the photosynthetic apparatus of western hemlock. The field experiment was conducted using a 5-year-old western hemlock plantation growing on a nutrient stressed site on northern Vancouver Island. The results of this experiment are reported in Chapter IV.

Chapter V reports the results of an experiment into P nutrition using nuclear magnetic resonance (NMR). An 18-year-old stand comprised of both Douglas-fir and western hemlock was fertilized with additions of N and P. Foliage collected from the experiment was used to study the distribution of P among various P metabolites. Foliage
was also used in an *in vivo* investigation that attempted to fractionate total P, at a cellular level, into vacuolar and cytoplasmic fractions.

Thesis conclusions are provided in Chapter VI and recommendations for future research are presented in Chapter VII for the benefit of future researchers interested in studying the nutrition of western hemlock
CHAPTER II
EFFECT OF FERTILIZATION ON GROWTH AND NUTRITION OF EIGHT IMMATURE WESTERN HEMLOCK STANDS

Introduction

White et al. (1999) investigated the response of a mixed species stand composed primarily of immature Douglas-fir and western hemlock to a number of nutrient additions. Their treatments included N at the conventional rate of 225 kg/ha, alone, and in combination with two levels of P (100 and 500 kg/ha). Their treatments also included a blend addition which included Mg, K, S, Fe, Zn and Cu. Douglas-fir responded only to N additions, with no further benefit being reported with the addition of the remaining elements including P. While the western hemlock trees were also reported to have responded to N additions, the best height growth response was following N additions in combination with P at the rate of 100 kg/ha and the blend fertilizer. A similar height growth response was achieved with the addition of N and P only, when P was applied at the rate of 500 kg/ha. These results indicated that the nutritional requirements for each species on this site differed, with western hemlock having a higher P requirement than Douglas-fir trees for maximum growth.

White et al. (1999) also reported that the addition of N increased the arginine concentrations in current-year foliage in both species approximately 250-fold from that in the foliage collected from the control plots. The amino acid arginine is generally accepted to be a primary form of N which accumulates in many plants and trees (Marshner, 1984). Arginine concentrations were reduced to near control concentrations with the further addition of P at the rate of 100 kg/ha. It was previously noted (Chapter I) that nutrient concentration, the conventional indicator of nutrient status, may not be a reliable indicator of the nutritional status in western hemlock and more physiologically meaningful indicators should be identified. The above findings by White et al. (1999) raises the question as to whether arginine concentrations in current-year foliage concentrations may represent such a parameter for assisting in the diagnosis of N status. For example, might the accumulation of arginine in the foliage from an unfertilized stand indicate that the
productivity of this stand is limited by nutrients other than N? Could the accumulation of arginine following N fertilization indicate that a stand’s response to N additions is limited by elements other than N? These questions remain unanswered because the study by White et al. (1999) was only replicated within a single stand. Further replication across a larger number of stands would be necessary to determine if the relationships between nutrient additions, growth and arginine concentrations are consistent.

The findings by White et al. (1999) also raise the question as to whether the lack of a response of western hemlock to N or conventional P applications is because this species has a high P requirement, relative to other conifers, which is often not satisfied with P additions at the rate of 100 kg/ha. Few researchers have tested the effect of P additions beyond 100 kg/ha, probably because this is the upper limit that can be cost-effectively applied in operational fertilization programs. An exception is a study by Radwan et al. (1991) who tested the role of P nutrition in limiting the response of western hemlock to N fertilization and included P additions of 0, 100, 300 and 500 kg/ha. However, their findings were inconclusive. The question as to whether the growth response of western hemlock following N additions is limited by P deficiencies that are not relieved by the addition of 100 kg/ha, has not yet been adequately addressed.

While the productivity of a particular stand may be limited by P deficiency, as noted in Chapter I, total P concentrations in current-year foliage do not appear to be a reliable indicator of the P status of western hemlock. The identification of a reliable indicator of P status, perhaps one that is more physiologically based than total P, would benefit the accurate diagnosis of nutrient status. In this regard, Marshner (1986) noted that there are essentially four primary fractions of P within plants and trees: ester-P, nucleic-P, lipid-P, and inorganic-P. A number of investigators have adopted this approach in studies of P requirement by fractionating total P into these four fractions (Chapin and Kedrowski, 1983; Chapin et al., 1982; Sun et al., 1992; Valenzuela et al. 1996). Hart and Jessop (1984) reported that the inorganic-P fraction in the legumes was increased the most with increasing P supplies, and increases in the lipid and ester fractions were smaller. Similarly, Caradus and Snaydon (1987) reported that white clover plants (Trifolium repens L.) grown in soils of high P supply had higher inorganic P concentrations than
plants grown on P-deficient soils. Batten and Wardlaw (1987) reported that P-deficiency in wheat plants (*Triticum aestivum* L.) resulted in an exhaustion of the inorganic P fraction. It is clear from these studies that the organic fractions are increased only moderately with increasing P supply, whereas the inorganic P fraction increases significantly with large changes in P supply. These observations are consistent with the generally held view that the inorganic P fraction includes P within the vacuolar compartment, which is a storage compartment for P in excess of current requirement. These findings raise the question as to whether inorganic P (Pi) may be a more sensitive indicator of P status in western hemlock than total P.

The objectives of this study were:

1) to investigate the effect of the addition of N, two levels of P, and a blend fertilizer on the three-year basal area increment.

2) to further investigate the relationship between arginine concentrations in the foliage of western hemlock and foliar nutrient concentrations.

3) to determine if foliar arginine and Pi concentrations could aid in the diagnosis of the nutritional status of western hemlock and its potential response to nutrient additions.

A fertilization experiment was initiated in the spring of 1995 to address each of these objectives.
Methodology

Experimental Approach

Eight immature western hemlock stands were each fertilized with six treatments. Each of these stands had been fertilized approximately 10 years earlier by Carter et al. (undated) and the growth response to a limited number of treatments had been documented. Four of these stands had responded to previous fertilization and four of these stands did not respond. As noted in Chapter I, this study reported that growth response was not correlated with foliar nutrient concentrations.

Stand Descriptions and Locations

Seven of the eight stands were on northern Vancouver Island with the remaining stand on the Sunshine Coast (Figure 2.1). Each of the eight pure hemlock stands had undergone pre-commercial thinning approximately twelve years prior to the commencement of this study. Site and stand characteristics are provided in Table 2.1.

Plot Establishment

A total of thirty-six single tree plots were established at each of the eight installations in the spring of 1995 (Figures 2.2 and 2.3). In most cases, these single-tree plots surrounded the plots that had been earlier fertilized by Carter et al. (undated). Only co-dominant trees with well-formed crowns were selected. Care was taken to ensure that the crowns of selected trees had the canopy space to respond to fertilization. The radius of each single-tree plot was 3.3 m from the base of each tree. An unfertilized buffer of 10 m separated all plot boundaries, including those plots that had been fertilized previously by Carter et al. (undated).
Table 2.1. Relative basal area response to previous fertilization, and site and stand characteristics. Adopted from Carter et al. (undated).

<table>
<thead>
<tr>
<th>Previous Response</th>
<th>Installation</th>
<th>Relative Response</th>
<th>Site Index</th>
<th>Mean Age</th>
<th>Subzone</th>
<th>SMR</th>
<th>SNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Responsive</td>
<td>Port Alice #1</td>
<td>1.08</td>
<td>30</td>
<td>34</td>
<td>vm1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Port McNeill #1</td>
<td>1.09</td>
<td>29</td>
<td>40</td>
<td>vm1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Port McNeill #2</td>
<td>1.16</td>
<td>30</td>
<td>42</td>
<td>vm1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Eve River</td>
<td>1.17</td>
<td>28</td>
<td>19</td>
<td>vm2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Responsive</td>
<td>Port Alice #2</td>
<td>N (1.23) Complete</td>
<td>30</td>
<td>31</td>
<td>vm1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Zebellos</td>
<td>N (1.22) Complete</td>
<td>27</td>
<td>34</td>
<td>vm1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Nimpkish</td>
<td>N (1.58) Complete</td>
<td>30</td>
<td>32</td>
<td>vm1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Sechelt</td>
<td>N (1.34) Complete</td>
<td>27</td>
<td>41</td>
<td>vm1</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 2.2. Typical single-tree plot located at the Port McNeill #1 installation.
Figure 2.3. Typical roadside view of an immature western hemlock stand used in the present study and located near Port McNeill installation.
Treatment Descriptions

The six treatments tested were: (1) control (no fertilizer applied), (2) N (224 kg/ha), (3) N (224 kg/ha) + P (100 kg/ha), (4) N (224 kg/ha) + P (500 kg/ha), (5) N (224 kg/ha) + P (100 kg/ha) + blend (230 kg/ha), and (6) N (224 kg/ha) + P (500 kg/ha) + blend (230 kg/ha). The treatments were applied in mid to late May of 1995. Nitrogen was applied in the form of urea. Phosphorus was applied in the form of triple-superphosphate. The blend application included 60 kg/ha K applied as potassium sulfate, 40 kg/h Mg applied as magnesium sulfate, 10 kg/h Cu applied as copper sulfate, and 20 kg/h Zn applied as zinc sulfate. This application resulted in the addition of approximately 100 kg/ha sulfur in the form of sulfate. The treatments numbered 1, 2 and 4 had been previously tested by Carter et al. (undated). The treatments that applied P at the rate of 500 kg/ha were included in the present study to address the question as to whether western hemlock has a higher P requirement than other conifer species, a requirement that is not met with an application of P at the rate of 100 kg/ha.

Foliage Collection

Current-year foliage was collected during October, 1995 (i.e. at the completion of the first growing season following fertilization) from each of the 288 individual trees. Samples were collected from the base of the upper one-third of the live crown using a 12-gauge shotgun. Current-year shoots were removed from the fallen branches in the field and placed in clear plastic bags. The individual tree samples were quick-frozen in the field within several hours of collection using dry ice and subsequently stored in dry ice using specially insulated coolers. The samples remained frozen (-68 °C) until they were returned to the laboratory at which time they were immediately transferred to a low temperature freezer (-30 °C). The samples were subsequently wrapped in aluminum foil, freeze-dried for 72 hours, cleaned and ground using a Braun blender, and returned to cold storage (-30 °C). At no time were the samples allowed to thaw before they were freeze-dried. This procedure ensured that the samples remained frozen from the time of collection until the
time of analysis, which was critical to obtaining meaningful estimates of the free amino acid concentrations. On one occasion, the foliage samples had thawed upon arrival at the laboratory. These samples were discarded, and the foliage was recollected from that specific installation.

Current-year foliage was also collected from each tree during the fall of 1996 (i.e. at the completion of the second growing season). Samples were again collected from the base of the upper one-third of the live crown using a 12-gauge shotgun. The individual tree samples were subsequently used for the determination of foliar nutrient concentrations.

**Conventional Foliar Analysis**

Total nutrient contents were determined for each of the 288 individual samples collected at the end of the first and second growing season. MacMillan Bloedel’s Soil and Plant Testing Laboratory located in Nanaimo carried out determination of total nutrient contents. The samples were wet digested with Caro’s acid, using a modification of Parkinson and Allen (1975), followed by colorimetric analysis for N (salicylate/nitroprusside) and P (absorvic acid/molybdate-antimony) and atomic absorption spectrophotometry for K, Ca, Mg, Mn, Cu, Zn, and Fe. Nitrogen content of foliage collected in the fall of the second growing season was determined by the Kjeldahl method of Bremner and Mulvaney (1982), modified for use with a Buchi/Brinkmann Kjeldhal nitrogen system. Contents of Ca, Mg and K were determined by atomic absorption spectrophotometry (Price, 1979) following dry ashing. Phosphorus contents were determined colorimetrically with a molybdo-vanadate reagent after dry ashing.

**Determination of Arginine Concentrations**

The concentrations of free amino acids in current-year foliage collected at the end of the first growing season were determined for each of the 288 trees.
**Extraction**

Approximately 100 mg of ground foliage was extracted in 5-ml of cold M:C:W (12:5:3). The solution was centrifuged and the supernatant recovered. The remaining pellet was washed twice with 4-ml and 3-ml of cold M:C:W, vortexing each time. The solutions were centrifuged and the supernatant recovered. The remaining pellet was then discarded. Three ml of water was added to the supernatant to achieve phase separation and centrifuged. The aqueous, clear, upper phase was recovered and placed into scintillation vials. The lower chloroform phase was discarded. The samples were then dried using a Savant Speed Vac equipped with a Savant Refrigerated vapor trap. Samples were subsequently stored at -30 °C to await purification.

**Purification**

Three ml. syringes were prepared containing 2 mL of Dowex -50W (H+) resin. The resin was previously washed three times with distilled, de-ionized water. It was then suspended in 80% methanol and allowed to stand for 30 min. If additional colour change occurred the washing was repeated with 80% methanol. The resin was then stored in 80% methanol until use.

The dried samples were reconstituted with 2 mL of 80% methanol and slowly added to the resin bed. The resin was then washed with approximately 10 mL of 80% methanol. The eluate was discarded. The resin was then washed twice with 5 mL of 2 M NH₄OH in 80% methanol. This eluate, containing the free amino acids, was retained. The ammonia was subsequently removed on a Brinkman rotary evaporator and the remaining solution was transferred to scintillation vials. The evaporator flasks were rinsed twice with 1 mL of 80% methanol, each time adding the rinsate to the sample in the scintillation vials. The samples were then dried in the speed vac to remove the remaining water and methanol. The samples were again stored at -30 °C to await determination of free amino acid concentrations.
Quantification

The amino acid concentrations were quantified by the Biotechnology Center at the University of British Columbia. The samples were reconstituted with 1 mL of distilled, de-ionized water, frozen, thawed, centrifuged, and subsequently filtered with 0.45-micron filters into micro centrifuge tubes. The samples were then stored at -30 °C to await final analysis. The method incorporated pre-column 0-phthaldiadehyde (OPA) derivatization and flourometric detection (Puchata et al., 1994). A Beckman 507 autosampler was used for the derivatization process. Fluorometric detection was used to measure OPA derivatives (excitation 338 nm, emission 425 nm). The following amino acids were quantified in the foliage from each of the 288 trees: aspartic acid, glutamic acid, asparagine, glutamine, arginine, glycine, histidine, tyrosine, valine, phenylalanine, leucine, lysine, serine and threonine. Only arginine concentrations were significantly affected by the fertilization treatments and the remaining amino acids will not be considered in this thesis.

Inorganic Phosphorus Analysis

The procedure of Bouma and Dowling (1982) was adopted. Approximately 40 mg of freeze-dried foliage was extracted in 3 N H2SO4. Distilled and de-ionized water was added, then centrifuged. An aliquot of the supernatant was transferred to a 50-ml volumetric flask. Approximately 5-mL of John’s reagent (John, 1970) and 0.075 g of ascorbic acid were added. After 10 minutes of colour development, 0.5 mg of citric acid was added (Irving and Bouma, 1984) and the solution was made to volume. Colour development at 60 minutes was determined using a spectrometer at both 660 nm and 882 nm.

Measurement of three-year basal-area response

In the fall of 1997 (i.e. after the completion of three growing seasons), two
increment corers were extracted at breast height from each of the 288 trees. Care was taken to avoid radical stem deformities and to ensure that each core passed through the center of the stem. The increments for each of the three growing seasons following fertilization, as well as the three growing seasons prior to fertilization, was carefully measured. These measurements were facilitated by using a Horizon Regent Scanning unit coupled with Windendro version 6.0.4.

**Statistical Analysis**

The foliar results were analyzed by ANOVA using a SAS General Linear Model (GLM) for a balanced design. Installation and treatment were each considered fixed variables, therefore, Zar’s (1984) ANOVA type I was used for the analysis of data. Duncan’s multiple range test compared means. Basal area response was normalized by a log transformation prior to covariate analysis where the previous three-year basal area increment prior to fertilization was considered the covariate.
Results

Growth Response

The three-year basal area increment was significantly affected by treatment (Table 2.2). No interaction between site and treatment was evident at this measurement period, owing perhaps to high between-tree variation. The response was attributed solely to N additions (Table 2.3). The further addition of P, at either application rate, or the further addition of the elements contained within the blend fertilizer, had no detectable effect on the three-year increment.

While each of the eight installations showed evidence of a significant response to N additions, the response to N was greatest in the stands Nimpkish, Zebellos, Port Alice #2 and Sechelt (Table 2.4). As noted earlier, Carter et al. (undated) had previously identified these four stands as responsive. In this study, the response at the Port McNeill #2 installation was also strong, though Carter et al. (undated) had earlier reported it as being unresponsive. The remaining three installations showed evidence of a statistically significant response to N additions but the magnitude of their response was low. This marginal response may not have been evident had it at a six-year re-measurement period.

Foliar nutrient concentrations

The concentrations of foliar nutrients were significantly affected by the various treatments and also by installation (Table 2.5). The foliar N and P concentrations in foliage collected from the control plots indicated that all stands were deficient in both nutrients (Table 2.6). Foliar N concentrations were significantly increased with N fertilization, but the concentration of N following N addition was still within a range considered deficient. For example, the foliar N concentration in the foliage from the Port McNeill #2 and Sechelt installations was 1.07 and 1.15% respectively. The P concentrations decreased at most installations following N-only additions. The effect
Table 2.2. Covariate Analysis of basal area increment: F ratios and level of significance for covariate and treatment, installation, and their interaction.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of freedom</th>
<th>F</th>
<th>Pr.&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covariate</td>
<td>1</td>
<td>1153.77</td>
<td>0.0001</td>
</tr>
<tr>
<td>Installation</td>
<td>7</td>
<td>9.60</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>13.32</td>
<td>0.0001</td>
</tr>
<tr>
<td>Installation*Treatment</td>
<td>35</td>
<td>0.99</td>
<td>0.48</td>
</tr>
</tbody>
</table>
Table 2.3. Basal area response by treatment relative to control: means were adjusted for covariate. Adjusted means with the same letter not significantly different at 5%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 (a)</td>
</tr>
<tr>
<td>N</td>
<td>1.31 (b)</td>
</tr>
<tr>
<td>NP100</td>
<td>1.33 (b)</td>
</tr>
<tr>
<td>NP100B1</td>
<td>1.39 (b)</td>
</tr>
<tr>
<td>NP500</td>
<td>1.33 (b)</td>
</tr>
<tr>
<td>NP500B1</td>
<td>1.36 (b)</td>
</tr>
</tbody>
</table>
Table 2.4. Basal area response relative to control by treatment and installation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nimpkish</th>
<th>Zebellos</th>
<th>Sechelt</th>
<th>Port McNeill #2</th>
<th>Eve River</th>
<th>Port Alice #2</th>
<th>Port Alice #1</th>
<th>Port McNeill #1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>N</td>
<td>1.54</td>
<td>1.41</td>
<td>1.49</td>
<td>1.29</td>
<td>1.11</td>
<td>1.34</td>
<td>1.19</td>
<td>1.18</td>
</tr>
<tr>
<td>NP100</td>
<td>1.26</td>
<td>1.41</td>
<td>1.71</td>
<td>1.28</td>
<td>1.20</td>
<td>1.62</td>
<td>1.18</td>
<td>1.09</td>
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<td>NP100B1</td>
<td>1.51</td>
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<td>1.90</td>
<td>1.34</td>
<td>1.19</td>
<td>1.49</td>
<td>1.38</td>
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<tr>
<td>NP500</td>
<td>1.37</td>
<td>1.41</td>
<td>1.73</td>
<td>1.39</td>
<td>1.19</td>
<td>1.46</td>
<td>1.12</td>
<td>1.11</td>
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<tr>
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<td>1.81</td>
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<td>1.19</td>
<td>1.61</td>
<td>1.13</td>
<td>1.09</td>
</tr>
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</table>
Table 2.5. Analysis of first year foliar nutrient concentrations, inorganic P (Pi), phosphorus to nitrogen ratio (P:N) and arginine: F values and probability.

<table>
<thead>
<tr>
<th></th>
<th>arg</th>
<th>N</th>
<th>P</th>
<th>P:N</th>
<th>Pi</th>
<th>K</th>
<th>Ca</th>
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<tbody>
<tr>
<td>Treatment</td>
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<td>31.67</td>
<td>60.69</td>
<td>11.45</td>
<td>2.97</td>
<td>4.30</td>
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</table>
Table 2.6. Mean and SE of arginine (µmoles/g), nutrient and inorganic P (Pi) concentrations, and the P:N ratio by site and installation. Means with the same letter do not differ at 5% confidence.

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>Arg</th>
<th>%N</th>
<th>%P</th>
<th>P/N</th>
<th>Pi</th>
<th>%K</th>
<th>%Ca</th>
<th>%Mg</th>
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<td>1.14</td>
<td>0.15</td>
<td>0.13</td>
<td>0.06</td>
<td>0.49</td>
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</tr>
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<td>0.02</td>
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Table 2.6 con’t. Mean and SE of arginine (µmoles/g), nutrient and inorganic P (Pi) concentrations, and the P:N ratio by site and installation. Means with the same letter do not differ at 5% confidence.

<table>
<thead>
<tr>
<th>Site</th>
<th>Treatment</th>
<th>Arg</th>
<th>%N</th>
<th>%P</th>
<th>P/N</th>
<th>Pi</th>
<th>%K</th>
<th>%Ca</th>
<th>%Mg</th>
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<td>1.47 (0.09)</td>
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<td>0.61 (0.04)</td>
<td>0.25 (0.01)</td>
<td>0.14 (0.01)</td>
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<td>1.34 (0.07)</td>
<td>0.13 (0.01) b</td>
<td>0.10 (0.01) ab</td>
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<td>0.14 (0.01)</td>
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<td>1.26 (0.03)</td>
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<td>0.04 (0.01) a</td>
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<td>0.10 (0.01) ab</td>
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<td>0.57 (0.02)</td>
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<td>0.11 (0.01) ab</td>
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<td>0.16 (0.02)</td>
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<td>0.46 (0.02)</td>
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<td>1.23 (0.13)</td>
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</tr>
<tr>
<td>Sechelt</td>
<td>NP500B</td>
<td>0.0 (0.03) a</td>
<td>1.20 (0.04)</td>
<td>0.26 (0.03) a</td>
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<td>0.61 (0.04)</td>
<td>0.26 (0.02)</td>
<td>0.15 (0.00)</td>
</tr>
</tbody>
</table>
of N additions on P status was reflected in the reduction in foliar P:N ratio. The addition of P at the rate of 100 kg/ha increased the foliar P concentration at each installation, with the exception of the Zebellos installation, though the concentrations remained below a concentration of 0.20%. The addition of P at the rate of 500 kg/ha significantly increased the foliar P concentrations at most installations. Interestingly, the highest mean P concentration following the high application rate of P was 0.27% in foliage collected from the Eve River installation. This treatment was targeted to increase the foliar P concentrations to a mean of approximately 0.40%.

The addition of the blend fertilizer failed to increase foliar K or Mg concentrations. However, uptake may have been increased if there had been an increase of foliage biomass produced in response to this treatment. Similarly, Fe, Cu and Zn concentrations (not shown) also were not increased with the addition of the blend fertilizer.

Arginine Concentrations

Arginine concentrations also varied strongly with treatment (Table 2.5). The foliage collected from the control trees at each of the eight installations had little or no detectable arginine concentrations (Table 2.6). The addition of N resulted in a significant increase in the concentration of arginine. The arginine concentrations also varied with installation (Table 2.5). There appeared to be a difference among the installations in arginine response following N additions, with the lowest concentration in foliage from the Port Alice #2 installation, though the between tree variation was large. The addition of P at 100 kg/ha in addition to N significantly reduced arginine concentrations to a level not significantly different from the control.

Arginine concentrations were significantly correlated with each measure of P nutrition: total P, Pi and the P:N ratio (Table 2.7). A closer examination of the relationship between arginine and the P:N is provided in Figure 2.4. Arginine concentrations do not appear at foliar P:N ratios above 0.14. However, it is also apparent that though total N and arginine were not correlated, arginine will not increase at low N concentrations, even if the P:N ratio is below 0.14.
P fractionation

The concentration of inorganic P (Pi) also varied significantly with treatment (Table 2.5). The concentration of Pi in the foliage from the unfertilized trees ranged from 0.01 to 0.06%, being higher at the installations that also had higher total P concentrations. The addition of N alone significantly reduced the Pi concentrations at each of the installations with the exception of Zebellos. The concentrations of Pi appeared to be more sensitive to N additions than total P concentrations. The addition of P increased the magnitude of the Pi fraction, and the increase was greatest with the two treatments that included P at the rate of 500 kg/ha.

The relationship between Pi, the organic P fraction (Po), and total P is provided in Figure 2.5. The organic fraction is the more stable of the two P fractions. The concentration of the Po fraction increases from a mean of approximately 0.06 in western hemlock foliage with very low total P concentrations, and increases to a concentration of between 0.12-0.13%. Further increases in the concentration of total P have little further effect on the proportion of total P that is allocated to the organic fraction. In contrast, Pi is more sensitive to changes in the total P concentration. For example, foliage that has a total P concentration of 0.10% has a corresponding Pi concentration of approximately 0.01%. A doubling of the total P concentration increases the concentration of the Pi fraction approximately 8-fold.

The concentrations of N, P, K and Mg in foliage collected at the end of the second growing season also varied with both treatment and installation (Table 2.8). The effect of N additions in maintaining high foliar N concentrations appears to have limited success as N concentrations declined in the second year. For example, the average N concentration in foliage collected from trees at the Port Alice #1 installation that received N only additions, decreased from 1.46% during the first growing season (Table 2.6) to 1.15% by the end of the second year (Table 2.9).
Table 2.7. Matrix of correlation coefficients for arginine (arg), nutrient, and inorganic P (Pi) concentrations and the P:N ratio.

<table>
<thead>
<tr>
<th></th>
<th>Arg</th>
<th>N</th>
<th>P</th>
<th>P:N</th>
<th>Pi</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>Fe</th>
<th>Cu</th>
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<td>0.06</td>
<td>0.20</td>
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<tr>
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<td>-0.07</td>
<td>-0.22</td>
<td>-0.14</td>
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<td>0.08</td>
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<tr>
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<td>0.10</td>
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<td>-0.11</td>
<td>-0.07</td>
<td>-0.05</td>
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<td>0.1</td>
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Figure 2.4. Arginine concentrations (μm/g dw) in current-year foliage at the end of the first growing season as a function of P:N for 288 individual determinations.
Figure 2.5. Effect of total P (%) in current-year foliage on the inorganic P (Pi) fraction (diamonds) and the organic P (Po) fraction (squares) for a total of 288 individual trees.
Table 2.8. Analysis of second year foliar data: F ratios and level of significance.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
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<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
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<td>1.45</td>
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<td>0.001</td>
<td>0.001</td>
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<td>9.47</td>
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</table>
Table 2.9. Mean foliar nutrient concentrations and SE at the end of the second growing season by treatment and installation. Each value is the mean of six individual measurements. Means with the same letter do not differ at the 5% level.

<table>
<thead>
<tr>
<th>Installation</th>
<th>Treatment</th>
<th>N (%)</th>
<th>P (%)</th>
<th>K (%)</th>
<th>Ca (%)</th>
<th>Mg (%)</th>
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<tbody>
<tr>
<td>Eve River</td>
<td>Control</td>
<td>0.97 (0.05) a</td>
<td>0.14 (0.02) a</td>
<td>0.43 (0.03) abc</td>
<td>0.28 (0.01)</td>
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<tr>
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<td>Control</td>
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<td>0.09 (0.00) b</td>
<td>0.42 (0.03) abc</td>
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<td>0.14 (0.01) a</td>
<td>0.33 (0.03) de</td>
<td>0.23 (0.01)</td>
<td>0.15 (0.01)</td>
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<tr>
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<td>0.44 (0.01) ab</td>
<td>0.23 (0.03)</td>
<td>0.16 (0.02)</td>
</tr>
<tr>
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<td>0.09 (0.00) b</td>
<td>0.24 (0.02) e</td>
<td>0.23 (0.02)</td>
<td>0.13 (0.01)</td>
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<tr>
<td>Eve River</td>
<td>N</td>
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<td>0.41 (0.04) a</td>
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<tr>
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<td>0.18 (0.01)</td>
<td>0.13 (0.01)</td>
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<td>Port Alice #2</td>
<td>N</td>
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<td>0.11 (0.01) c</td>
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<tr>
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<td>0.09 (0.00) d</td>
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<td>0.21 (0.02)</td>
<td>0.12 (0.01)</td>
</tr>
<tr>
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<td>0.07 (0.00) d</td>
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<td>0.22 (0.02)</td>
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</tr>
<tr>
<td>Sechelt</td>
<td>N</td>
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<td>0.09 (0.00) d</td>
<td>0.38 (0.03) abc</td>
<td>0.22 (0.02)</td>
<td>0.12 (0.01)</td>
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<td>0.14 (0.01) ab</td>
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<td>0.14 (0.01)</td>
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<tr>
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<td>0.13 (0.00) b</td>
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<td>0.33 (0.02) a</td>
<td>0.20 (0.02)</td>
<td>0.14 (0.01)</td>
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</tbody>
</table>
Table 2.9 con't. Mean foliar nutrient concentrations and SE at the end of the second growing season by treatment and installation. Each value is the mean of six individual measurements. Means with the same letter do not differ at the 5% level.

<table>
<thead>
<tr>
<th>Installation</th>
<th>Treatment</th>
<th>N (%)</th>
<th>P (%)</th>
<th>K (%)</th>
<th>Ca (%)</th>
<th>Mg (%)</th>
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</thead>
<tbody>
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<tr>
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<td>0.22 (0.02) bc</td>
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<td>0.21 (0.01) abc</td>
<td>0.46 (0.03) a</td>
<td>0.27 (0.03)</td>
<td>0.12 (0.01)</td>
</tr>
<tr>
<td>Port Alice #1</td>
<td>NP500BL</td>
<td>1.13 (0.06) b</td>
<td>0.15 (0.01) c</td>
<td>0.38 (0.01) a</td>
<td>0.19 (0.02)</td>
<td>0.12 (0.01)</td>
</tr>
<tr>
<td>Port Alice #2</td>
<td>NP500BL</td>
<td>1.28 (0.03) a</td>
<td>0.16 (0.01) bc</td>
<td>0.43 (0.02) a</td>
<td>0.21 (0.02)</td>
<td>0.13 (0.00)</td>
</tr>
<tr>
<td>Port McNeill #1</td>
<td>NP500BL</td>
<td>1.10 (0.05) b</td>
<td>0.15 (0.01) bc</td>
<td>0.37 (0.05) a</td>
<td>0.21 (0.02)</td>
<td>0.10 (0.01)</td>
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<td>0.22 (0.03) abc</td>
<td>0.38 (0.02) a</td>
<td>0.25 (0.03)</td>
<td>0.13 (0.01)</td>
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<tr>
<td>Sechelt</td>
<td>NP500BL</td>
<td>0.94 (0.01) c</td>
<td>0.17 (0.01) bc</td>
<td>0.38 (0.03) a</td>
<td>0.26 (0.02)</td>
<td>0.13 (0.00)</td>
</tr>
<tr>
<td>Zeballos</td>
<td>NP500BL</td>
<td>1.27 (0.04) a</td>
<td>0.19 (0.02) abc</td>
<td>0.39 (0.02) a</td>
<td>0.21 (0.03)</td>
<td>0.14 (0.01)</td>
</tr>
</tbody>
</table>
Discussion

It was earlier questioned if the growth response of western hemlock following N additions is limited by P deficiency, which may not be relieved by the addition of 100 kg/ha. This experiment addressed the latter half of this question by including treatments that applied P at the rate of 500 kg/ha. The three-year basal area response results, however, indicated that none of the eight stands responded to P additions applied at either rate. Indeed, the three-year measurement indicated that none of the stands had responded to any nutrient addition other than N within this three-year time frame. This would seem to indicate that the P requirement in these trees was met from existing sources without the need for further P additions through fertilization. However, the question that must be posed and addressed is whether the three-year basal area increment in the present study represented a “critical test”. This question is particularly relevant given the low P concentrations in current-year foliage of either the control or N-treated plots at all installations.

The six-year basal area increment, beyond the scope of this thesis, would certainly provide a growth response data set from which more definitive conclusions could be drawn. Indeed, since an increase in basal-area response must be preceded by one or more years of crown expansion, six-year response data would be more sensitive in detecting not only the magnitude of a response but also the duration. In this regard, it is possible that some of the stands that have responded to N additions over a three-year period may not show such evidence when their growth response is measured over a six-year period if their responses to N additions were short.

The finding that the organic fraction of P (Po) fluctuated little over a wide range of external P supplies is in agreement with similar studies reported in the plant nutrition literature (e.g. Sun et al., 1992; Caradus and Snaydon, 1987; Batton and Wardlow, 1987; Hart and Jessop, 1984; Bouma and Dowling, 1982). The stability of the organic fraction, as determined for 288 trees, indicates that further fractionation of P in western hemlock by other investigators, working within different western hemlock stands, is probably not warranted. What would be interesting, and has yet to be measured, is the size of the
organic fraction in other conifer species. These changes, if they exist, could then be compared to species differences in P requirement.

It was also earlier questioned as to whether the inorganic P (Pi) fraction in current-year foliage might be a more sensitive indicator of P status in western hemlock than total P. The lack of a P response at any of the eight installations precludes any discussion as to whether Pi can be used to diagnose nutritional status with an aim to predict P response. However, what this experiment has clearly shown is that small reductions in the total P concentration can have significant impact on the size of the inorganic P fraction, which represents the metabolically active form of P. Hence, it can certainly be concluded that this pool is a more sensitive indicator of P status. Changes in this P fraction can have strong physiological effects related to strategies that deal with enhanced P acquisition, photosynthesis, and cellular compartmentation. The relationship between the inorganic fraction and each of these factors will be discussed in detail in later chapters.

The low concentrations of arginine in foliage collected from unfertilized trees are consistent with the view that these trees were N-deficient. The precise cause of the high arginine concentrations in trees that had received N only additions is more difficult to ascertain but similar increases in arginine following N additions have been documented by other researchers (Edfast et al., 1996; Ericsson et al., 1993; Edfast et al., 1990; Nasholm and Ericsson, 1990 and Kim et al., 1987). If these particular trees had reached their maximum growth rate following the alleviation of N stress with N fertilization, then the arginine represents "accumulation" of N in excess of requirement (Millard, 1988). Alternatively, the N additions may have increased N supplies beyond the tree's N demand, but its growth remained retarded by secondary deficiencies. In this case, the high arginine concentrations would represent a "storage" form of N (Millard, 1988). Arginine is thought to be the primary form of storage for N in many plants and trees because of its low C to N ratio (Marshner 1986).

The fact that arginine concentrations were reduced to near control concentrations with the addition of P and the blend elements, indicates that the high arginine concentrations may have been due to a condition of unbalanced nutrition induced by the addition of N. In this regard, Ericsson et al. (1993) had reported that elevated arginine
concentrations in Norway spruce (*Picea abies* [L.] Karst.) were associated with low P:N ratio and may also have been associated with K deficiency. Lambert (1986) reported that the high concentration of arginine following N fertilization of radiata pine plantations in New South Wales was the result of induced S deficiency.

Rabe and Lovatt (1984; 1986) have proposed an alternative explanation for high foliar arginine concentrations. They argued that the increase in arginine synthesis is induced by P deficiency. The accumulation of arginine was attributed to an increase in *de novo* synthesis as a mechanism to detoxify accumulating ammonium. As later noted by Rabe (1990), the source of ammonium is thought to be from the conversion of accumulating nitrate, which may be of more limited application in conifers, and from the degradation of amino acids normally incorporated into proteins. The reduction in arginine concentrations in the present investigation following the additions of P agrees with the hypothesis put forth by Rabe and Lovatt (1984; 1986). However, it is also possible that the reduction in arginine concentrations was due to increased internal N demand following the addition of other elements. Regardless, ammonium toxicity following N-only additions in western hemlock, and conifers in general, is an interesting hypothesis that warrants further investigation.

It was earlier questioned whether or not arginine concentrations in the foliage collected from unfertilized trees could be used to identify stands in which nutrients other than N limited growth. In the present study, there was little variation in the concentrations of arginine in the unfertilized trees, so this does not appear to be an application of amino acid determinations.

It was also earlier questioned as to whether arginine concentrations were a more sensitive indicator of N status in western hemlock. Arginine concentrations in the present investigation were not related to either N concentration nor to the P:N ratio. This would seem to be strong evidence that arginine concentrations are a true measure of internal N utilization rather than mere N content. The fact that arginine concentrations decreased with the addition of P and the blend fertilizer, as noted above, would seem to also substantiate this conclusion. Hence, arginine concentrations would appear to be a more sensitive indicator of N status. It was also questioned if arginine concentrations following
fertilization could be used to aid in the identification of responsive stands. Unfortunately, each of the eight installations showed some evidence of responding to N additions. Lacking a number of unresponsive stands makes addressing this question impractical.

The addition of N at the conventional application rate of 225 kg/ha appeared to have limited success at increasing the N concentration in these western hemlock stands. In contrast, N additions to an 18-year-old western hemlock and Douglas-fir stand increased the foliar N concentrations over 2%. In this latter study, N was applied in the form of ammonium nitrate rather than urea. It is also worth noting that the P additions at 500 kg/ha, five times the conventional application rate, also had only a small effect on foliar P concentrations. These shortcomings should be considered by other investigators in the design of new experiments involving immature or mature stands of western hemlock.
Conclusions

1) Each of the eight installations showed evidence of a growth response to N additions but further increases in growth were not observed with the addition of P, at either of two rates, or with the additional application of a blend fertilizer.

2) The precise role of P in limiting the response of western hemlock remains inconclusive and should be further studied using mid-rotation stands and application rates similar to those used in the present study.

3) Fractionation of total P into organic and inorganic fractions indicated that the organic fraction is the more stable fraction and peaks at approximately 0.13%. The inorganic fraction is the more sensitive fraction to external P supplies. In the present study, N-only additions resulted in a strong reduction in the size of the inorganic pool though changes in the total concentration changed much less.

4) The addition of N alone resulted in a significant increase in the concentration of the amino acid arginine at all installations. The increase in arginine concentrations was attributed to a condition of unbalanced nutrition, but the role of ammonium toxicity cannot be dismissed.

5) The addition of P resulted in a significant decrease in the concentrations of arginine.

6) Arginine concentration was determined to be a sensitive indicator of internal N utilization in the foliage collected from the N-only treated plots. The lack of a clear difference in the response of the installations to N fertilization, however, precludes conclusive statements regarding the application of this amino acid to predicting long-term growth responses to fertilization.
7) It is recommended that the six-year basal area increment be determined to obtain a superior indicator of the long-term growth response of these trees to the various nutrient additions.
CHAPTER III
32P UPTAKE IN EXCISED ROOTS AS AN INDICATOR OF NUTRIENT STATUS AND PREDICTOR OF FERTILIZATION RESPONSE

Introduction

Harrison and Helliwell (1979) investigated the uptake of 32P in roots of birch (Betula verrucosa Ehrh.) seedlings grown in sand culture, which had received differing supplies of P. They reported that uptake was negatively correlated with the rate at which P had been previously supplied to the growing medium. The authors also studied the effect of P fertilization on uptake. Mineral soil was collected from thirteen sites and was either left unfertilized or fertilized with a phosphate fertilizer. The birch seedlings that had been grown in soil that had their P supply augmented had greatly reduced levels of P uptake. The authors also grew birch seedlings in mineral soil that had been collected from twenty-five different sites of known varying fertility. The 32P uptake in two-year-old birch seedlings grown on these soils was negatively correlated with the P content of the seedlings. The authors argued that their results indicated that this bioassay technique was a reliable indicator of both P supply from the soil and an indicator of the degree to which the seedling demand for P was satisfied.

The above research by Harrison and Helliwell (1979) has been recognized as a valuable contribution to the field of tree nutrition. However, their research did not address the question as to whether this technique was a superior indicator of a tree’s P status than that indicated by conventional foliar analysis. This, and related questions, have been addressed, to some degree, by subsequent studies. Dighton and Harrison (1983) determined uptake rates in excised roots collected from two immature lodgepole pine and two immature Sitka spruce stands growing on three sites. Each of the stands included treatments which consisted of P additions at the time of planting and/or near crown closure. Most notable was the finding that uptake was a more sensitive indicator of previous fertilizer treatments than total P in the current-year foliage. For example, trees at one site that had received a total of 71 kg/ha had uptake rates of 187 pg P mg⁻¹ root. Roots collected from the control plots had a measured uptake rate of 425 pg P mg⁻¹ root. However, the foliage from the unfertilized plots had a mean P concentration of
0.15% compared to 0.16% to the fertilized plots. Uptake was also better related to mean tree height than to total P in foliage. Dighton and Harrison (1990) measured the uptake of $^{32}$P in excised roots collected from a number of young Sitka spruce plantations growing in upland Great Britain varying in age from between one and thirty-three years of age. They reported that uptake varied with the stage of stand development, being lowest in stands recently established, and highest in stands that had recently achieved stand closure. Uptake was lower in older stands. While P uptake was related to the stages of stand development and crown closure, foliar P concentrations were not related to stand development. Seven closed-canopy stands, which had the highest uptake of P in excised roots, were subsequently fertilized with P at the rate of 100 kg/ha. The addition of P to these stands resulted in a significant growth response after three growing seasons, confirming that the P uptake in the excised roots prior to fertilization was an indicator of P demand which was not revealed by foliar P concentrations nor soil chemical properties. A subsequent study by Steven et al. (1993) reported that the fertilization of a mature Sitka spruce plantation with P and K at rates of 120 and 200 kg/ha, respectively, decreased the leaching of nitrate from the soil profile. The authors argued that the reduction in leaching following the application of P and K was due to increased uptake of N by the stand. Furthermore, the authors reported that $^{32}$P uptake in excised roots was dramatically reduced in the roots from fertilized plots. More importantly, these researchers reported that $^{32}$P uptake in excised fine roots of Sitka spruce was a more sensitive indicator of P demand than foliar P concentrations. This finding led the authors to conclude that the uptake of $^{32}$P in excised fine roots was a superior technique for identifying the nutritional status of Sitka spruce stands than conventional foliar analysis. Jones and Dighton (1993) reported that uptake of $^{32}$P in excised roots collected from a newly established Eucalyptus plantation (Eucalyptus grandis Hill ex. Maiden) was lowest in plots that had received 80 kg/ha of P at, or shortly after time of planting. Consistent with the findings of Dighton and Harrison (1990; 1983), $^{32}$P uptake did not appear to be related to foliar P concentrations. The lack of a consistent relationship between P uptake in excised roots and foliar P concentrations, as noted above, should not be surprising. Concentrations of a particular nutrient in foliage are a reflection not only of the rate of uptake of this particular nutrient
from the environment, but also a reflection of the growth rate of the tree or plant and the
degree of nutrient retranslocation. Since foliar nutrient concentrations and rate of growth
are hopelessly confounded, researchers have long been wary of basing conclusions
pertaining to nutrient uptake ability or uptake strategies merely using nutrient
concentrations in foliage. In contrast, the uptake rate of a nutrient, where the rate of
uptake of the nutrient in question can be regulated by the plant or tree, is assumed to be a
more direct measure of nutrient demand at a particular point in time. The demand
essentially represents the difference between the previous supply and requirement, a
difference that can be augmented with fertilization additions.

Many researchers studying the nutrition of western hemlock have reported that
foliar concentrations, including that of total P, have not assisted in the diagnosis of the
nutritional status of this species (Chapter I, II). Accordingly, the research findings
pertaining to uptake in other tree species cited above raise the interesting question as to
whether this biological estimate of P status could be used to accurately and consistently
diagnose the P status of western hemlock and predict its response to fertilization. For
example, do high rates of uptake in roots collected from a particular stand indicate that
this stand is P deficient and will respond positively to P additions, and further, that this
stand’s response to N-only additions will be limited by this pre-existing P deficiency?
Do increased uptake rates in excised roots of hemlock following N fertilization indicate
that N fertilization induced a secondary deficiency in P? Could increased uptake in roots
collected from N-fertilized stands explain the lack of a specific stand’s response to N
fertilization (i.e. this stand failed to respond to N additions because P was a primary
growth-limiting factor), or indicate that the additions of P, in combination with N, may
have resulted in a further increase in growth? These questions have yet to be tested by
researchers interested in the nutrition of western hemlock.

Accordingly, the objectives of this study were:

1) To determine if the rate of $^{32}$P uptake in excised fine roots collected from
unfertilized western hemlock trees is related to their subsequent growth
response following N and P fertilization.
2) To determine the effect of N and P fertilization on the rate of $^{32}$P uptake in excised fine roots.

3) To investigate the relationship between the rate of $^{32}$P uptake and P concentrations in current-year foliage and the fine roots.

A root bioassay study was carried out in the fall of 1996 to address each of these questions.
Methodology

Sampling of fine roots

Fine roots were collected from each of the eight single-tree screening installations during October and early November of 1996 (i.e. two growing seasons following the application of fertilizer treatments). Samples were collected from each of the six-single-tree plots representative of the control, N (225 kg/ha), N (225 kg/ha) + P (100 kg/ha), and N (225 kg/ha) + P (500 kg/ha) treatments. Roots were not collected from the two treatments that included the blend fertilizer.

The fine roots were collected from the L, F and upper H horizons within a 3-m radius plot using a square-pointed spade. Two square blocks of forest floor (Figure 3.1) measuring approximately 20 cm$^2$ were removed from opposite sides of each plot. An assumption was made that all roots contained within the forest floor samples were western hemlock. This assumption was deemed to be acceptable because of the lack of vegetation within the ground and shrub layers due largely to the presence of dense thinning debris (Figure 3.2). In addition, tree species other than hemlock had been removed during the previous pre-commercial thinning operation. McDonald et al. (1991) had earlier reported that microsite variability had a significant affect on uptake. Consequently, to reduce the effects of site variability, samples were not collected from rotting wood material or from poorly drained depressions.

The sampling procedure adopted assumed that the root sample collected from within the 3 m plot represented the same tree from which growth measurements were subsequently obtained and from which foliage samples had been earlier collected. While the roots that were sampled were always from within the fertilized area, they may have originated from a neighbouring tree. The only way it could be ensured that the root sample was of the proper sample tree would have been to physically trace the fine root back to the base of a particular tree by way of lateral roots. This procedure had been adopted in an earlier root sampling experiment conducted by this author but not reported in this thesis. Fine roots had been collected from a mixed species experiment (White et al., 1999) during the summer of 1996 and this approach was successful in accurately
Figure 3.1. Fine roots of western hemlock within a forest floor sample collected with a square spade. Note the white root tips indicating recent root growth, characteristic of the fall season.
Figure 3.2. Single-tree plot within the Port McNeill #1 installation. Note the presence of heavy slash from the previous pre-commercial thinning operation and the lack of vegetation within either the ground of shrub layer.
identifying roots (Figure 3.3). However, this approach was physically demanding and only 8 to 10 samples could be collected per day. Adopting this latter approach in the current study would have greatly reduced the number of samples that could have been collected from a given installation and, realistically, would most likely have reduced the number of installations that roots could have been sampled. Consequently, the approach of root tracing was not adopted in favor of increasing the number of samples that could be collected from each installation as well as ensuring that roots were sampled from all eight installations.

A major impediment in studies of root morphology, distribution, function or mortality is the significant spatial variation in each of these parameters that can be attributed to the variation, both horizontal and vertical, in the physical and chemical characteristics of the rooting environment. Hence, the purely physical problems related to the collection of root samples are compounded by the need to ensure adequate sample replication. In the present study, the initial sampling plan specified that a total of two samples was to be collected from the base of each tree. This would result in the collection of 12 fine roots per treatment, per installation, for a total of 384 total samples. Following the collection of these samples, a second sampling of each installation was undertaken to further increase the sample size. Fine roots were collected until the second week of November. Field sampling abruptly ended with an early snowfall on northern Vancouver Island. At this point, in excess of 630 samples had been collected.

The collection of the fine roots in the field and the subsequent determination of uptake in the laboratory were undertaken by different personnel. In order to ensure that the samples collected on a given day in the field could be analyzed the following day by staff in the laboratory at the University of British Columbia, the collection and analysis were closely coordinated. If it could be predicted that samples could not be analyzed on a given day, collection was not undertaken during the previous day.

The root bioassay method is an estimate of the active transport of P across cellular membranes; hence, collection and handling procedures were adopted to minimize the possibility of desiccation, overheating or physical damage to the fine roots. The roots and forest floor material were collected during the mid-morning hours of each sampling day. The forest floor samples were wrapped in moistened paper towels and placed within
Figure 3.3. Fine root collection from a mixed species trial (White et al., 1996) during the summer of 1996. Roots collected near the base of the Douglas-fir tree above left were traced to a hemlock tree, right, a distance of 4.5m. In the background are red ingrowth bags, remnants of an earlier fine root ingrowth study.
sealed plastic bags within minutes of initial collection. The samples were then returned to laboratory facilities in Port McNeill, generally within two to four hours of collection. The samples were carefully soaked and washed with cool water at which time roots greater than 1 mm in diameter and dead fine roots were discarded. Criteria for distinguishing live and dead roots were adopted from McClaugherty et al. (1982), Persson (1983), and Keyes and Grier (1981). A root was classified as live if it was resilient, translucent, or white and tan in color. Actively growing root tips were light colored and translucent. Dead roots were typically dull and grey to black in appearance and could often be easily fragmented. Dead roots also typically lacked cohesion between the cortex and periderm. The remaining fine roots were wrapped in moistened paper towels and transported to Vancouver on the day of collection by air transport from Port Hardy. To prevent high respiration rates, the samples were picked up nightly from the Vancouver airport and stored at 4°C overnight in the laboratory at the University of British Columbia.

The following morning, the samples were removed from refrigeration and again washed under tap water. Adhering organic matter was carefully removed and dead roots were further identified and discarded. A single fine root weighing approximately 150-200 mg was selected from each of the two forest floor samples that had been initially collected from each tree. Hence, a total of two fine roots were analyzed from each tree. Each root was placed in a numbered plastic vial with a perforated bottom, which was subsequently submerged in the labeled solution. The remaining root samples collected from a given tree were combined and dried at 70 °C for 48 hours for later determination of root P concentrations.

The color of the root was noted though Harrison and Helliwell (1979) reported that root color did not affect the $^{32}$P uptake rate in birch and sycamore seedlings. It is well recognized that mycorrhizae can greatly enhance the uptake of P from the rooting environment (e.g. Jones et al., 1999, 1990; Cress et al., 1979), though this ability varies significantly with mycorrhizal species (Van Tichelen and Colpaert, 2000; Cumming, 1996; Mejstrik and Krause, 1973; Langlois and Fortin, 1978). Accordingly, the presence or absence of mycorrhizae and their colour were noted for each fine root. Mycorrhizae were typically restricted to the slower growing lateral roots since the fine roots were
collected during a period of active growth. Mycorrhizal colour or frequency did not appear to vary with treatment or installation and will not be considered further.

The uptake of $^{32}$P was determined in each of 630 individual fine roots. Roots from a particular tree were not paired to form composite sample representing that tree. Though this would have reduced the number of samples for which uptake was measured, the within-tree variation with respect to uptake was not known prior to the experiment. The uptake values for individual roots for a given tree were averaged to obtain a single measure of the trees "demand" for P.

$^{32}$P bioassay procedure

The measurement of $^{32}$P uptake was undertaken within 36 hours from the time of initial collection. Pennell et al. (1990) had reported that $^{32}$P uptake in excised roots of loblolly pine remained unchanged when measured at 5, 26 and 50 hours following collection.

The procedure of Harrison and Helliwell (1979) was adopted with minor modifications. Roots were first placed in a primer solution containing 0.5mM calcium sulfate at 18°C for a period of 30 minutes. The roots were then transferred to the labelled solution containing 0.5mM calcium sulfate, 0.005 mM KH$_2$PO$_4$, and approximately 20uCi/l of $^{32}$P. Roots remained in the labelled solution for a period of 15 minutes and the temperature of this solution was maintained at 20°C. The roots were washed under running tap water immediately after removal from the labelled solution for approximately 5 seconds. The roots were then dipped in a room temperature solution of 0.5 mM calcium sulfate and 0.005mM KH$_2$PO$_4$ and subsequently placed in a cold shock solution consisting of 1mM KH$_2$PO$_4$ at 4°C for a period of 5 minutes (Topa and Cheeseman, 1993; Lefebvre and Glass, 1982). The steps following the removal from the labelled solution were intended to remove $^{32}$P from the root surface and Donnan free space, i.e. to remove the P that had not been actively transported across cellular membranes. These steps were viewed as being critical to ensure that the $^{32}$P measured in the root sample was a true measure of uptake and not partially a measure of surface contamination.
The roots were dry-ashed at 450°C using a muffle furnace located in the Department of Botany. Approximately 15 ml of distilled water was added to the ash and counts were obtained using a liquid scintillation counter located in the Department of Animal Science. Counts were corrected for decay, weights, and solution activity.

Following the recommendation of Dighton and Harrison (1983), an additional sub-sample of the roots was pretreated with a metabolic inhibitor to ensure that the bioassay procedure was a true measure of active uptake and not significantly affected by the adsorption of $^{32}$P within the root free space. The roots were pre-treated in a solution of 5mM potassium cyanide for a period of 2 hours. Following this pre-treatment, the rate of $^{32}$P uptake in these roots was determined as noted above. Uptake in these roots (results not shown) was reduced by approximately 90% confirming that the measured uptake was the result of active uptake.

**Determination of nutrient P concentrations**

The fine roots collected were analyzed for P concentrations using standard analytical techniques described in Chapter II.

**Statistical Analysis**

The results were analyzed by ANOVA using a SAS General Linear Model (GLM) for a balanced design. Installation, tree and treatment were each considered fixed variables, therefore, Zar’s (1984) ANOVA type I was used for the analysis of data. A matrix of Pearson correlation coefficients was determined for root and foliage nutrient content and uptake. Means were compared by Duncan’s multiple range test.

Twelve roots collected from trees representing the control and N treatments had levels of uptake markedly lower than other within-tree replicates or other control or N-treated trees. Indeed, their uptake values were in the range of roots that had been treated with cyanide. An assumption was made that these low values were indicative of dead roots that had not been previously identified and the uptake data for these samples were discarded.
Results

The rate of $^{32}$P uptake varied significantly with treatment and installation (Table 3.1). Most notably, the N treatment resulted in a significant increase in the rate of $^{32}$P uptake in roots collected from the Sechelt, Port Alice #1 and Eve River installations (Table 3.2). For example, the rate of uptake in roots collected from the N-fertilized trees at the Eve River installation was increased by a factor of 73% over that of similar roots collected from the control trees. In contrast, the rate of uptake at the Port McNeill #1 installation decreased slightly following fertilization, while the rate of uptake at the remaining four installations was not affected by N additions. The additions of P, at either of the application rates, resulted in a strong reduction in the rate of uptake (Table 3.2).

Rate of uptake in the N-fertilized trees did not appear to be related to the three-year basal area increment (Figure 3.4). One would have expected that the rate of uptake would be greatest in trees that had limited response to N additions if uptake was a predictor of N response. Clearly, this was not the case. Furthermore, the Zeballos installation appeared to respond strongly to N-only additions (Chapter II) but these trees also have the highest rate of uptake in roots collected from the control trees at this installation (Table 3.2).

The fact that the three-year basal area increment measurement failed to demonstrate that any of the eight installations responded to P additions (Chapter II), prevents an objective test of the relationship between uptake and response to P additions. The increased rate of P uptake following N additions at the three installations noted above would also seem to suggest that N fertilization of these installations resulted in a condition of “induced deficiency” of P. However, none of these three installations showed any evidence of a response to P additions (Chapter II).

The concentration of total P (%) in the fine roots varied significantly with both installation and treatment (Table 3.1). Concentrations of P were greatest in roots that had received the addition of P at the rate of 500 kg/ha and were lowest in the two treatments that did not receive P additions (Figure 3.5).

Concentrations of total P (%) in the current-year foliage collected within weeks of the root collection were weakly correlated ($r = -0.51$) with rate of uptake (Figure 3.6). Uptake increased in the majority of fine roots below a threshold of 0.18%. Similarly, the
P:N ratio was weakly correlated (r = -0.53) with uptake (Figure 3.7). Uptake appeared to increase below a P:N ratio of 0.17.

The inorganic fraction of total P had not been determined in the current-year foliage collected at the end of the second growing season (i.e. at the time of fine root collection). As the role of this metabolically active fraction in regulating uptake is of interest, the size of this fraction in the foliage was calculated by regression using the values determined in foliage that had been collected in the previous year (Chapter II). The relationship between the calculated PI fraction and uptake is provided in Figure 3.8 and a general trend is apparent (r = -0.51). Below a threshold of 0.06%, uptake increases dramatically. Root P concentration was poorly related to uptake (Figure 3.9).
Table 3.1. Uptake of $^{32}$P and P concentration of roots: F ratios and levels of significance. Significance levels: *, 5%; **, 1%; ***, 0.1%; ns, not significant.

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<th>Source of Variation</th>
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<th>Root % P</th>
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<tr>
<td></td>
<td>***</td>
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<td>ns</td>
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</table>
Table 3.2. $^{32}$P uptake (nmoles/mg/hr) in excised fine roots and SE by installation and treatment. Each value is the mean of six individual trees. Means with the same letter are not significantly different at the 5% level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sechelt</th>
<th>Port Alice #1</th>
<th>Port Alice #2</th>
<th>Eve River</th>
<th>Port McNeill #1</th>
<th>Port McNeill #2</th>
<th>Nimpkish</th>
<th>Zebellos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>79 (4) a</td>
<td>81 (11) a</td>
<td>93 (5) a</td>
<td>105 (5) a</td>
<td>152 (8) a</td>
<td>126 (7) a</td>
<td>117 (7) a</td>
<td>148 (8) a</td>
</tr>
<tr>
<td>N (225 kg/ha)</td>
<td>119 (9) b</td>
<td>117 (10) b</td>
<td>94 (8) a</td>
<td>182 (9) b</td>
<td>121 (8) b</td>
<td>135 (8) a</td>
<td>104 (9) a</td>
<td>164 (8) a</td>
</tr>
<tr>
<td>N (225 kg/ha) +P 15 (1) c (100 kg/ha)</td>
<td>36 (5) c</td>
<td>26 (4) b</td>
<td>35 (2) c</td>
<td>46 (7) c</td>
<td>46 (5) b</td>
<td>41 (8) b</td>
<td>59 (8) b</td>
<td></td>
</tr>
<tr>
<td>N (225 kg/ha) +P 13 (1) c (500 kg/ha)</td>
<td>36 (5) c</td>
<td>40 (4) b</td>
<td>37 (3) c</td>
<td>21 (4) c</td>
<td>18 (2) c</td>
<td>15 (2) b</td>
<td>27 (3) b</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.4. Relationship between mean uptake in N fertilized trees at each of the eight installations and the relative basal area response to N only additions.
Figure 3.5. Mean P concentration (%) in fine roots by treatment. Each value is the mean of 48 determinations. Means with the same letter are not significantly different at 5%.
Figure 3.6. $^{32}$P uptake in excised roots as a function of the total P concentration in current-year foliage, each collected in the fall of 1996, i.e. at the end of the second growing season ($r = -0.51$). Values based on a total of 192 individual trees. Note the decrease in uptake as P concentrations in the foliage increase above 0.18%.

Figure 3.7. $^{32}$P uptake in excised roots as a function of the P/N ratio in the current-year foliage ($r = -0.53$). Values are based on a total of 192 individual trees. Note the increase in uptake as the P:N ratio decreases below a value of approximately 0.17.
Figure 3.8. $^{32}$P uptake in excised roots as a function of the concentration of inorganic P in current-year foliage ($r = -0.51$). Values are based on a total of 192 individual trees. Note the increase in uptake as the Pi concentration in the foliage decreases below a value of approximately 0.06%.

Figure 3.9. $^{32}$P uptake in excised roots as a function of the total P concentration in the root ($r = -0.42$).
Discussion

The rate of P uptake did not accurately predict the growth response to fertilization. There are several possible explanations for this conclusion. The first problem that may mask a connection between uptake and basal area increment is the fact that in the present study a three-year response period was utilized. Since an increase in crown biomass precedes stem response, a six-year response period might have been a superior indicator of each tree's response. Another possibility is that basal area response to P additions was prevented or limited by another nutrient deficiency. However, the blend treatment did not seem to have any effect on growth. It cannot be dismissed though, that N concentrations in the present experiment were only moderately increased. It may be possible that the N application at 225 kg/ha was not sufficient to eliminate N as a growth-limiting factor in these stands and a P response might have been more evident had the N addition been increased. An additional source of variation may have been due to the assumptions originating from the sampling procedure. The fine roots were collected from within a three-meter radius of the sample tree and were assumed to have belonged to that sample tree. It is possible that some of the roots may have originated from a neighbouring tree. Yet another consideration that must be considered is the fact that samples were only collected at a single time during the growing season which may, or may not, have been during a period of high P demand from the soil environment (McDonald et al., 1991). Notwithstanding these concerns, it may be argued that the most likely explanation for the lack of correlation between uptake and basal area increment is that western hemlock's response to P deficiency is complex and not limited to the regulation of the P uptake apparatus in fine roots.

Lynch and Brown (1998) recently noted low P availability during the evolution of higher plants has given rise to a wide array of plant responses to overcome or cope with periods of P deficiency. As documented in the present investigation with western hemlock, one such mechanism is to increase the P uptake ability of fine roots on a fresh weight basis. However, higher plants have also developed a wide array of mechanisms, which may also be induced concomitant to increased uptake ability.
Perhaps the most documented adaptive response of plants and trees to P deficiency is an increase in root growth at the expense of shoot growth (Romer and Fahning, 1998; Topa and Sisak, 1997; Bates and Lynch, 1996; Baon et al., 1994; Romer et al., 1988; Foehse and Jungk, 1983; and Schenk and Barber, 1979) as well as changes in root architecture (Lynch and Brown, 1998; and Bonser et al., 1996). Increased root growth increases the exploration of the soil volume by the plant and increases the surface area of the root system, which serve to enhance P acquisition. Changes in root architecture are thought to be important in reallocating fine root growth to the upper portions of the soil profile, particularly to within the forest floor of forest soils. Increased soil exploration is also accomplished under conditions of P deficiency by an increase in the colonization of fine roots by mycorrhizal fungi, which can greatly increase P uptake at the expense of C (e.g. Cumming, 1998). It is also well recognized that many plants and individual genotypes increase P acquisition by accessing fixed P or organic sources of P (Po) through the production and secretion of organic acids (Keerthisinghe et al., 1998; Johnson et al., 1994; Jungk et al., 1993; Hoffland, 1992; Hoffland et al., 1992, and Fox et al., 1990) and acid phosphatase (Richardson et al., 2000; Wasaki et al., 1997; Ascencio, 1997; Tadano et al., 1993). Plaxton (1998) and Duff et al. (1994) noted that acid phosphatases can also serve as internal scavenging agents for Pi from P-esters and may be particularly important in scavenging Pi from P-choline contained within xylem. An equally important mechanism to relieve P-deficiency stress may be the utilization of alternative metabolic pathways (Plaxton, 1998).

Nutritional Implications

Uptake in the fine roots appeared to increase when the foliar P concentration was reduced below a concentration of 0.18% and a foliar P: N ratio of approximately 0.15. These values are suggested to represent the critical concentrations for western hemlock, a concentration below which the uptake apparatus is enhanced in this species. These biologically-based values for P requirement are in general agreement with those of Ballard and Carter (1983).
How Do Fine Roots of Western Hemlock Regulate P Uptake?

The fine roots of western hemlock clearly have the ability to regulate P uptake from the rooting environment; enhancing uptake when P was supposedly growth or function limiting, and reducing its uptake when the P status of these trees had been presumably improved by previous P fertilization. The ability of hemlock, and presumably that of other conifers, to coordinate the presumably complex mechanisms that involve P uptake raises a number of interesting questions. Morgan and Drew (1997) recently discussed the perception and signal transduction of plant stresses, nutrient or otherwise, in the context of the hormone ethylene. They noted that a plant must first be able to detect and quantify the nature of a stress such as P deficiency. The plant must then be able to communicate the nature and severity of the stress to a receptor located in the region of the plant where the plant's response is observed. Finally, once the receptor has received the message, the observed response must be induced. In the present investigation, the question that one must ask is how does western hemlock detect and quantify its P status? How does this species, and conifers in general, communicate this status to the cells at the soil/root interface where uptake is ultimately enhanced, and how do fine roots actually increase the rate of P uptake from their soil environment?

How is Uptake in Fine Roots of Western Hemlock Increased?

Advances in molecular biology have allowed for the study of the genetic basis for many aspects of plant and tree nutrition including the regulation of P uptake. From this recent body of research it has become evident that regulation of P uptake is the result of genes whose expression is enhanced when Pi becomes limiting, many of which encode proteins that directly, or indirectly, enhance the synthesis of high-affinity Pi transporters in the fine roots.

Poirier et al. (1991) undertook a molecular based study to further the understanding of how P uptake is regulated and their findings are particularly notable. These researchers investigated the effect of a specific mutation in Arabidopsis (Arabidopsis thaliana), identified as being at the pho1 locus, on growth, P uptake and P
translocation from root to shoot. Mutant plants had reduced growth, a four-fold reduction in total P content, and exhibited visual symptoms indicative of P deficiency. Interestingly, root uptake rates of P were not affected by the mutation. However, the translocation of P from the root to shoot was greatly reduced in mutant plants. The wild plants translocated 35% of the P to the shoots whereas the mutant plants translocated only 0.9%. Furthermore, Pi transport through the hypocotyl did not differ between the wild and mutant plants. The researchers concluded that the mutation resulted in a defect in a Pi specific transporter, most likely located in the xylem parenchyma cells. Also noteworthy was the finding that the reduction in translocation, attributed to the mutation, could be overcome if the mutant plants were grown with a supply of high P concentrations. This finding indicated that the mutation was most likely restricted to a high affinity P transporter and did not affect a second low affinity transport system supposedly located in the xylem parenchyma cells.

Burleigh and Harrison (1998) were able to demonstrate that the gene identified as GvPT encoded a functional P transporter in the hyphae of the arbuscular mycorrhizal fungi Glomus versiforme. They speculated that a second gene was responsible for regulating P efflux from the mycorrhiza fungus to the host. These authors also identified two genes (MtPT1, MtPT2) in the legume Medicago truncatula as being responsible for the regulation of the P transporters which were thought to be responsible for P uptake from the soil and perhaps may have been involved in regulating xylem-phloem loading. The expression of these genes was induced with P starvation in the roots but not in leaves. An additional gene (Mt4) was identified in M. truncatula, which was downregulated following either P starvation or, interestingly, the mere colonization of the root by arbuscular mycorrhizae.

More recently, Dong et al. (1999) investigated the relationship between the expression of two phosphate transporter genes (APT1 and APT2) in the roots of Arabidopsis, which had been earlier isolated by Smith et al. (1997), with P uptake, and internal P concentrations. The plants were grown in full-strength nutrient solution for 23 days and then transferred to a P-deficient solution for a period of 7 days. During the latter period, the inorganic fraction of P decreased dramatically in both the root and shoot tissue. Within four days of the interruption of P supply the relative expression of the two
genes increased five-fold. Phosphorus uptake increased during this period, but there was evidence of a two-day lag following the increase in gene expression until which time P uptake was increased. The authors argued that the latter was evidence of the need for an increase in the synthesis of transport proteins prior to the observed increase in uptake. When the plants were re-supplied with P, the inorganic P concentrations in the shoots increased significantly during the first day which coincided with a 70% reduction in the relative expression of the two genes for the same 24-hour time period. Two additional genes thought to control P uptake in arabidopsis, identified as PHT2 and PHT3 were reported by Mitsukama et al. (1997).

Daram et al. (1998) reported that a gene (LePT1), isolated from a root hair cDNA library, and thought to be responsible for encoding H⁺/Pi co-transporter in tomato plants, increased the uptake of P in a yeast mutant which was defective in a high-affinity Pi transport. When the yeast was grown in the presence of m-chlorophenylhydrazone (CCCP), which reduces or eliminates the pH gradient across plant membranes, uptake was reduced to 2% of the control levels. The addition of vanadate, an inhibitor of H⁺-ATPases, reduced uptake by 40%. The authors concluded that the H⁺/Pi co-transporter relied on a pH gradient across the yeast cell membrane that was maintained by a plasma membrane H⁺-ATPase.

Liu et al. (1998) also investigated the genes LePT1 and LePT2 in tomato plants grown under a range of P supplies. As reported by Daram et al. (1998), the expression of LePT1 was greatly increased under conditions of P deficiency, as was LPT2. Interestingly, the expression of LePT1 was increased not only in root tissue but also in the stem, petioles and foliage, whereas LePT2 was only evident in root tissue. Similar to the recent findings of Dong et al. (1999) and Liu et al. (1997), the activity of these genes was increased within 24 hours of inducing P deficiency, and their expression was maximized after 5 days. Gene expression was greatly decreased within 24 hours of the re-supply of P. Also noteworthy, is the fact that the activity of the two genes was increased when the supply of P was 100 μM or less and further, that LePT1 was more sensitive to P supply concentrations than that of LePT2. This finding that gene expression in tomato is closely related to P supply is in agreement with the findings by
Liu et al. (1997). In the latter experiment, the gene TPS11 in tomato plants was induced at P concentrations of 50 μM or less.

Liu et al. (1997) suggested that the degree of gene expression over a range of P supplies might be useful in the identification of P-efficient plants. Though P specific genes have yet to be identified in western hemlock, the concept of using genetic markers to identify P-efficient genotypes is most interesting and could be expanded to include other elements. Molecular approaches have yet to be applied to the study of western hemlock nutrition or to the study of tree nutrition in general. While the response of hemlock and other conifers to P deficiency is complex, research into identifying genes that regulate P uptake in hemlock, and their relationship to P supply, may offer an alternative approach to understanding the P requirements of this and other species. It should be cautioned however, that each of the molecular studies noted above consisted of the use of plant material that was exposed to short-term P deficiency. In the present investigation, it would have been expected that where a condition of P existed, this condition would have been pronounced for a period of time exceeding one growing season, and in many cases, for the duration of their life. Conifers may well have different genes that respond to short-term as opposed to long-term P stresses.

Delhaize and Randall (1995) alluded to the fact that there remains much genetic-based research to be undertaken in additional areas of P nutrition. This would of course include mechanisms related to P use efficiency, including translocation of P from root to shoot and retranslocation within shoot biomass, as well as in the area of intra-cellular P transport. Perhaps of more immediate importance, however, we still do not know what factor(s) (i.e. foliar P concentrations, root P concentrations) regulate the expression of genes, which in turn, control P uptake in western hemlock.

It is interesting to note the similarity in the molecular composition of the various proteins regulated by the genes noted above. For example, the P transporters in the arbuscular mycorrhizal fungi Glomus versiforme (Burleigh and Harrison, 1998), tomato (Daram et al., 1998; Liu et al., 1998), Arabidopsis (Smith et al., 1997), potato (Leggewie et al., 1997) and Catharanthus roseus (Kai et al., 1997) are thought to be composed of integral membrane proteins composed of 12-membrane spanning regions which are separated into two distinct groups by a single large hydrophilic charged region.
Raghothama et al. (1998) concluded that the functional and structural similarities in P transporters across the life forms cited above indicates that these genes have been highly conserved through evolution. Indeed, it may be argued that since P concentrations in soil solution are very low, particularly in the region immediately surrounding fine roots, the low affinity system in many of our conifer species might be argued by some to be an evolutionary remnant. In this regard, it is interesting to speculate that the only time in the life of these western hemlock trees that they were able to utilize their low affinity transport system to any significance for P acquisition may have only been during the months immediately following P additions.

How is P Status Perceived and Quantified in Western Hemlock?

Since P deficiency can elicit numerous plant and tree responses, many of which represent considerable carbon costs, it is imperative that both plants and trees be able to accurately identify their respective P status. The latter, in this case, would represent a relative measure of current P reserves versus current demand and possibly, future anticipated requirements. To understand how this status is perceived, one has to first identify where the perception mechanism is located (i.e. whole plant or root level), and secondly, how P status is actually perceived.

Delhaize and Randall (1995) reported that a mutation at the locus designated pho2 in Arabidopsis resulted in the accumulation of Pi in the foliage to levels considered toxic. They suggested that the mutation might have deregulated the control that would have been normally exerted by the plant in the maintenance of Pi concentrations in the shoot. This was thought to be due to a Pi transporter, located in either the root or shoot, that fails to respond to high Pi concentrations in the shoot and continues to transport Pi beyond the requirements. The defective gene may have been responsible for regulating a Pi transporter or for encoding a protein that somehow senses the Pi concentration, and in turn, regulates the activity of a Pi transporter.

Liu et al. (1998) were also interested in the question as to the origin of the signals that regulate uptake. In their study of gene expression in tomato plants, these researchers undertook a split root experiment in which one half of the root system was supplied with
P and the second half grown without. There was no difference in the expression of the LePT1 and LePT2 gene in either root system, nor was the expression of these two genes different from that in plants grown entirely with an adequate supply of P. Liu *et al.* (1998) and Raghothama *et al.* (1998) concluded that the signals for P uptake are most likely linked to changes in the internal P status of the plant as a whole, and further, that adequate foliar concentrations of P repress phosphate-induced gene expression in the entire root system.

In many of the studies noted above, induced P deficiency increased the expression of genes not only in root tissue but also commonly in stem and foliage tissue (*e.g.* Liu *et al.*, 1998; Poirier *et al.*, 1991; Leggewie *et al.*, 1997; Liu *et al.*, 1997; Howard *et al.*, 1998). The fact that gene expression is not restricted to roots may be argued as evidence that the plant’s response to P deficiency is indeed complex and not under the exclusive control of fine roots. Cogliatti and Clarkson (1983) also investigated whether P uptake was controlled at the root or whole plant level using a split-root approach with potato plants. They found that uptake was related to the general P demand for the plant as a whole, and not related to the nutritional status of the root in which uptake was measured. This finding is in agreement with the molecular studies cited above, carried out some fifteen years later.

In sharp contrast to the above studies, Mimura *et al.* (1998) reported that neither cytoplasmic nor vacuolar Pi concentrations were involved in the regulation of Pi uptake in *Chara corallina*. These authors argued that uptake rate was determined by the supply of P in the external medium which regulates the induction or repression of genes responsible for the synthesis of Pi transporters located within the plasma membrane. This proposed model for the regulation of Pi uptake would allow for uptake to be up-regulated in areas of the soil where P was in high supply. This finding, though interesting in the context of spatial variability of P supply in forest soils, is not in agreement with the findings of Cogliatti and Clarkson (1983), nor with the molecular based studies noted above.

Lefebvre and Glass (1982) proposed that uptake in barley roots was better correlated with the organic-P fraction in the roots and that changes in this fraction signal the transport system to regulate uptake. While they would seem to suggest that uptake is
regulated entirely at the root level, they did caution that shoot influences, perhaps via hormone production, should not be overlooked.

Uptake in the present investigation was weakly correlated with both the concentration of P and the P/N ratio in current-year foliage, conventional indicators of P status. This finding would seem to indicate that the regulation of uptake of P in the roots is related to the P status of the foliar biomass, i.e. regulated at the crown or whole tree level. While it is inviting to conclude that the mechanism is crown-based, the precise mechanism that may be involved has not yet been identified. It would also be inviting to speculate that a critical concentration of inorganic-P within the cytoplasmic compartment may be somehow involved in this regard (Liu et al., 1998), but there is no firm evidence at this time to support this idea. However, as noted by Dong et al. (1999) and Metzenberg (1998), the fact that the cytoplasmic fraction of Pi is relatively stable, at the expense of the vacuolar compartment (Chapter V), would appear to make this feedback mechanism less tenable. Dong et al. (1999) speculated that perhaps P uptake in the roots was mediated by an intermediate of P metabolism that responds to Pi concentrations within the vacuolar compartment, but this intermediate product has yet to be identified.

The above studies relating to the perception of P status may imply that this is accomplished by a single mechanism. This may not be the case. Indeed, as the P demand changes over the course of a growing season, a number of factors may be involved in perceiving P status and their relative importance in this regard may change with the season. While there seems to have been much interest in speculating that the cytoplasmic Pi pool is involved in regulating uptake, this hypothesis does not appear to have been critically tested. In this regard, the experiment of Heldt et al. (1977) is interesting to note. These investigators supplied plants with mannose to reduce Pi concentrations in the cytoplasm through phosphorylation, which reduces Pi, but not total P. Artificially regulating cytoplasmic Pi and the simultaneous measurement of uptake through a time series depletion experiment may further our understanding of the relationship, if any, between this metabolically active pool and the regulation of P uptake. It should be cautioned, however, that while researchers have noted that the cytoplasmic pool is a relatively stable fraction, as a static parameter, it may not be the most useful reflection of a tree's internal demand for P. The latter may be in constant flux, both in
terms of magnitude, but also in terms of a changing sink (i.e. shoot demand versus root demand). It may prove that future studies into the regulation of P uptake must involve some consideration of this internal demand. In this regard, it is interesting to hypothesize that uptake is partially regulated by protein sensors located at the point of phloem loading. The latter would represent the above ground fraction of P that exceeds the current demand for P by the crown and that would be available for phloem transport. Alternatively, xylem-based transporters located in either the roots or foliage, might regulate uptake by being in position to measure the rate of P flux to vegetative tissue, an indirect indicator of current demand. Undoubtedly, future uptake studies that incorporate both conventional and molecular-based approaches will further our understanding of the factors that regulate P uptake.

**How are Signals for Increased P Uptake Recognized and Transduced in Western Hemlock?**

Borch *et al.* (1999) noted that the ability of plants to concentrate root growth in areas of high P availability suggests that plants possess a signaling mechanism to regulate changes in plant growth. Similarly, the ability of western hemlock to regulate uptake following changes in nutrient supply or internal P status also implies that a signaling mechanism must be involved. If the signal originates in the foliage, as argued above, then this signal must somehow be transported to the root, presumably via the phloem, where the signal is received and the desired effect upon the uptake apparatus is effected. As emphasized by Raghothama *et al.* (1998), signals for enhanced uptake must be tightly regulated in plants and trees growing under conditions of nutrient limitation in order to conserve valuable carbon reserves. While the signaling mechanisms that are involved in communicating this nutrient stress are unknown at this time, they have been the subjects of recent research.

Lynch and Brown (1998) recently posed the question as to whether ethylene production in fine roots, growing under a condition of P deficiency, might represent one mechanism that triggers changes in root growth. Borch *et al.* (1999) investigated the effect of ethylene on the growth response of the roots of bean (*Phaseolus vulgaris* L.)
plants to P deficiency. They reported that P-deficient roots produced twice as much ethylene per unit weight of dry matter as P-sufficient plants. They also reported that ethylene promoted main root extension but reduced lateral root development, a response that would aid in the exploration of soil for areas of high P availability. Interestingly, the authors reported evidence to suggest that P-deficiency also increased the sensitivity of these roots to ethylene. Drew et al. (1989) reported that P-deficiency in maize might have resulted in an increased susceptibility of roots to ethylene. However, in contrast to the findings by Borch et al. (1999), ethylene production was decreased in roots of maize plants when P supply was withheld though the latter induced the formation of aerenchyma close to the root apices. In a follow-up study using maize plants, He et al. (1992) confirmed that P deficiency increased the sensitivity of roots to ethylene and further, attributed the aerenchyma noted in the earlier study to ethylene. While there is strong evidence to conclude at this time that ethylene has a role in altering root development and is linked to P deficiency, its role in regulating rates of P uptake, as in the present investigation with western hemlock, has yet to be investigated. In their recent review, Lynch and Brown (1997) wondered if ethylene could be involved in the development of ectomycorrhizal associations in soils of limited P supply. This question also has yet to be fully explored. Bates and Lynch (1996) reported that root-hair production in Arabidopsis was partially due to auxin production under conditions of P deficiency.

Malboobi and Lefebvre (1995) studied differential gene expression in Brassica nigra suspension cells. They reported that P deficiency resulted in a significant increase in the synthesis of the enzyme β-glucosidase but its precise role, if indeed any, during P deficiency has yet to be clearly identified. Malboobi et al. (1998) later speculated that β-glucosidase might be involved in the regulation of phosphatases, which in turn, may be involved in the ameliorative response to P deficiency.

There has been interest in understanding the molecular mechanisms governing gene expression involved in regulating the plant’s response to P deficiency, including that of increased uptake. Much of this research has been directed towards the study of both transcriptional regulatory mechanisms and transcription binding sites, though this research is considered to be in its early stages (Malboobi et al., 1998). The latter authors
have proposed a model concerning the synthesis of β-glucosidase. Synthesis of the enzyme is thought to be down-regulated in the presence of adequate P by repressors which bind to the gene's DNA and cause either a reduction or inhibition of the gene's expression. When P is deficient, activators bind to cognate sites on the gene's DNA and induce or enhance the expression of the gene, and hence, cause an increase in the synthesis of the enzyme. Burleigh and Harrison (1998) have proposed that regulators have a central role in driving the expression of genes that control the plant's response to P deficiency and also in monitoring the effectiveness of this response. Walker (1998) proposed that receptor-like protein kinases (RLKs) might have a role in signal recognition and transduction, including those concerned with P deficiency, but that further research in these areas is required.
Conclusions

1) $^{32}$P uptake in excised roots offered little utility in identifying stands of western hemlock that would respond to either N or P additions.

2) Previous additions of N increased P uptake only in roots collected from three of the eight installations, indicating that the P uptake apparatus in these roots was up-regulated in the years following N fertilization. The latter was attributed to the synthesis of an increased number of P-transporters in these fine roots.

3) The previous addition of P, at either 100 kg/ha or 500 kg/ha, significantly reduced the rate of P uptake indicating that the improved P status of these trees resulted in a strong down-regulation of the P uptake apparatus in these roots.

4) Reductions in P uptake in trees that had earlier received P additions was attributed to a reduction in the number of P-transporters in the fine roots. The latter was most likely the result of a reduction in the number of P-transporters that were synthesized in the fine roots, which had developed during the two years following fertilization.

5) The precise physiological mechanisms that regulate uptake in these western hemlock trees are not known. They are thought to involve a series of complex mechanisms that include those responsible for the perception and quantification of P stress, transduction of this message to the fine roots, and finally, the regulation of the uptake apparatus. Many of these mechanisms are thought to be regulated by gene expression, which, in turn, may be regulated directly, or indirectly, by Pi concentrations within the tree.

6) $^{32}$P uptake was weakly correlated with both the total P (%) and Pi (%) in the current-year foliage, indicating that the perception portion of the above sequence of events that regulates P uptake may be based in the crown, but again, the precise mechanisms remain unknown at this time.
7) Much has been recently learned regarding P nutrition via the rapid advances of molecular-based science. Future studies into tree nutrition should encourage the incorporation of this approach to compliment the more conventional approaches.
CHAPTER IV
PHOTOSYNTHETIC AND NUTRITIONAL RESPONSE OF A 5-YEAR-OLD WESTERN HEMLOCK PLANTATION FOLLOWING FERTILIZATION

Introduction

White et al. (1999) reported that fertilization of an 18-year-old stand of western hemlock and Douglas-fir resulted in a strong reduction in carbon isotope discrimination in current-year foliage during each of three years following fertilization. Nitrogen additions had the greatest effect on reducing discrimination, but the addition of P at each of two application rates also had a significant effect. White et al. (1999) argued that the reduction in discrimination indicated that the improved nutritional status increased the photosynthetic rate in fertilized trees. The researchers concluded that the gain in photosynthetic rates was, concomitant with an observed increase in leaf area, a significant factor that contributed to the observed three-year growth responses.

The relationship between carbon isotope discrimination and the nutritional status of conifers, particularly that as affected by P nutrition, has received only limited attention by researchers in the field of tree nutrition. Furthermore, while increased photosynthetic rates in conifers following N fertilization has been well documented, the effect of P nutrition on this process has not been as widely studied. Indeed, a study of the effect of P nutrition on photosynthesis rates per leaf unit area in western hemlock would also assist in understanding the P requirement for this species.

As noted by Pessarakli (1997), photosynthesis is one of the most spectacular processes within the fields of tree and plant physiology, which are concerned with productivity and growth. Carbon fixed through the process of photosynthesis is the basis for growth, energy transduction, and amino acid and protein synthesis. Indeed, the subject of photosynthesis is perhaps the most studied field in plant and tree physiology. Wilson (1981) introduced the concept of incorporating light capture and utilization as components of crop growth analysis. Essentially, crop growth rate is the product of (1) incident photon flux density, (2) the light intercepting efficiency, and (3) the light utilizing efficiency. Similarly, researchers in the field of tree nutrition have long been interested in the relative contribution of increased foliage efficiency to increased growth
following forest fertilization. Helms (1964) studied the effect of N fertilization on photosynthetic rates during the first growing season following treatment in a 38-year-old Douglas-fir plantation growing in the state of Washington. He reported that photosynthetic rates in fertilized trees were not increased over those of control trees during the spring or summer months. A significant increase in photosynthetic rates was observed in fertilized trees during autumn measurements. Nevertheless, the author concluded that future observations of diameter growth would be the result of increases in foliage biomass and not due to an increase in needle efficiency. Unfortunately, the author did not provide the results of foliar analysis, which might have improved the interpretive value of the results. Fagerstrom and Lohm (1977) reported that the principal mechanism of increased growth for Scots pine was increased leaf area. In a more limited study, Brix and Ebell (1969) reported that N additions to a 20-year-old Douglas-fir stand had no effect on photosynthetic rates despite that fact that both N and chlorophyll concentrations were significantly higher in fertilized trees. However, the photosynthetic measurements were only determined in the summer and fall of the second growing season following fertilization. While working in the same stand as Brix and Ebell (1969), Brix (1971) reported that N fertilization resulted in a small, but significant, increase in photosynthetic rates in current-year needles during the first two growing seasons following fertilization. Brix (1972) and Kellomaki et al. (1982) have reported similar results in Douglas-fir and Scots pine (Pinus sylvestris L.) respectively. A more dramatic effect of nutrient additions on photosynthetic rates was provided by Keay et al. (1968) for a 14-year-old Pinus pinaster stand in western Australia. They reported that N and P fertilization resulted in a two-fold increase in photosynthesis during a six-month period following fertilization. Sheriff et al. (1986) investigated the effect of nitrogen and phosphorus additions on a seven-year-old radiata pine plantation in Australia using a factorial experiment. They reported that, though conventional nitrogen additions were effective in increasing the foliar N concentrations from a mean of approximately 0.96 % to that of above 1.22 %, these additions had little effect on the photosynthetic rate. However, interestingly, the addition of P alone at a rate of 80 kg/ha increased the foliar P concentrations from a control concentration of approximately 0.07% in control trees to above 0.11%.
Concomitant photosynthetic rates were increased by a factor of two over that of the control trees following the P additions.

While the above studies into the relationship between tree nutrition and photosynthesis are noteworthy, several researchers have expressed that their findings be interpreted with caution. For example, Linder and Rook (1984) noted that in situ measurements of photosynthetic rates in forest stands following silvicultural treatments are limited by the fact that such studies typically employ measurements that are taken over a period of a relatively few days and usually only include the current-year age class of foliage. Unfortunately, more comprehensive field studies that entail measurements, well replicated throughout the entire growing season and across a wide range of environmental conditions, are usually not feasible. Physiologically based studies are also of somewhat limited value in furthering our understanding of the origin of gains in yield as they typically are limited to studying only a small component of a complex system (Brand et al., 1987). The latter investigators used the relative production rate and unit leaf production to understand the factors responsible for changes in yield following spacing in red pine and brush control in a Douglas-fir plantation. Brix (1983) had previously used unit leaf production per foliage area (E) in understanding the effects of thinning and fertilization on stemwood production in a Douglas-fir stand. While growth analysis has shown to be a promising tool to understanding how plants and trees respond to changing environments, it has not been widely applied in forestry studies due to the extensive data collection required.

An alternative approach to the understanding of the effect of nutritional status on the photosynthetic apparatus of plants and trees is the examination of changes in leaf ultrastructure across a range in nutritional status. As noted by Hecht-Buchholz (1983), changes in cell structure and anatomy caused by changes in the nutritional status of plants have improved the diagnosis of nutritional status but more importantly, have led to a further understanding of the role of nutrition in plant metabolism. For example, Vesk et al. (1965) conducted an extensive study into the effects of various nutrient deficiencies on changes in chloroplast structure in tomato, spinach and maize plants. Similar studies into the effects of nutrition on chloroplast structure have also been reported by numerous investigators (e.g. Homann, 1967; Whatley, 1971; Hall et al., 1972; Spiller and Terry,
Several investigators have also used microscopy to examine potential differences amongst genotypes with respect to chloroplast ultrastructure which in turn, may be evidence of adaptive mechanisms under conditions of mineral stress (Hecht-Buchholz, 1983). For example, Laza et al. (1993) investigated the differences in chloroplast size, number of grana and starch grains per chloroplast, and stroma density amongst rice cultivars across a range of N treatments and related these observations to differences in growth performance. Similar approaches to investigating differences in chloroplast structure amongst genotypes were reported by Park and Tsunoda (1979), also working with rice cultivars, and by Yelle et al. (1989) while working with tomato species. Rock et al. (1992) used electron microscopy to determine differences in chloroplast ultrastructure amongst different genotypes of arabidopsis. More recently, investigators have used electron microscopy to investigate the possible effects of elevated CO₂ and its interaction with nutritional status (Pritchard et al., 1997; Kutik et al., 1995). Researchers in plant and tree physiology have also used electron microscopy to investigate the effects of low temperature (Nie and Baker, 1991; Nie et al., 1995), simulated acid rain (Bach and Huttunen, 1991; Holopainen and Nygren, 1989; Wulff et al., 1996; Ebel et al., 1990), elevated SO₂ (Karenlampi and Houpis, 1986; Schifffgens-Gruber and Lutz, 1992) and elevated ozone (Miyake et al., 1989) on the photosynthetic apparatus.

Objectives

The primary objective of this experiment was to examine how nutrient additions, and N and P in particular, affected the photosynthetic capacity of western hemlock. This experiment also provided for an additional opportunity to address the question as to what are the nutritional requirements of this species and the question related to the utility of nutrient fractionation in aiding the interpretation of nutritional status of this species. Adopting an experimental approach that integrates growth, nutrition, and physiological parameters in a single investigation may further our understanding of the response mechanisms of hemlock to each of the six fertilizer applications and enhance our understanding of the nutritional requirements of this species.
The specific objectives were:

1) To determine the effect of N, P and blend additions on the photosynthetic capacity of western hemlock where the latter consisted of a determination of photosynthetic rates per leaf area basis, carbon isotope discrimination, chlorophyll a and b concentrations, chlorophyll fluorescence, and an examination of the structure of chloroplasts following fertilization using electron microscopy.

2) To document the effect of N, P and blend additions on the three-year height increment.

3) To determine the effect of N, P and blend additions on the nutritional status of western hemlock as determined by conventional foliar analysis and nutrient utilization as determined by fractionation of total N and P.

The experiment was conducted within a five-year-old plantation located on northern Vancouver Island. The site is typical of many of the cutovers of the area and where growth of newly planted stands is limited by what is believed to be deficiencies in N and P.

Background

The forests of northern Vancouver Island are composed largely of two forest types. The first of these consists of old-growth stands composed of western red cedar and western hemlock, commonly referred to as Ch stands (Figure 4.1). These stands are characterized as being long lived, with individual trees often exceeding 1000 years of age, and have multiple-layered canopies with low to moderate crown closure. They are typically situated in the low-lying areas across the landscape and are generally thought to be more resistant to the catastrophic wind events that are the dominant form of
Figure 4.1. Typical old-growth Ch stand on northern Vancouver Island composed of over-mature western hemlock and western red cedar.
disturbance in this region. The soils are characteristically poorly to moderately well drained and have a thick mor humus indicative of incomplete decomposition. The ground vegetation is dominated by salal (*Gaultheria shallon* Pursh). The second stand type, generally referred to as Ha, is composed of a mixture of western hemlock and amabilis-fir. These stands are typically even-aged and have developed under dense stocking conditions with standing volume often exceeding 1000 m$^3$ at 80 years of age (Figure 4.2). Crown closure is typically very high and the ground vegetation is predominately made up of a variety of mosses owing to the low light conditions in the Ha understory. Soils are typically well drained and have a pit and mound appearance owing to the frequent mixing of soil and humus following windthrow.

While the dominant feature of Ch stands is the lack of disturbance, Ha stands typically occupy upper slope positions across the landscape, which are more prone to catastrophic wind disturbance (Figure 4.3). Most current Ha stands in the region are thought to have regenerated following windstorms earlier in this past century.

During the past two decades, many of these Ha and Ch stands have been clear-cut and subsequently planted with western hemlock and/or western red cedar. Initially, growth of the planted stock met acceptable standards but between the ages of 5 and 10 years of age, the trees planted on sites that had previously supported Ch stands began to enter a period of growth check (Figure 4.4). At this time, the foliage began to take on a chlorotic appearance and subsequent height growth was dramatically reduced. The timing of the growth check and chlorotic foliage coincided with the dense regeneration of the salal on these sites, which had been initially kept in check with prescribed burning prior to planting. In sharp contrast, regeneration established on cutovers that had previously supported Ha stands was not affected by the problem of growth check (Figure 4.4).

Needle chlorosis of western hemlock on Ch sites was clear evidence that the poor growth was in part the result of nutrient deficiency. Subsequent fertilization studies conducted on Ch cutovers have confirmed that the growth check was due to a combination of N and P deficiencies. For example, Weetman *et al.* (1989) studied the
Figure 4.2. Mature Ha stand composed of western hemlock and amabilis fir growing on northern Vancouver Island.
Figure 4.3. Landscape view of a northern Vancouver Island forest. The mixed western hemlock and amabilis fir stands (Ha) appear dark green and occupy the higher portions of the landscape that increases their exposure to wind. The old-growth western hemlock and western red cedar stands (Ch) occupy the remaining landscape and are characterized here by the dominance of large dead cedar tops.
Figure 4.4. Regenerating western hemlock plantation on a recently harvested cutblock. In the background is a mature Ha stand composed of western hemlock and amabilis fir. The regenerating trees appear vigorous in the rear portion of the cutblock, the portion of the site that had previously supported both western hemlock and amabilis fir (Ha). In sharp contrast, the regenerating trees in the foreground are severely chlorotic and their growth is stunted. This portion of the cutblock had previously supported an old-growth stand composed of western hemlock and western red cedar (Ch).
effect of numerous combinations of N, P and K on the first-year growth of western red cedar and western hemlock using vector analysis. The study confirmed that both N and P were deficient. A later study by Weetman et al. (1993) also confirmed N and P deficiency as the cause of the poor growth. In this study, first year leader growth of western hemlock was tripled over that of the control plots following the addition of N at 225 kg/ha and P at 75 kg/ha. In a similar investigation, Thompson et al. (1993) studied the effect of N, P and K fertilization on the 5-year height growth of hemlock trees. They reported that 5-year height growth was improved by approximately 140 % or 1.37 meters over that of the control trees. In sharp contrast, fertilization of trees on an adjacent Ha cutover yielded no significant gain in growth following fertilization, confirming that the growth on these cutovers was not nutrient-limited. As further evidence that N and P deficiencies were the primary causes of reduced growth, conventional foliar analysis in each of the above experiments revealed N and P concentrations in current-year foliage to be well below those considered to be deficient for most commercial conifer species. As a consequence of the above research, fertilization of plantations on Ch cutovers with N and P fertilizers has now become a silvicultural option to forest managers. Indeed, during the spring of 1999, approximately 2000 hectares of young western hemlock and cedar plantations growing on Ch cutovers were operationally fertilized on northern Vancouver Island.

While the primary cause of the growth check and a management strategy to overcome this problem have been identified, the exact mechanisms which lead to N and P deficiencies have yet to be clearly identified. Weetman et al. (1990) investigated N and P availability on a number of Ch sites that ranged in years since burning. They reported that both mineralizable and extractable N and extractable P declined steadily since harvest and were lowest in the eighth year since harvesting, which coincided with the time that growth check typically occurs. In a well-replicated study that compared N and P availability in adjoining Ch and Ha cutovers, Prescott et al. (1993) reported that extractable N was greatest in the forest floor horizons collected from the Ha cutover. Similar results were reported for levels of extractable P. A 40-day incubation study indicated that mineralization rates for N were dramatically greater in forest floor material.
collected from the HA cutover compared to that collected from the Ch cutover (Prescott et al., 1993).

While the poor quality of the forest floor between the two sites noted above might have a role in causing the nutrient deficiencies, a number of researchers have directed their attention towards salal as a factor that impedes tree growth. The suspicion that salal may have a role to play in contributing to N and P deficiency in the regenerating trees seems in large part to be the result of three observations. The first is that the appearance of the growth check at 5 to 8 years coincides with the rapid re-growth of salal following burning. Secondly, salal is a significant component of the understory of Ch stands and rapidly invades Ch cutovers. In contrast, salal is relatively absent from the understory of Ha stands owing to the high crown closure and has difficulty in invading Ha cutovers due to the rapid time to crown closure. The third, and more convincing observation, is that manual salal removal on Ch cutovers has been shown to result in increased N and P uptake in western hemlock and improved height growth. For example, Weetman et al. (1989) reported that the N concentration of western red cedar was improved from a control level of 1.04% to 1.44% after two growing seasons. A similar increase in N uptake was observed in western hemlock, though cedar showed a greater height response to salal removal than did that of hemlock. In a similar study of the effect of salal removal on N uptake, Chang et al. (1996) reported that N uptake from applied fertilizers was increased by a factor of eight in western hemlock over a period of two growing seasons. deMontigny (1992) hypothesized that salal had an allelopathic effect on western hemlock, cedar and spruce but results were inconclusive. Xiao (1994) investigated the interaction of the ericoid mycorrhizae of salal and three forms of ectomycorrhizae from western hemlock in pure cultures. He was able to clearly demonstrate in the laboratory that the growth of the three ectomycorrhizal fungi were inhibited by the ericoid mycorrhizae. Exactly how pertinent these latter results are remains to be seen as this study was conducted in pure culture and not under field conditions and, furthermore, a limited number of mycorrhizae tested.

In a well cited review of studies into nutritional disorders using light and electron microscopy, Hecht-Buchholz (1983) noted that some nutritional disorders cause not only a reduction in growth but also an impairment of plant metabolism and damage to root and
shoot tissue. While the low nutrient concentrations and reductions in growth have been well documented in the trees growing on the Ch sites, the effect of poor nutrient levels or damage to plant tissues on a microscopic level has yet to be identified.
Methodology

Experimental Approach

Six fertilization treatments were tested in a randomized complete block design in which each of the six treatments was randomly tested within a total of six blocks (Figure 4.5). The experiment was conducted using a five-year-old plantation (Figure 4.6) that had been established earlier by researchers with the Research Branch, British Columbia Ministry of Forests.

Location

The plantation was located on northern Vancouver Island within Western Forest Products TFL #25. It is approximately 20 km north of the town of Port McNeill and 2.5 km north of the junction of Misty Lake Road and Rupert Main (Figure 4.7).

Site Description

The plantation is located within the very wet maritime subzone of the Coastal Western Hemlock biogeoclimatic zone. The study site is within 10 kilometers of the nearest weather reporting station at Port Hardy airport where the mean annual precipitation is approximately 1870 mm, primarily in the form of rainfall. Mean daily temperature ranges from a low of 3.0 °C in January to a high of 13.9 °C in August (Environment Canada, 1993).

The original stand consisted primarily of old-growth western red cedar and western hemlock. The stand was harvested in 1990 and the site was subsequently broadcast burned. The site was planted in November of 1991 with western hemlock at 3 x 3 m spacing (using 1 + 0 615s stock type) and interplanted with western red cedar (using 1 + 0 313s stock type). The soil was classified as a Humo-Ferric Podzol. The ground vegetation included salal and fireweed (Epilobium angustifolium).
Figure 4.5. Plot and block layout where I: control, II: N (225 kg/ha), III: N (225 kg/ha) + P (100 kg/ha), IV: N (225 kg/ha) + P (100 kg/ha) + Blend (230 kg/ha), V: N (225 kg/ha) + P (300 kg/ha), and VI: N (225 kg/ha) + P (500 kg/ha).
Figure 4.6. View of study site with five-year-old western hemlock trees growing on a Ch cutover two years following fertilization. The orange posts mark individual plot boundaries. In the background is the unfertilized portion of the cutover. The ground vegetation is dominated by salal.
Figure 4.7. Location of study site on northern Vancouver Island, approximately 20 km north of the town of Port McNeill.
The hemlock trees were approximately 1.2 m in height prior to fertilization. The current mean annual height increment was approximately 15 cm and the foliage was severely chlorotic (Figure 4.8) characteristic of severe nutrient deficiency. These observations are consistent with those of other plantations in the area that have also undergone growth check at this stage of stand development.

**Plot Establishment**

Each of the blocks established by the Ministry of Forests were sub-divided into six plots measuring 9 by 15 m (Figure 4.5), each to randomly receive one of six treatments. This plot size ensured a minimum of 15 western hemlock trees within each plot. The plot corners were permanently marked with 1 m cedar stakes and buffers of between 3.7 m and 4 m width were established between all plots.

**Treatments**

The treatments used were identical to those used in the eight-single-tree screening installations with some modification. The use of the same fertilizer additions and rates would allow for extrapolation of results in this experiment to accompanying studies reported in this thesis and elsewhere. The treatments included: (1) control, (2) N (225 kg/ha), (3) N (225 kg/ha) + P (100 kg/ha), (4) N (225 kg/ha) + P (100 kg/ha) + Blend (230 kg/ha), (5) N (225 kg/ha) + P (300 kg/ha), and (6) N (225 kg/ha) + P (500 kg/ha). Nitrogen was applied in the form of urea and phosphorus in the form of triple-superphosphate. The blend application included magnesium (40 kg/ha) applied as magnesium sulfate, potassium (60 kg/ha) applied as potassium sulfate, copper (10 kg/ha) applied as copper sulfate, and zinc (20 kg/ha) applied as zinc sulfate. All fertilizers were broadcast applied by hand during the second week of May 1996. To ensure even application, each plot was divided into four quadrants and the appropriate fertilizer was pre-weighed for each quadrant.
Figure 4.8. Typical western hemlock tree prior to fertilization in the spring of 1996. Note the poor leader increment, low crown biomass, and chlorotic appearance of current-year foliage.
Height Growth Measurement

The three-year height increment was measured in the fall of 1998. The height of 15 trees per plot was determined using an 8 meter height pole (10 trees per plot x 6 plots per block x 6 blocks = total of 360 trees). The height increment of each tree was then determined by subtracting total tree height from the tree height prior to fertilization.

Determination of Nutrient Concentrations

Current-year foliage was collected in the fall of the first and second growing seasons for conventional foliar nutrient analysis. Foliage was collected from six trees within each plot and combined to form a single composite sample for a total of 36 samples (1 composite sample per plot x 6 plots per block x 6 blocks). The foliage was dried at 70°C for 48 hours, cleaned, and ground using a Braun blender. MacMillan Bloedel’s Soil and Plant Testing Laboratory, located in Nanaimo, carried out the determination of total nutrient contents. Standard analytical techniques described in previous chapters were employed.

Nitrogen Fractionation / Free Amino Acid Determination

A separate collection of current-year foliage at the end of the first growing season was also made for the determination of free amino acid concentrations. Current-year foliage was collected from three trees randomly selected within each plot for a total of 108 samples (3 trees per plot x 36 plots). The current-year foliage from each individual tree was carefully wrapped in aluminum foil and fixed within an hour of collection in the field using dry ice located in specially designed cold storage containers. The use of dry ice and the insulated containers insured that the samples were properly fixed and remained frozen (-68°C) until returned to the laboratory where they were subsequently transferred to a low temperature freezer (-30°C). The samples were then freeze-dried within a week of collection and subsequently cleaned and ground using a Braun blender and returned to cold storage to await extraction.
The procedures previously described for extraction and purification of free amino acids were followed (Chapter II). The amino acids were quantified using the AccQ Tag procedure for derivatization (Cohen et al., 1993). Analysis was carried out using a Waters 474 Scanning Fluorescence Detector. The following amino acids were quantified: aspartic acid, glutamic acid, asparagine, glutamine, arginine, glycine, histidine, tryosine, valine, phenylalanine, leucine, lysine, serine and threonine. Only arginine concentrations were significantly affected by the fertilization treatments and the remaining amino acids will not be considered.

**P Fractionation**

Total P was fractionated into organic and inorganic P fractions in foliage that had been collected at the end of the first growing season for conventional foliar analysis. The procedure described in Chapter II was adopted.

**Gas Exchange Measurements**

Photosynthetic rates were monitored during the first and second growing seasons following fertilization. During the first growing season, a total of three measurements were undertaken: late July, late August and early October. During the second growing season, photosynthetic rates were determined on two occasions. The first measurements were undertaken during the month of August and the second during the month of September. At each of the five measurement periods, foliage was collected from the base of the upper one-third of the crown of five trees in each plot (or a total of 30 trees per treatment and a total of 180 trees per measurement period). The foliage samples were initially placed in plastic bags during the collection period. The stems of each branch were then carefully cut under water approximately 3 cm from the initial cut within 10 minutes of the initial collection and immediately placed in plastic containers with water until measurement. This procedure was adopted to prevent water stress and subsequent stomatal closure. Samples were only collected during the late morning hours. Consequently, each measurement period was of a three-day duration with two blocks
sampled on each of the three days. The order in which each block was sampled within the three-day period was randomized, as was the order in which the treatments were sampled within a given day.

The measurement of photosynthetic rates using intact branches on each tree in the field is a difficult task, and in the present study made particularly difficult by the rough terrain of the plantation. Hence, the measurement of photosynthetic rates on cut branches was adopted following the procedure suggested by Brix and Ebell (1969). In the latter study, the validity of this method was tested measuring the photosynthetic rates before and following excision. Following the excision, the branches were re-cut under water approximately 0.5 cm from the end. Measurements indicated that the rate of photosynthesis in these excised twigs was relatively stable over a period of 7 hours (±5%). Brix (1971) adopted this procedure in a later study, while also working with Douglas-fir, as did Kellomaki et al. (1982) while working with Scots pine. In the later study, the authors re-cut each branch under water approximately 5 to 10 cm from the end of the original cut to ensure an uninterrupted flow of water. Lange et al. (1986) reinvestigated the effect of removing branches on the measurement of photosynthetic rates using Norway spruce. The results were in agreement with that of Brix and Ebell (1969) insofar that photosynthetic rates were stable over a measurement period of 2 hours.

An ADC LCA3 gas exchange unit was used within a tent equipped with artificial lighting. Variables monitored included photosynthetic rates, stomatal conductance, relative humidity, internal (calculated) and external CO₂ concentration, and temperature. The PPFD was maintained at 800 μmoles m⁻² s⁻¹. Needle areas were determined in the laboratory using a Licor-3100 leaf area meter.

**¹³C Isotope Discrimination Analysis**

Current-year foliage was collected from six trees within each plot, for a total of 216 samples (6 trees per plot x 6 plots per block x 6 blocks), during the fall of both 1996 and 1997. The foliage samples were dried at 70°C for 48 hours, cleaned, and subsequently pulverized in a stainless steel planetary ball mill. The samples were then
sent to the University of Saskatchewan for determination of $\delta^{13}$C (‰). The latter was
determined using a calibrated sample of acetanilide as a standard. The isotopic
composition in the samples was calculated as:

$$\delta^{13}$C (‰) = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1000$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the ratios of $^{13}$C/$^{12}$C respectively in the original foliage
samples and the standard.

**Chlorophyll Fluorescence**

Chlorophyll fluorescence was measured during the first and fourth week of the
month of August, 1997 (i.e. during the second growing season following fertilization).
Shoots containing current-year foliage were collected from six trees within each plot for a
total of 216 individual samples at each measurement period. The needles were dark
adapted for a period of 30 minutes using dark adaptation clips. Fluorescence of the upper
needle surface was determined using an Optiscience 400 fluorometer where $F_0$ was
determined (PAR = 0.006 $\mu$mol m$^{-2}$ s$^{-1}$) followed by a saturating pulse (3000 $\mu$mol m$^{-2}$ s$^{-1}$
for 0.8s) to determine $F_m$.

**Pigment Analysis**

Current-year foliage was also collected at the completion of the first and second
growing seasons and used for the determination of chlorophyll a and b concentrations.
Foliage was collected from each of five trees within each plot, for a total of 180 samples
(5 samples per plot x 6 plots per block x 6 blocks). Consistent with the recommendation
of Young et al. (1997), care was taken to ensure that only fresh biological tissues were
used. The foliage samples in this study were returned to the laboratory within one day of
collection and promptly analyzed. Fresh weight samples of approximately 40 mg were
extracted with N,N-dimethylformamide following the procedure of Moran (1982). The
concentrations of chlorophyll a and b were calculated according to formulae provided by Porra et al. (1989).

**Electron Microscopy**

Current-year needles were collected in late September of 1997 from two trees in each of the plots representing the control and the blend treatments for a total of 12 samples for each of the two treatments. The fresh samples were returned to the laboratory within a day of collection and fixed using a solution of 2.5% paraformaldehyde glutaraldehyde in a 200-mM phosphate buffer for 1 hour under vacuum. The samples were then rinsed several times in the buffer solution and again fixed in a solution of 2% osmium tetroxide. Finally, the samples were dehydrated through an ethanol series and embedded in epoxy resin (Spurr, 1968). Nine ultra-thin sections were cut longitudinal from each needle using a diamond knife (Figure ) and stained with uranyl acetate and lead citrate. The samples were mounted on 200-square copper mesh coated with 0.2% formvar.

Chloroplasts were viewed using a transmission electron microscope (10C/CR, Zeiss) located in the Department of Botany at the University of British Columbia. The number of grana per chloroplast and the number of thylakoid membranes per granum were determined. In addition, the presence and absence of starch grains was determined and the approximate size of the starch grains was estimated as the volume that the grain comprised as a percentage of total chloroplast volume using a grid plot approach. Approximately 50 chloroplasts were viewed per needle and contact sheets were made of 10 chloroplasts per needle.

**Destructive Sampling for Measurement of Foliage Biomass**

The effect of fertilization on foliage production was determined. A total of 24 trees from the control plots and 24 trees from the plots that had received the addition of N (225 kg/ha) + P (100 kg/ha) + blend (270 kg/ha) were destructively sampled. Foliage production in trees representing the additional four treatments was not measured due to
Figure 4.9. Western hemlock needle cross section stained with toluene blue and similar to that used for electron microscopy.
time constraints. Consequently, a total of 48 trees were sampled (4 trees x 2 treatments x 6 blocks).

The trees were felled and transported to laboratory facilities at the University of British Columbia in the fall of 1997. Twelve trees were harvested during each of four collection times over a two-week period. This collection schedule ensured that the trees could be processed in the laboratory within four days from the time of collection. This procedure ensured that the opportunity for the crowns to dry out, and the accompanying loss of foliage, was minimized. The crowns were carefully dissected into three crown classes (1997, 1996 and <96). The samples were then dried at 70°C for 48 hours.

**Statistical Analysis**

The data were analyzed using an ANOVA Model Type I (Zar, 1984) where block and treatment were considered fixed variables. Means were compared by way of Duncan's multiple range test. The three-year height response was analyzed by covariant analysis where the tree height before fertilization was considered the covariant.
Results

Effect of fertilization on leaf area production

As earlier noted, the planted western hemlock trees on this cutover, represented by those within the control plots, had limited height increment and also limited branch production and extension, with a concomitant limitation in foliage production. Fertilization had a dramatic effect on the production of foliage biomass, and hence needle area, during each of the two growing seasons following fertilization (Figure 4.10). During the first year, foliage production was increased approximately four-fold over that of the control trees. Foliage production in response to fertilization increased further during the second growing season. At the end of the second growing season, fertilized trees had approximately six times the foliage biomass as the unfertilized trees. Visual observations indicated that the enhanced foliage production was attributed mainly to the addition of N rather than P or the elements contained within the blend fertilizer.

Effect of fertilization on photosynthetic rates on a unit leaf area basis

The addition of N alone resulted in a nearly two-fold increase in the mean photosynthetic rate for each of the measurement periods during the first growing season (Table 4.1). There was also evidence that the photosynthetic rate of the current-year foliage was increased during September and October with the additional application of P at the rate of 100 kg/ha. There was no evidence during the first growing season that the blend addition had any effect on photosynthetic rates. The addition of P at the rate of 300 kg/ha further increased the photosynthetic rate during the September and October measurements. The addition of P at the rate of 500 kg/ha had no significant effect on increasing the photosynthetic rates above that of other treatments.

During the second growing season, mean photosynthetic rates were also increased approximately two-fold in plots that had received the N only treatment, but further increases with the addition of P or the elements within the blend fertilizer were not observed.
Figure 4.10. Foliage biomass prior to fertilization (<96), and that produced during the first (96) and second (1997) growing seasons following fertilization for the control and the N (225 kg/ha) + P (100 kg/ha) + blend (230 kg/ha) treatment. Each value is the mean of 12 trees. Means with different letters differ at 5%.
Table 4.1. Mean photosynthetic rate (μmol m⁻² s⁻²) and SE by treatment for each of five measurement periods. Each value is the mean of 30 individual measurements.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>August 96</th>
<th>September 96</th>
<th>October 96</th>
<th>August 97</th>
<th>September 97</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.86 (0.23) c</td>
<td>4.10 (0.17) d</td>
<td>3.30 (0.13) d</td>
<td>2.78 (0.11) b</td>
<td>4.28 (0.26) b</td>
</tr>
<tr>
<td>N</td>
<td>5.46 (0.41) b</td>
<td>6.34 (0.34) c</td>
<td>6.39 (0.26) c</td>
<td>5.42 (0.29) a</td>
<td>7.30 (0.30) a</td>
</tr>
<tr>
<td>NP100</td>
<td>5.98 (0.35) ab</td>
<td>7.74 (0.30) b</td>
<td>7.52 (0.27) b</td>
<td>5.32 (0.16) a</td>
<td>7.61 (0.22) a</td>
</tr>
<tr>
<td>Blend</td>
<td>6.52 (0.40) ab</td>
<td>8.22 (0.28) ab</td>
<td>7.60 (0.21) b</td>
<td>5.63 (0.28) a</td>
<td>7.41 (0.30) a</td>
</tr>
<tr>
<td>NP300</td>
<td>6.93 (0.36) a</td>
<td>8.93 (0.41) a</td>
<td>8.40 (0.28) a</td>
<td>4.87 (0.15) a</td>
<td>7.63 (0.26) a</td>
</tr>
<tr>
<td>NP500</td>
<td>7.13 (0.49) a</td>
<td>8.54 (0.27) a</td>
<td>7.72 (0.24) ab</td>
<td>5.85 (0.23) a</td>
<td>7.83 (0.34) a</td>
</tr>
</tbody>
</table>
$^{13}$C isotope discrimination analysis

The mean $\delta^{13}$C value for control samples was $-31.30$ in current-year foliage collected at the end of the first growing season (Table 4.2). The addition of N alone resulted in a strong reduction in discrimination as indicated by an increase in the mean $\delta^{13}$C to a value of $-29.27$. The mean $\delta^{13}$C value was further increased, i.e. discrimination was reduced, during the first growing season with additions of P at the rate of 100 kg/ha but was unaffected by further additions of P at the rate of either 300 or 500 kg/ha. The addition of the blend fertilizer also resulted in a further reduction in discrimination. The lowest $\delta^{13}$C value was in foliage that had received this treatment with a mean $\delta^{13}$C value of $-27.75$.

The mean $\delta^{13}$C value for control samples was $-30.99$ in current-year foliage collected at the end of the second growing season (Table 4.2). The strong reduction in discrimination that was noted with N additions during the first growing season remained evident, and indeed, was more pronounced in current-year foliage collected at the end of the second growing season. The remaining treatments had no additional effect on discrimination above that of N additions, with the exception of the plots that had received P at the rate of 500 kg/ha.

**Chlorophyll $a$ and $b$ concentrations**

Nitrogen additions resulted in an approximate two-fold increase in the chlorophyll $a$ and $b$ concentrations in current-year foliage collected at the end of the first growing season (Table 4.3). The addition of P, at any of the three application rates, or the addition of the blend fertilizer had no further effect on the chlorophyll concentrations. The addition of N also resulted in a reduction in the chlorophyll $a:b$ ratio owing to the fact that chlorophyll b concentrations were increased more by the N additions than that of chlorophyll a. The effect of the N fertilization on chlorophyll concentrations remained evident in the current-year foliage that had been collected at the end of the second growing season (Table 4.4).
Table 4.2. Mean $\delta^{13}$C ($^{0/oo}$) value and SE for current-year foliage collected at the end of the first and second growing season by treatment. Each value is the mean of 18 individual measurements. Values with the same letter are not significantly different.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\delta^{13}$C ($^{0/oo}$) 1st year</th>
<th>$\delta^{13}$C ($^{0/oo}$) 2nd year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-31.30 (0.15) d</td>
<td>-30.99 (0.12) c</td>
</tr>
<tr>
<td>N</td>
<td>-29.27 (0.11) c</td>
<td>-28.77 (0.12) b</td>
</tr>
<tr>
<td>NP100</td>
<td>-28.45 (0.09) b</td>
<td>-28.68 (0.15) ab</td>
</tr>
<tr>
<td>Blend</td>
<td>-27.75 (0.12) a</td>
<td>-28.41 (0.11) ab</td>
</tr>
<tr>
<td>NP300</td>
<td>-28.33 (0.08) b</td>
<td>-28.53 (0.12) ab</td>
</tr>
<tr>
<td>NP500</td>
<td>-28.26 (0.08) b</td>
<td>-28.38 (0.09) a</td>
</tr>
</tbody>
</table>
Table 4.3. Mean concentration and SE of chlorophyll a and chlorophyll b, and their ratio by treatment in current-year foliage collected at the end of the first growing season. Each value is the mean of 30 individual determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>319 (17) a</td>
<td>81 (5) a</td>
<td>4.1 (0.1) a</td>
</tr>
<tr>
<td>N</td>
<td>706 (33) b</td>
<td>205 (9) b</td>
<td>3.5 (0.1) b</td>
</tr>
<tr>
<td>NP100</td>
<td>785 (33) b</td>
<td>224 (9) b</td>
<td>3.5 (0.1) b</td>
</tr>
<tr>
<td>Blend</td>
<td>774 (32) b</td>
<td>226 (9) b</td>
<td>3.4 (0.1) b</td>
</tr>
<tr>
<td>NP300</td>
<td>776 (29) b</td>
<td>220 (10) b</td>
<td>3.6 (0.1) b</td>
</tr>
<tr>
<td>NP500</td>
<td>774 (42) b</td>
<td>218 (14) b</td>
<td>3.6 (0.1) b</td>
</tr>
</tbody>
</table>
Table 4.4. Mean concentration and SE of chlorophyll a and chlorophyll b, and their ratio by treatment in current-year foliage collected at the end of the second growing season. Each value is the mean of 30 individual determinations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chl a</th>
<th>Chl b</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>360 (22) a</td>
<td>103 (7) a</td>
<td>3.5 (0.1) a</td>
</tr>
<tr>
<td>N</td>
<td>632 (28) b</td>
<td>184 (9) b</td>
<td>3.4 (0.2) ab</td>
</tr>
<tr>
<td>NP100</td>
<td>618 (26) b</td>
<td>188 (8) b</td>
<td>3.3 (0.1) b</td>
</tr>
<tr>
<td>Blend</td>
<td>671 (43) b</td>
<td>205 (12) b</td>
<td>3.3 (0.1) b</td>
</tr>
<tr>
<td>NP300</td>
<td>695 (46) b</td>
<td>203 (15) b</td>
<td>3.4 (0.1) ab</td>
</tr>
<tr>
<td>NP500</td>
<td>738 (42) b</td>
<td>220 (12) b</td>
<td>3.4 (0.1) b</td>
</tr>
</tbody>
</table>
Chloroplast structure via electron microscopy

An examination of chloroplasts representative of trees from the control and the N (225 kg/ha) + P (100 kg/ha) + blend (230 kg/ha) treatment indicated that nutritional status, as affected by fertilization, had a profound effect on the chloroplast structure. Most noteworthy was the observation that chloroplasts from unfertilized trees typically had a single large starch grain (Figure 4.11), which, on average, occupied approximately 80% of the cross sectional area of the chloroplast (Table 4.5). The presence of these large starch grains resulted in a physical distortion of the chloroplast shape, from that of the typical lens shape, to a more elliptical or football shape. The thylakoid membranes within these chloroplasts appeared to have been mechanically distorted, occupying a small portion of the plastid volume and typically occurring only along the plastid membrane. In sharp contrast, starch grains appeared to be absent from the chloroplasts representing seven of the twelve trees that had received the fertilization treatment (Figure 4.12). In the remaining trees, the starch grains that were observed were typically smaller in size from those observed in the control chloroplasts.

The total number of grana per chloroplast was only slightly increased from a mean of 34 in chloroplasts representing the control trees to a mean of 41 in chloroplasts representing the fertilized trees (Table 4.5). However, it is meaningful to note that the mean number of thylakoid membranes per granum stack was increased from 2.4 to 4.3 with fertilization. There did not appear to be any changes in stroma density between the two treatments, nor were changes observed in the number or density of plastoglobuli.

Thylakoid efficiency as indicated by chlorophyll fluorescence

The mean Fv/Fm ratio for control trees was between approximately 0.660 and 0.670 for each of the two measurements (Figure 4.13 and 4.14). Nitrogen additions alone improved the photochemical efficiency as indicated by a significant increase in the Fv/Fm ratio to a mean of approximately 0.750. The addition of P, at any of the three application rates, or the blend fertilizer, had no effect on chlorophyll fluorescence during either measurement period. The Fo value remained relatively constant across treatments.
Figure 4.11. Chloroplasts typical of un-fertilized trees. Note the single starch grain within each chloroplast that appears to occupy the majority of the plastid’s volume. Thylakoids appear to be mechanically distorted and occur primarily along the plastid membrane.
Table 4.5. Mean number and SE of grana per chloroplast, cross sectional area (%) that starch grain(s) occupy, and the mean number of thylakoids per granum in chloroplasts in trees from control and N (225 kg/ha) + P (100 kg/ha) + blend (230 kg/ha) treatments. Values with the same letter are not significantly different at 0.05% confidence.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of grana per chloroplast</th>
<th>Percent of cross-sectional area occupied by starch grain(s)</th>
<th>Number of thylakoids per granum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.1 (1.8) a</td>
<td>79.2 (2.5) a</td>
<td>2.6 (0.1) a</td>
</tr>
<tr>
<td>Fertilized</td>
<td>40.2 (1.6) b</td>
<td>20.4 (3.2) b</td>
<td>4.5 (0.3) b</td>
</tr>
</tbody>
</table>
Figure 4.12. Chloroplasts typical of fertilized trees. Note the absence of starch grains and enhanced thylakoid stacking.
Figure 4.13. Chlorophyll fluorescence in current-year foliage during the first week of August, 1997 (i.e. during the second growing season). Each measurement is the mean of 30 individual measurements. Values with the same letter are not significantly different at 0.05% confidence.

Figure 4.14. Chlorophyll fluorescence in current-year foliage during the last week of the month of August (i.e. during the second growing season). Each measurement is the mean of 30 individual measurements. Values with the same letter are not significantly different at 0.05% confidence.
with a mean of 226 indicating that the increase in the Fv/Fm ratio at each of the two measurement periods was due to an increase in Fm.

**Effect of fertilization on foliar nutrient concentrations**

Mean nutrient concentrations in current-year foliage collected at the end of the first growing season are reported in table 4.6. The concentrations of N and P in current-year foliage from control plots were 0.73% and 0.10% respectively. These results confirm the visual symptoms of severe N and P deficiencies. Nitrogen additions alone increased N concentrations to 1.50%, which reduced the P/N ratio to 0.09 (data not shown) and lowered the \( \text{SO}_4^{2-} \) concentrations from 204 ppm in control foliage to 52 ppm. The addition of P at the rate of 100 kg/ha increased mean P concentrations to approximately 0.23 and an increase in the P/N ratio to 0.15 (data not shown). The addition of P at the rates of 300 and 500 kg/ha increased the mean P concentration to 0.32 and 0.30 respectively. The addition of the blend fertilizer had little effect on increasing the concentrations of K, Mg, Fe, Zn or Cu but increased the \( \text{SO}_4^{2-} \) concentration to a mean of 467 ppm.

The mean nutrient concentrations in foliage collected at the end of the second growing season are reported in table 4.6. While the N concentrations declined in the second growing season, the P concentrations remained elevated.

**Fractionation of Total P**

The relationship between the inorganic P fraction and total P is reported in figure 4.15 and followed a near linear relationship. The inorganic fraction of P in foliage collected from the control plots was stable at 0.01%. Nitrogen fertilization had only a small effect on reducing the size of inorganic fraction. The size of the latter was, however, increased significantly with the addition of P, and increased with increasing addition rates.
Table 4.6. Foliar nutrient concentrations and SE at the end of the first growing season by treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%N</th>
<th>%P</th>
<th>%K</th>
<th>%Ca</th>
<th>%Mg</th>
<th>Mn ppm</th>
<th>Fe ppm</th>
<th>Cu ppm</th>
<th>Zn ppm</th>
<th>%S</th>
<th>SO₄ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.73</td>
<td>0.10</td>
<td>0.76</td>
<td>0.20</td>
<td>0.09</td>
<td>1368</td>
<td>64</td>
<td>2.1</td>
<td>10.2</td>
<td>0.093</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.00</td>
<td>0.07</td>
<td>0.01</td>
<td>0.01</td>
<td>170</td>
<td>6</td>
<td>0.32</td>
<td>1.66</td>
<td>0.01</td>
<td>41</td>
</tr>
<tr>
<td>N</td>
<td>1.50</td>
<td>0.09</td>
<td>0.67</td>
<td>0.23</td>
<td>0.08</td>
<td>1354</td>
<td>66</td>
<td>2.4</td>
<td>13.7</td>
<td>0.112</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td>0.01</td>
<td>288</td>
<td>4</td>
<td>0.22</td>
<td>1.84</td>
<td>0.01</td>
<td>14</td>
</tr>
<tr>
<td>NP100</td>
<td>1.52</td>
<td>0.23</td>
<td>0.64</td>
<td>0.26</td>
<td>0.09</td>
<td>1241</td>
<td>74</td>
<td>2.6</td>
<td>16.5</td>
<td>0.124</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.02</td>
<td>0.05</td>
<td>0.00</td>
<td>0.01</td>
<td>114</td>
<td>5</td>
<td>0.35</td>
<td>0.92</td>
<td>0.01</td>
<td>18</td>
</tr>
<tr>
<td>NP100B1</td>
<td>1.50</td>
<td>0.19</td>
<td>0.71</td>
<td>0.30</td>
<td>0.09</td>
<td>1698</td>
<td>63</td>
<td>2.5</td>
<td>19.2</td>
<td>0.203</td>
<td>467</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00</td>
<td>172</td>
<td>5</td>
<td>0.21</td>
<td>0.91</td>
<td>0.02</td>
<td>63</td>
</tr>
<tr>
<td>NP300</td>
<td>1.51</td>
<td>0.32</td>
<td>0.65</td>
<td>0.30</td>
<td>0.10</td>
<td>1428</td>
<td>93</td>
<td>2.8</td>
<td>20.8</td>
<td>0.135</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.04</td>
<td>0.05</td>
<td>0.02</td>
<td>0.01</td>
<td>165</td>
<td>23</td>
<td>0.41</td>
<td>1.40</td>
<td>0.01</td>
<td>18</td>
</tr>
<tr>
<td>NP500</td>
<td>1.52</td>
<td>0.30</td>
<td>0.66</td>
<td>0.31</td>
<td>0.10</td>
<td>1238</td>
<td>68</td>
<td>2.5</td>
<td>16.5</td>
<td>0.146</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.03</td>
<td>0.07</td>
<td>0.02</td>
<td>0.01</td>
<td>103</td>
<td>5</td>
<td>0.5</td>
<td>1.3</td>
<td>0.012</td>
<td>55</td>
</tr>
</tbody>
</table>
Table 4.7. Foliar nutrient concentrations and SE at the end of the second growing season by treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%N</th>
<th>%P</th>
<th>%K</th>
<th>%Ca</th>
<th>%Mg</th>
<th>Mn ppm</th>
<th>Fe ppm</th>
<th>Cu ppm</th>
<th>Zn ppm</th>
<th>%S</th>
<th>SO₄ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.76</td>
<td>0.11</td>
<td>0.85</td>
<td>0.23</td>
<td>0.10</td>
<td>1497</td>
<td>134</td>
<td>2.8</td>
<td>10</td>
<td>0.102</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>0.01</td>
<td>0.05</td>
<td>0.02</td>
<td>0.00</td>
<td>191</td>
<td>43</td>
<td>0.2</td>
<td>1</td>
<td>0.00</td>
<td>13</td>
</tr>
<tr>
<td>N</td>
<td>1.41</td>
<td>0.09</td>
<td>0.73</td>
<td>0.29</td>
<td>0.09</td>
<td>2120</td>
<td>99</td>
<td>3.7</td>
<td>15</td>
<td>0.120</td>
<td>86</td>
</tr>
<tr>
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<td>0.07</td>
<td>0.00</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td>316</td>
<td>20</td>
<td>0.9</td>
<td>1</td>
<td>0.01</td>
<td>27</td>
</tr>
<tr>
<td>NP100</td>
<td>1.29</td>
<td>0.27</td>
<td>0.70</td>
<td>0.33</td>
<td>0.12</td>
<td>1437</td>
<td>144</td>
<td>3.1</td>
<td>15</td>
<td>0.112</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
<td>0.02</td>
<td>0.04</td>
<td>0.02</td>
<td>0.01</td>
<td>153</td>
<td>34</td>
<td>0.3</td>
<td>1</td>
<td>0.00</td>
<td>19</td>
</tr>
<tr>
<td>NP100Bi</td>
<td>1.17</td>
<td>0.21</td>
<td>0.73</td>
<td>0.38</td>
<td>0.11</td>
<td>1898</td>
<td>98</td>
<td>4.1</td>
<td>15</td>
<td>0.133</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.01</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>271</td>
<td>25</td>
<td>1.2</td>
<td>1</td>
<td>0.01</td>
<td>27</td>
</tr>
<tr>
<td>NP300</td>
<td>1.30</td>
<td>0.34</td>
<td>0.75</td>
<td>0.35</td>
<td>0.12</td>
<td>1662</td>
<td>111</td>
<td>3.5</td>
<td>17</td>
<td>0.136</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>0.02</td>
<td>0.06</td>
<td>0.03</td>
<td>0.01</td>
<td>141</td>
<td>17</td>
<td>0.5</td>
<td>2</td>
<td>0.01</td>
<td>41</td>
</tr>
<tr>
<td>NP500</td>
<td>1.35</td>
<td>0.37</td>
<td>0.77</td>
<td>0.36</td>
<td>0.12</td>
<td>1765</td>
<td>79</td>
<td>3.5</td>
<td>18</td>
<td>0.133</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>0.49</td>
<td>0.14</td>
<td>0.27</td>
<td>0.10</td>
<td>0.04</td>
<td>585</td>
<td>30</td>
<td>0.7</td>
<td>7</td>
<td>0.0543</td>
<td>47</td>
</tr>
</tbody>
</table>
Figure 4.15. Inorganic P (%) in current-year foliage collected at the end of the first growing season for each of the six treatments. Means with the same letter do not differ at 5%.
Arginine Concentrations

The concentration of arginine in control samples was negligible in all control samples, a finding similar to that documented chapter II for un-fertilized trees. Nitrogen additions alone increased the concentration of arginine approximately 160-fold over that of the control levels (Figure 4.16), a result also similar to that which was reported in chapter II. The concentrations of arginine were significantly reduced by the addition of P at the rate of 100 kg/ha but remained slightly elevated from control levels.

Three-year Height Growth Increment

Nitrogen fertilization alone increased the three-year height increment from approximately 2 m for the control trees to a mean of approximately 3.4 m. The further addition of P at the rate of 100 kg/ha had no additional effect on height growth, nor did the further addition of the blend fertilizer. However, a small but significant increase in height growth was observed with the addition of P at the rate of 300 kg/ha. Height growth was not further increased by the application of an additional 200 kg/ha of P.
Figure 4.16. Arginine concentrations (μm/g) in current-year foliage collected at the end of the first growing season by treatment. Means with the same letter do not differ at 5%.
Figure 4.17. Three-year height increment (m) by treatment. Means with the same letter do not differ at 5%.
Discussion

The primary objective of this investigation was to assess the effect of nutrient additions on the photosynthetic capacity of western hemlock trees growing on a nutrient stressed site. In this regard, Natr (1972) noted that the processes of nutrition and photosynthesis are complex. These sentiments were later re-emphasized by Dietz and Harris (1997), who added that a single nutrient deficiency might induce a number of primary and secondary biochemical and photochemical reactions spanning temporal orders of magnitude. Furthermore, and perhaps more importantly, the latter authors argued that it is very difficult to identify a specific rate-limiting factor for photosynthesis in response to a specific nutrient deficiency. Dietz and Harris (1997) also reminded us that nutrient deficiencies often result in a variety of complex responses on the part of the plant or tree in order to adapt to this particular stress and that it is often difficult to distinguish primary and secondary responses to specific nutrient stresses. Indeed, as noted by Marschner (1986), response mechanisms in plants and trees to conditions of low nutrient availability include a wide variety of plant strategies that enhance nutrient acquisition and metabolic adaptations including those related to enhancing nutrient use efficiency. It is the variation in these various strategies amongst different genotypes that has given rise to the field of science that pertains to the study of the genetic influences on plant and tree nutrition.

In the present experiment with western hemlock, there is evidence that the fertilizer additions, in particular, those of N and P, have played a significant role in enhancing the photosynthetic capacity of these trees. It can be argued that this increase in photosynthetic capacity is due to (1) an increase in the needle surface area, (2) an increase in the photosynthetic rate per unit needle surface area, and (3) an increase in the efficiency of the thylakoid membranes.

Effect of Fertilization on Needle Production

The addition of N increased the N concentration in current-year foliage from a mean of 0.70%, a level indicative of severe N deficiency, to a concentration of 1.60%, a
level generally thought to represent sufficiency. This improvement in N status, was concomitant with an approximate four-fold increase in foliage production in the growing season immediately following fertilization (i.e. 1996 growing season). This dramatic first season response was possibly due to the fact that western hemlock is a species exhibiting an indeterminant growth pattern. The significant increase in photosynthetic capacity of the fertilized trees during the first growing season would have lead to enhanced bud formation during the 1996 growing season and resulted in the six-fold increase in foliage biomass production during the second growing season. This significant increase in needle surface area would significantly increase the photosynthetic capacity of these trees in the years following fertilization and, arguably, was responsible for the increased height growth.

The observed increases in foliage biomass production in this experiment are similar to that documented by other researchers. For example, Weetman et al. (1993) studied the effect of N additions at the rate of 225 kg/ha and P at the rate of 75 kg/ha on the growth of an 18-year-old western hemlock stand growing on a similar site. They reported that the growth of the first year leader length was increased from 14.8 cm to 53.5 cm. Unit foliage weights were also increased with fertilization from a control level of 0.34 g/200 needles to 0.64 g/200 needles. In an earlier study, Miller and Miller (1976) reported that the addition of N at the rate of 336 kg/ha to a 36-year-old Corsican pine stand growing in Scotland increased the foliage biomass of their trees from a control level of approximately 5000 kg/ha to that of approximately 8000 kg/ha three years following fertilization. In a well-known experiment, Brix and Ebell (1969) studied the effect of N additions on the foliage production of a 20-year-old Douglas-fir stand. They reported that fertilization resulted in an increase in needle width and length, branch length, number of leaves and leaf area produced per shoot as well as the number of lateral branches. This increase in foliage production preceded an increase in basal area response. Chandler and Dale (1995) reported that N fertilization of 3-year-old Sitka spruce (Picea sitchensis [Bong] Carr.) seedlings increased the leader length, number of needles per shoot, and the projected needle area. Kellomaki et al. (1982) reported similar results while working with Scots pine.
Effect of Fertilization on Photosynthetic Rates Per Unit Leaf Area

The increase in the rate of photosynthesis per unit leave area and decreased carbon isotope discrimination clearly indicating that the observed increases in height growth were also the result of an enhanced photosynthetic capacity as measured on a unit leaf area. This enhancement in function would appear to be the result of improved nutrition, particularly that of N and P.

Effect of N status of photosynthetic rates

The addition of N significantly increased the rate of photosynthesis on a unit leaf area basis in these trees more so than the addition of P or the elements in the blend fertilizer, confirming the N was the primary element limiting photosynthesis on this site. This finding was expected insofar as the relationship between foliar N concentrations and the rate of photosynthesis has been well documented in the literature (e.g. Ellsworth and Liu, 1994; Karlsson, 1994; Baddeley et al., 1994; Cromer et al., 1993; Kirchbaum and Tompkins, 1990; Reich and Schoettle, 1988; Seeman et al., 1987; Evans, 1983; and Brix, 1981). The N status of plants and trees has more of an effect on the process of photosynthesis due to the fact that N is the fundamental building block for macromolecules (Dietz and Harris, 1997). With specific reference to N nutrition and photosynthesis, Evans (1989) noted in his well-cited review that foliar N could be divided up into two principle components. The first is the soluble protein fraction, which is composed primarily of the enzyme ribulose 1,5-bisphosphate (RuBP) carboxylase. The second fraction includes the various proteins contained within the thylakoid membranes including that of chlorophyll which was significantly increased in this study following N fertilization.

Concomitant with the increase in chlorophyll concentrations following the addition of N was a reduction in the chl a:b ratio. This reduction was in general agreement with the findings of Dryer et al. (1994). In the latter study, it was reported that Mg and Ca additions to a 10-year-old stand of Norway spruce, which was chlorotic, increased the chlorophyll concentrations and reduced the chl a:b in both current and year-
old needles. In an earlier study, Nielsen et al. (1979) reported that a reduction in the chl a:b in barley mutants was associated with an increase in the proportion of lamellae that was organized in grana. Mutant plants that had chl a:b ratios that exceeded 5.2 were found to have fewer lamellae associated with the grana. In the present study, the reduction in the chl a:b ratio following N fertilization was also associated with an increase in grana stacking. These findings are, however, in contrast to those Stocking (1975) and Spiller and Terry (1980) who reported that Fe deficiency in maize grains (Zea mays L.) and sugar beet (Beta vulgaris L.) respectively resulted in a strong reduction in grana stacking but did not affect of chl a:b ratio.

Effect of P status on photosynthetic rates

The determination of gas exchange rates during the first growing season indicated that the addition of P at the rate of 300 kg/ha or more increased the photosynthetic rates above that of trees that had received additions of N alone or N in combination with the application of P at the conventional rate of 100 kg/ha. Similarly, the addition of P at a rate above that of 100 kg/ha resulted in a reduction in the carbon isotope discrimination. These findings would seem to support the conclusion that the addition of N induced a condition of P deficiency in these trees, and further, that the addition of P at the conventional rate did not alleviate this deficiency. The increase in photosynthetic rates during the first growing season following the application of P at the rate of 300 kg/ha in addition to N corresponded to the modest increase in height growth at the end of three growing seasons.

Reich and Schoettle (1988) reported that foliar N and P concentrations were correlated with both dry matter production and photosynthetic rates in seedlings of eastern white pine (Pinus strobus). More importantly though, they reported that at P:N ratios below 0.10-0.14, low P interferes with the ability of the seedlings to photosynthetically utilize N and concluded that both N and P and their ratios, should be considered when considering photosynthesis-nutrient relations. Kirschbaum and Tompkins (1990) and Cromer et al. (1993) also reported significant relationships between
foliar P concentrations and photosynthetic rates while working with seedlings of *Eucalyptus grandis* and *Gmelina arborea* Roxb. respectively.

The precise mechanism of P deficiency and a decline in photosynthesis has been of much interest to researchers in the field of plant nutrition. As noted in more detail below, the chloroplast is a P importing and demanding organelle and accordingly, reductions in P supply will have a pronounced on its efficiency. Rao and Terry (1995) reported that a 25% decline in photosynthetic rates in sugar beet following the onset of P deficiency coincided with a reduction in the concentration of RuBP (ribulose-1,5-bisphosphate) by an amount of 60%. The re-supply of P to the plants increased the photosynthetic rates to pre-treatment levels within a period of 4 hours which coincided with an increase in the concentrations of RuBP indicating that photosynthesis in these plants was linked to RuBP regeneration. Fredeen *et al.* (1990) reported the P deficiency reduced the concentration of RuBP by 47% in hydroponically grown soybean plants. Plesnicar *et al.* (1994) reported that reduced photosynthetic rates in sunflower (*Helianthus annuus* L.) grown with limited P supply was due to the reduced efficiency of RuBP regeneration. Lewis *et al.* (1994) reported that the photosynthetic rate was reduced in loblolly pine seedlings grown with elevated CO₂ rates and limited P supplies. They too, attributed this nutritional reduction in photosynthesis to a reduction in RuBP regeneration. Jacob and Lawlor (1992) reported that P deficiency reduced RuBP regeneration in both sunflower and maize plants and was a greater factor in reducing photosynthetic rates than an observed reduction in Rubisco activity. Jacob and Lawlor (1993) reported that sunflower and maize plants grown under long-term conditions of P deficiency had low ATP concentrations which, in turn, was the principle factor that contributed to reduced rates of RuBP regeneration. Sawada *et al.* (1992), Rao and Terry (1989) and Brooks (1986) have reported similar findings of P deficiency on the regeneration of RuBP.

The effect of fertilization on chloroplast ultrastructure and rate of photosynthesis per unit leaf area

The investigation into chloroplast development revealed strong differences in
chloroplast structure between the control and fertilized trees, which in turn, corresponded with the various physiological, nutritional and growth measurements undertaken in this study. For example, the chloroplasts of control trees typically had fewer thylakoid membranes per granum, fewer grana per chloroplast and large starch grains. These visual observations corresponded with the chlorotic appearance of the foliage owing to lower concentrations of chlorophyll, lower photosynthetic rates, increased carbon isotope discrimination, reduced chlorophyll fluorescence, and pronounced N and P deficiency. These factors, in turn, corresponded to the poor height increment and limited foliage production in these trees. In sharp contrast, the chloroplasts of the trees that had received the blend treatment typically had enhanced thylakoid stacking, an overall greater number of grana per chloroplast of trees, and in general, a lack of starch grain. These visual observations corresponded with a doubling of photosynthetic rates, a reduction in carbon isotope discrimination and a significant increase in chlorophyll concentrations. These factors, in turn, corresponded to an improvement in the nutritional status of these trees and with the dramatic increase in height increment and foliage production.

The observed relationship between thylakoid stacking, the number of thylakoids per chloroplast and nutritional status has been well documented in the literature. For example, Pritchard et al. (1997) reported that longleaf pine (Pinus palustris) seedlings grown under conditions of low N had smaller chloroplasts than those seedlings fertilized with N. More importantly though, chloroplasts of the seedlings grown under conditions of low N supply had approximately half the number of thylakoids per granum than those chloroplasts of fertilized seedlings. They also reported that N deficiency resulted in a higher percentage of chloroplasts that contained either swollen or disintegrated thylakoid systems. Kutik et al. (1995) recently studied the relationship between N status and the ultrastructure of sugar beet (Beta vulgaris L.). Similar to the findings by Pritchard et al. (1997), they reported that improved N status resulted in a near two-fold increase in the number of thylakoid membranes per granum and also a small increase in the total number of grana. Kutik et al. (1995) reported that N deficiency in sugar beets reduced the electron density of the stroma. They attributed this observation to a possible reduction in the concentration of the Rubisco protein due to N deficiency. Laza et al. (1993) grew rice cultivars under four levels of N supply. Similar to the finds noted above, increasing
N supply resulted in an increase in chloroplast size as well as an increase in the number of grana per chloroplast. As reported above by Kutik et al. (1995), high supplies of N also resulted in an increase in the density of the stroma. The increased density of the stroma was accompanied by a significant increase in the concentrations of total proteins. Vesk et al. (1966) had earlier reported that N deficiency resulted in a reduction in the number of grana and the number of thylakoid membranes per granum in both spinach and tomato plants. In contrast to the above findings, Hall et al. (1972) reported that maize plants grown under conditions of N deficiency did not have any effect on the number of grana per chloroplast though the size of the chloroplasts was reduced.

Spiller and Terry (1980) reported that Fe deficiency resulted in a strong reduction in the number of grana per chloroplast as well as the number of thylakoids per granum. Stocking (1975) had earlier reported that maize (Zea mays L.) plants grown under conditions of low Fe supply had chloroplasts with poorly developed grana, often with the lamellar system extending the length of the chloroplast. Vesk et al. (1966) also reported a lack of grana in spinach plants grown under conditions of Fe deficiency.

Vesk et al. (1966) reported that deficiencies in K and Ca in tomato plants resulted in a general reduction in the number of grana per chloroplast. Deficiencies in Zn, Cu, and Mo, however, did not have an apparent effect chloroplast ultrastructure. In a subsequent study by Whatley (1970), chloroplasts from Phaseolus vulgaris grown under conditions of S and P deficiency also did not have specific ultrastructure effects. Hall et al. (1972) reported maize plants grown under conditions of low Ca supply were often swollen and many chloroplasts appeared to have a ruptured envelope. The chloroplasts of maize plants grown under a condition of low Mg supply were reported to have a reduction in grana and also a disorganization and disintegration of the chloroplast membranes. Sulfur deficiency caused the shape of the chloroplasts in these maize plants to become irregular, while those grown under with low P supply did not appear to have pronounced effect on the structure of the chloroplasts.

Rock et al. (1992) investigated the structure of chloroplasts of four genotypes of Arabidopsis thaliana (L.) Heynh., three of which were mutants. They found that the mutants had significantly fewer thylakoid lamellae per granum stack. Interestingly, the
mutants seemed to compensate for the reduction in thylakoid stacking by increasing the number of grana per chloroplast, as well as the number of chloroplasts per cell.

In the present investigation, the study of chloroplast structure was restricted to that of the control and the fertilizer treatment that consisted of N, P and the blend fertilizers that consisted of Mg, K, Fe, Zn Cu and S. While significant improvements in chloroplast development were evident with the addition of this fertilizer it is not possible to discern which elements may have had a contributing factor and those which did not. Most certainly, the improvement is structure would have been mainly attributed to the improvement in N status. In the absence of additional sampling, the relative contribution of the remaining elements is unknown.

**Why are starch grains present in the chloroplasts of unfertilized trees?**

The precise reasons for the formation of large starch grains in the chloroplasts of the trees from the control plots is not clear. It is most likely the result of a combination of two factors that are inter-related with one another. The first of these factors is related to the internal demand for fixed carbon by the vegetative portions of the plant or tree. The second factor is the availability of inorganic P within the chloroplast.

The fertilized trees in this experiment dramatically increased both their height growth and their foliage and branch production during each of the three growing seasons following fertilization. Accordingly, these trees could be considered to be source limited under which condition the demand for fixed carbon is greater than that supplied by the photosynthetic apparatus. Under this condition, Madore (1997) suggested that the growth of the tree is limited or regulated to prevent an excessive loss of carbon from the chloroplast. This internal mechanism would prevent the risk of a decline in Rubisco availability during a period of high vegetative growth. Hence, this explanation may have in part serve to explain the lack of starch grains in the majority of chloroplasts in the trees that had been fertilized and had exhibited superior growth rates. In sharp contrast, the control trees in this experiment could be categorized as being sink limited in so far as their growth on this site was severely stunted. Therefore, the demand for fixed carbon
may be less than that in the fertilized trees, and it could be argued that the risk of a decline in Rubisco availability in the control trees would not be as high as it would be in the fertilized trees. In this regard, Fredeen et al. (1989) concluded that the accumulation of starch in the chloroplasts of soybean under conditions of P deficiency was most likely due to the production of fixed carbon in excess to that required by the plant. In an earlier study, Hurewitz and Janes (1983) studied the relationship between starch accumulation in the leaves of tomato (*Lycopersicon esculentum* Mill.) plants and the sink represented by root growth. As the temperature of the rooting medium was reduced from 30.0 °C to that 15.6 °C, thereby retarding root growth, the starch concentrations in the leaves increased from a concentration of 64.3 μg/mg dry wt to 123.3 μg/mg dry wt. The authors attributed this change in internal carbohydrate allocation was mainly due to the change in C sink as represented by the demand for C by the root biomass.

The second factor that may have contributed to the formation of starch grains in the chloroplasts of the control trees pertains to aspects of P nutrition. The control trees, which had low photosynthetic rates, were also found to be P deficient. There is significant evidence in the literature to support the conclusion that the availability of inorganic P within these trees may have been a prominent contributor to the formation of starch grains in the chloroplasts of control trees. The chloroplast is well known to be a P demanding organelle. Inorganic P is transported from the cytoplasm into the chloroplast via the phosphate transporter in exchange for triose-P, which is pumped from the chloroplast to the cytoplasm (Figure 4.18). The triose-P is subsequently converted in the cytoplasm into sucrose, a readily soluble carbohydrate that is transported for use throughout the plant (Marschner, 1986). The conversion of triose-P into sucrose regenerates inorganic P, which can then be made available for exchange with triose-P again. According to Dietz and Harris (1997), it is this partitioning of the organic and inorganic P fractions between the cytoplasm and the chloroplast that determines the production of starch or sucrose. They suggest the rate of efflux by the translocator is related to the Pi concentration within the cytoplasm and the stromal trios P concentration. As the concentration of available P decreases within the cytoplasm, the trios P within the chloroplast will become converted to starch. Hence, P deficiency may be the principal factor controlling starch formation in the control trees by limiting both the growth and
Figure 4.18. Pathway of sucrose and starch synthesis in the chloroplast and cytoplasm during photosynthesis. Note that starch formation occurs within the chloroplast and sucrose formation occurs within the cytoplasm. Also note the one to one exchange of Pi from the cytoplasm to the chloroplast in exchange for triose-P via the triose-P transporter located within the chloroplast membrane (Adopted from Madore, 1997).
hence, the vegetative demand for fixed carbon, and secondly, by limiting the flux of triose P across the chloroplast membrane.

Rao and Terry (1995) reported that sugar beets grown under conditions of limited P allocated more photosynthate to the production of non-P containing carbohydrates such as starch than to the production of sugar phosphates. When the P supply was increased, the starch concentrations in the foliage decreased rapidly with a concomitant increase in sucrose concentrations. The authors referred to this alteration in internal P allocation under conditions of P deficiency as an adaptive mechanism by which plants and trees "free-up" Pi to maintain the process of photosynthesis. Indeed, while the P concentrations in the foliage of sugar beets were reduced by 88%, the rate of photosynthesis was only reduced by approximately 25%. In an earlier study of sugar beets, Rao et al. (1990) had concluded that this adaptive mechanism to conditions of low P supply also permitted the plant to continue photosynthesizing while at the same time conserving photosynthate in the form of starch that could be made available when supplies of P increased at a latter time. Fredeen et al. (1990) had also concluded that the increased production of starch in soybean (Glycine max [L.] Merr.) plants grown hydroponically under conditions of P deficiency may also be a strategy to conserve stromal Pi. In yet another study, Heldt et al. (1977) reported that starch synthesis increased in the leaves of spinach (Spinacia oleracea L.) under conditions of P starvation or when the cytoplasmic Pi concentrations were artificially induced through the addition of mannose. The findings of Foyer and Spencer (1986) are particularly noteworthy. These researchers investigated the effect of cytoplasmic Pi concentrations on the ratio of starch and sucrose production in pea (Pisum sativum cv. Kelvedon Wonder), barley (Hordeum vulgare L. cv. Sonja) and spinach (Spinacea oleracea L. cv. Virtuosa). The ratio of sucrose to starch in barley plants grown under conditions of P deficiency was 3.47 but increased to 11.14 in similar plants grown without P deficiency. The change in carbohydrate allocation with changes in P status was less evident in spinach plants and absent in soya plants. These findings indicated that different plant species, and presumably tree species, might vary their response to conditions of low P availability. Radin and Eidenbock (1986) reported that P deficiency increased the allocation of carbohydrate production to starch in cotton (Gossypium hirsutum L.) plants. In yet
another study, Fredeen et al. (1989) reported that the starch and sucrose concentrations in the foliage of soybean plants which were P sufficient were approximately 0.4 and 0.7 g/m\(^2\) respectively. Similar plants grown under the condition of P deficiency had starch and sucrose concentrations of 12.9 and 0.20 g/m\(^2\) respectively clearly indicating a shift in C allocation under different P conditions. In the current experiment with western hemlock, the ratios of starch to sucrose were not determined. Future analysis is recommended to ascertain the effect of nutritional status, and in particular that of P, on the internal carbohydrate allocation that may shed light on the strategies employed by western hemlock to adapt to growth under P limitation. Conroy et al. (1990) reported that the starch content of radiata pine (Pinus radiata D. Don) seedlings increased concomitantly with P deficiency.

Conifer species typically found within Canadian forests, including that of western hemlock, are generally thought to grow under conditions of limited P supply owing to the acidity of soils on which they occur. While there has been some research attention to strategies on the part of conifers to increase P acquisition and use efficiencies, there has been little if any efforts made toward understanding the physiological aspects that actually define nutrient efficiency of these same conifer species. Consequently, though the control trees in this experiment may have employed the strategy suggested by Rao and Terry (1995) and others, research into this interesting strategy for our conifer species is currently lacking but certainly warranted.

The addition of N alone decreased the concentration of both total P and Pi below that in the needles of control trees and therefore, as noted above, further aggravated an existing condition of P deficiency. Unfortunately, the structure of the chloroplasts in the needles from these trees was not examined so the presence or absence of starch grains in these the chloroplasts of these trees remains unknown. It would seem prudent however, to expect that these trees may also have employed a strategy similar to that of control trees to effectively utilize the available P. It could be argued that this adaptive strategy might have, in part, contributed to the ability of these plants to maintain substantially higher photosynthetic rates than the control trees.
Possible effect of starch grains on photosynthetic capacity

In the present study, the vast majority of chloroplasts examined in the needles of control trees had a single large starch grain that occupied approximately 90% or more of the plastid’s volume. These same trees also had significantly lowered photosynthetic rates than the fertilized trees. In sharp contrast, the trees that had received the blend treatment had chloroplasts that often had no starch grains or, when present, occupied a much smaller portion of the plastid’s volume. Several researchers have raised the question as to whether the large size of such starch grains may have physically impeded the ability of these plastids to function effectively.

A number of researchers have questioned if large starch grains, such as those in the present study, may cause a mechanical disturbance and hence, may in part, be responsible for reducing the photosynthetic rates of these trees. For example, Ariovich and Cresswell (1983) studied the effects of N and P nutrition on starch accumulation in Panicum maximum, a C4 plant. They reported a significant accumulation of starch in plants that had been deprived of N and P and further, that the thylakoid membranes were disrupted following de-starching in the dark. An observed reduction in the photosynthetic rates of these plants was related to the degree of starch accumulation and the authors argued that this reduction was the result of the disruption to the thylakoid structure. In an earlier study, Cave et al. (1981) reported that the large starch grains in Trifolium subterraneum cv. Dinninup following CO₂ enrichment appeared to disrupt the chloroplast structure. In their study of seasonal effects on starch grain size and presence, Senser et al. (1975) reported that fully developed Norway spruce needles sampled during the early growing season often had several large starch grains within their chloroplasts that resulted in what the authors termed a strong deformation of the thylakoid system. As noted earlier, Park and Tsunoda (1979) investigated the effect of low temperatures of the chloroplast structure of rice. They reported significant starch accumulation in the form of numerous starch grains with a reduction in temperature, which resulted in the deformation of the chloroplasts and disorganization of the thylakoid structure. Carmi and Shomer (1979) reported that de-topping of bean (Phaseolus vulgaris L.) plants resulted in the accumulation of starch grains, which appeared to result in a deformation and
disorientation of the thylakoid membranes and grana. More recently, Pritchard et al. (1997) concluded that N deficiency in longleaf pine seedlings resulted in the accumulation of starch grains, which in turn, caused mechanical damage to the chloroplast structure. Sasek et al. (1985) concluded that the accumulation of large starch grains in cotton (*Gossypium hirsutum* L.) coincided with low photosynthetic rates that mechanical damage may have been inflicted on the chloroplast structure. They further stated that before the photosynthetic rates could be increased, such as in the current study with western hemlock following N additions, the thylakoid membrane system would have to be repaired as the size of starch grains is reduced. If the presence of large starch grains in the western hemlock trees caused a mechanical injury to the thylakoid membranes then the injury must have been reversible as evidenced by the increased rates of photosynthesis in these trees following the addition of N and other nutrients. Sasek et al. (1985) suggested that the length of time that would be required to increase photosynthesis rates following an improvement in the nutritional status of trees may be indicative of the extent of damage to the chloroplast structure that had existed prior to fertilization.

Several investigators have speculated that the presence of large starch grains in chloroplasts reduces the photosynthetic rates of plants and trees by reducing light interception. In his early review of chloroplasts and key enzymatic systems, Wildman (1969) wrote that the presence of large starch grains often distorts the shape of the chloroplast. As was the case in the present study, chloroplasts that do not have starch grains are typically flat or disc shaped. As starch grains increase in size the shape of the chloroplast becomes distorted and becomes sphere or football shaped, as was the case for chloroplasts representing the control trees. The resulting change in position of the chlorophyll containing grana with respect to the available light could be argued to result in a reduction in the light absorption capability by these chloroplasts with the resulting reduction in photosynthetic rates. Park and Tsunoda (1979) agreed with the above argument by Wildman (1969), stating that the large starch grains observed in their study must have prevented or reduced the light penetration to the photosynthetic active site.

The presence of large starch grains may also have a negative effect on the photosynthetic rates of plants and trees by increasing the resistance of CO₂ diffusion from the ambient air to the site of fixation along the stomata pathway, also known as the
mesophyll resistance (Jarvis, 1971). As noted by Rackman (1966), the grana in chloroplasts that contain large starch grains are often located some distance from the cell walls that abut the intercellular spaces. This increases the distance, and hence, the resistance to CO₂ diffusion. In this regard, Nafziger and Koller (1976) reported a statistically significant correlation between stomata resistance to CO₂ diffusion and starch concentrations in leaves of soybean. In this same study, stomata resistance was not correlated with concentrations of soluble sugar and that photosynthetic rates declined as the starch concentrations increased.

As emphasized by Madore (1997), the relationship between photosynthesis and carbohydrate accumulation is not a simple cause and effect relationship. Nevertheless, and in consideration to the comments by Madore (1997), there appears to be an argument that the significant accumulation of starch grains in the chloroplasts of the control trees may, in part, have contributed to the low photosynthetic measurements in these western hemlock trees.

**Seasonal and diurnal variation in starch grain presence**

The needles collected for examination of chloroplast ultrastructure in this investigation were collected during late September of the second growing season. Given the potentially adverse affect the large starch grains on photosynthesis, as noted above, limiting the sampling to the end of the growing season raises several interesting questions. Were the large starch grains in the chloroplasts of the control trees only present during the end of the growing season, or were the starch grains in these trees present for much of the growing season? The presence of starch grains during the middle of the growing season, as opposed to that only to that of the end in early fall, would indicate that they may have had a more significant effect on the ability of these trees to fix carbon on an annual basis. Furthermore, knowing their presence or absence during the growing season would also provide more information as to the physiological status of these trees during mid season. Secondly, the present sampling did not include nighttime or early morning sampling. The latter sampling may have provided us with an insight into the diurnal dynamics of the starch grains. For example, if the changed in size
significantly following the period of dark respiration could have damage to the membrane system of the chloroplast be evident?

Senser et al. (1975) examined the changes in starch concentrations in the needles of a 50-year-old Norway spruce tree over the course of three growing seasons. Their analysis was complemented with an examination of the chloroplast ultrastructure in needles collected during the course of shoot elongation, mid summer, fall and winter. They reported that the concentrations of starch were highly variable and two patterns became prevalent. The first phase was that of early spring when starch concentrations were high and starch grains were evident. They referred to this phase as a period at which time starch represented a reserve pool for future needle growth, which was subsequently depleted during the period of shoot and needle elongation following bud burst. They termed the second phase as the accumulation of starch in needles during mid to late summer, which was correlated with a period of high production of assimilated carbon. They do not report on the presence or lack thereof, of starch grains during the latter period of the growing season as reported in the present study with western hemlock. However, it is noteworthy that they found that during the mid summer, electron microscopy of needles collected during the day showed large starch grains similar to those reported in this study and which subsequently disappeared upon darkening. This raises the question as to whether the presence of starch grains varies over the course of a few days or within a 24-hour period. Unfortunately, the authors provided no information regarding the nutritional state of the tree from which needles were collected, nor estimates of its productivity which would have made a comparison of the results contained in their study to that of the present study more definitive.

In a similar study, Soikklei (1978) documented the presence of starch grains and their characteristics in the chloroplasts in needles of Norway spruce growing in central Finland. He reported that the chloroplasts in needles that had been collected during the month of May and during the summer months had a single large starch grain that occupied most of the chloroplast volume. The starch grains disappeared by the end of September but reappeared as early as March of the following year. Pritchard et al. (1997) recently reported that longleaf pine seedlings were found to have large starch grains during the spring months but the former were rarely found in chloroplasts sampled in the
autumn. Chabot and Chabot (1975) also studied the changes in the chloroplast ultrastructure of balsam fir needles, including the presence and characteristics of starch grains, over the course of a 12-month period. Chloroplasts in tissue collected from within the bud prior to bud burst had what the authors termed as rudimentary chloroplasts which contained small starch grains and a poorly developed membrane system. The small starch grains were also evident in the examination of immature and young needles. During the mid season, chloroplasts were found to have starch grains near the cell wall on what the authors referred to as favorable days. During the period of November to March, no starch grains were evident. The largest starch grains were present in 1-year-old needles collected during the month of April indicating that they were photosynthesizing. However, as in the case of Senser et al. (1975), no information is provided by the authors with respect to the nutritional status of their experimental trees nor their growth rates.

Barnes et al. (1995) recently studied the effect of elevated CO₂ concentrations and K deficiency on the photosynthetic rate and carbohydrate content in the needles of 2-year-old Norway spruce seedling. While they too only measured starch concentrations by electron microscopy in needles collected at the end of the growing season, they reported the seasonal changes in the concentrations on sucrose, glucose and fructose from the period of early May to late October. Sucrose concentrations were found to generally increase over the course of the growing season while the concentrations of fructose were found to decrease. More interesting, the concentrations of glucose were reported to initially decrease in concentration during the periods that followed bud burst. Concentrations of glucose were then reported to have begun to increase have way through the growing season and reached a maximum by late October. Little and Loach (1973) reported on dynamic nature of starch concentrations in needles over the course of a single growing season collected from 6-year-old balsam fir trees. Starch concentrations were found to increase several-fold during the spring and peaked in concentration shortly after budburst then declined in concentration to lowest level in late August.

Unfortunately, in the present study, conclusive statements cannot be made regarding the duration which the chloroplasts of the control trees contained starch grains that occupied the majority of the plastids volume. Limited time and monies prevented a more comprehensive study in this regard. While Chabot and Chabot (1975) noted that
the mere presence of starch grains and their size cannot be used as a quantitative estimate of photosynthetic rates as translocation may be as much a factor that determines the accumulation of starch within chloroplasts as the rate of production. Nonetheless, the analysis of chloroplast ultrastructure of these western hemlock trees had indicated clearly that nutritional status, as affected by fertilization has resulted in significant changes in chloroplast ultrastructure that may be the driving factor for the observed growth rates.

Effect of fertilization on the efficiency of thylakoid membranes

As noted by Krause and Weis (1991) in their review of chlorophyll fluorescence and photosynthesis, the Fv/Fm variable has become an important measurable parameter of the photosynthetic apparatus and reductions in this ratio are indicative of environmental stresses that affect PS II efficiency. Bjorkman and Demmig (1987) have reported that the Fv/Fm ratio remains within a generally constant among a wide range of C3 species with a mean value of approximately 0.832 (± 0.004). In the present experiment with western hemlock, nutrient deficiency was found to have a pronounced effect on the efficiency of PS II, which was alleviated with the addition of N. This increase in the Fv/Fm ratio with N fertilization was concomitant with the increase in chlorophyll concentrations as well as the improvement in chloroplast ultrastructure as noted above.

The relationship between nutrition and chlorophyll fluorescence has been well documented in the literature. In sharp contrast to the findings reported in this experiment, Schaberg et al. (1997) reported that N additions made to a mature red spruce stand did not have any effect on chlorophyll fluorescence. This stand was considered to be N deficient with the N concentration in foliage collected from the control plots being approximately 0.85 %. Fertilization had increased the N concentrations to concentrations between 1.3 and 1.8%. It is noteworthy to report that the lack of any change in chlorophyll fluorescence in this investigation was accompanied by the lack of any change in either photosynthetic rates or chlorophyll concentrations. Similarly, Ciompi et al. (1996) reported that foliar N concentrations in sunflower had no effect on chlorophyll fluorescence in sunflower Helianthus annuus L.). Strand (1997) recently studied the
effect of fertilization and irrigation treatments replicated within a 19-year-old Scots pine plantation growing in northern Sweden. The Fv/Fm ratio in 1-year-old foliage increased from a value of 0.71 for the control foliage to 0.78. Irrigation had no effect on chlorophyll fluorescence. Wang and Kellomaki (1997) studied the effect of elevated N supply on the photosynthetic rates and chlorophyll fluorescence in foliage of a 25-year-old Scots pine stand located in Finland. In contrast to Strand (1997) and Schaberg et al. (1997), but in agreement with the results of this study with western hemlock, Wang and Kellomaki (1997) reported the photochemical efficiency of the PSII was affected by N additions. The Fv/Fm ratio was increased from 0.78 in control plots to a value of 0.83 in fertilized plots. Unfortunately, these authors did not report the foliar N concentrations in the control and treated plots.

In the present experiment with western hemlock, the P additions had no effect on the Fv/Fm ratio. This finding is similar to that reported by Fay et al. (1990). That latter reported that Fv/Fm ratio was not affected by foliar P concentrations in barley (Hordeum vulgare L. cv. Manitou) plants. In contrast, Loustau et al. (1999) recently reported on a study into the effects of P deficiency on the processes limiting photosynthesis in maritime pine (Pinus pinaster Ait) seedlings. They reported that even a small change in the foliar P concentrations, from 0.05% to 0.6%, resulted in change in the Fv/Fm ratio from 0.74 to 0.83. Jacob (1995) and Guidi et al. (1994) reported near identical Fv/Fm values for P deficient and P fertilized foliage in greenhouse experiments using sunflower and maize plants, and sunflower and soybean plants respectively.

The addition of the blend fertilizer also did not result in any change in the photochemical efficiency as reflected by the Fv/Fm ratio in the western hemlock trees. Strand and Lundmark (1995) had earlier reported similar findings while working within the same stand. Dreyer et al. (1994) studied the effect of Ca and Mg additions to a 12-year-old Norway spruce plantation growing in the Vosges Mountains. The older needle classes on many of the trees within this young plantation appear yellow consistent with the visual symptoms of Mg deficiency. The Fv/Fm value for 1-year-old needles from the control plots was approximately 0.686 compared to a value of 0.740 for the plots that had received Ca and Mg additions. The authors also reported that a negative correlation between Fv/Fm and foliar N concentrations was evident. Maksymiec and Baszynski
(1996) had reported that Cu deficiency of bean (*Phaseolus coccineus* L., cv. Piekny Jas) resulted in a 15% reduction in the Fv/Fm ratio. Jung and Winter (1992) reported that exposure of 4-year-old *Abies nordmanniana* to SO2 resulted in a decline in the photochemical efficiency of PSII. The Fv/Fm ratio declined from a value of 0.8 before treatment to a low of 0.77 in trees that were both Mg and N deficient.

**Effect of Fertilization on Nutrient Fractionation**

Fractionation of total P into organic and inorganic components revealed a similar pattern to the allocation of P as documented in Chapter 3. While the pattern of allocation did not change, the determination of the metabolically active fraction of P indicated that this pool was decreased following N fertilization and may have been responsible for limiting photosynthesis following N additions on this site. The determination of the free amino acid concentration also revealed a similar pattern to that which had been documented in Chapter 2 and 3 indicating that the fractionation of externally supplied N would seem to follow a similar pattern across a range of site types and age classes of western hemlock.

**Management Implications**

The growth results of this study confirmed that the growth of this plantation could be significantly increased with the addition of N at the conventional rate of 225 kg/ha. Indeed, the response of this stand, and those other western hemlock stands growing on similar sites in the region must certainly represent some of the most dramatic and impressive fertilization responses in Canadian forests. The results of this research also indicate that P was also a factor limiting productively on this site, though the small additional height growth achieved does not appear to justify the extra costs of applying P.
Conclusions

1) The increased photosynthetic rates, as measured on a unit leaf area basis, and the reduction in carbon isotope discrimination during each of the first two growing seasons following fertilization indicated that nutrient deficiency significantly reduced the photosynthetic capacity of the western hemlock trees growing on this site.

2) The reduction in photosynthetic capacity was most strongly affected by severe N deficiency. The latter limited foliage production and the accompanying needle surface area. The alleviation of N deficiency following N fertilization resulted in a dramatic increase in the needle area during each of the three growing seasons following fertilization. In addition, there is evidence to suggest that N deficiency was predominantly responsible for the reduction in the number of thylakoid membranes per granum and the number of grana per chloroplast and a concomitant decrease in chlorophyll concentrations. Furthermore, N deficiency reduced the internal sink for carbon, which may have contributed to the formation of large grains in the chloroplasts of control trees. These starch grains may have reduced the photosynthetic capacity of these trees by reducing light interception, increasing the resistance to CO₂ diffusion, and by causing a mechanical damage to the thylakoid membranes. Finally, N deficiency resulted in a reduction in the photosynthetic properties of the thylakoid membrane itself as indicated by the lower photochemical potential of the control trees as compared to the trees that had received N additions.

3) The photosynthetic capacity of these trees was also limited by a secondary deficiency in P. The addition of P at the rate of 300 kg/ha, in combination to N, increased photosynthetic rates, as measured on a unit leaf basis, during two measurement periods in the first growing season. Carbon isotope discrimination was also reduced by P additions during each of the two growing seasons following fertilization. P deficiency may have limited the photosynthetic capacity on these trees by reducing the regeneration of RuBP (ribulose-1,5-bisphosphate).
4) The three-year height increment was significantly increased with the alleviation of N deficiency and the concomitant improvement in the photosynthetic capacity of these trees. The addition of P at the rate of 100 kg/ha had no effect on the three-year height increment but the latter was improved when applied at the rate of 300 kg/ha. The small, though significant increase in the three-year height increment following the latter P addition was attributed to an increase in the photosynthetic capacity of these trees due to the elimination of P deficiency.

5) The addition of N alone resulted in a dramatic increase in the arginine concentrations. The latter decreased with the addition of P and the blend elements indicating that the addition of these nutrients may have increased the internal utilization of applied N.

6) The inorganic P fraction was limited in foliage from the control and N treatment plots. This fraction increased with concomitantly with increasing total P concentrations in current-year foliage. The increase in the inorganic pool of P, the metabolically active fraction of P, in the trees that received P additions coincided with a reduction in carbon isotope discrimination. This result indicates that future investigations into the relationship between nutrient supply and photosynthetic capacity should include a determination of the inorganic fraction of P in addition to that of mere total P concentrations.
CHAPTER V  
A STUDY OF P NUTRITION USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY  

Introduction  

White et al. (1996) reported that western hemlock and Douglas-fir differed in their growth response to additions of P, and further, suggested that western hemlock may have a higher P requirement than that of Douglas-fir for a given level of productivity. The fertilization of the five-year-old western hemlock plantation with varying levels of P (Chapter IV) also seemed to support the assertion that western hemlock has a high P requirement.  

Marschner (1986) suggested that differences in nutrient use efficiency among species or genotypes, as reflected by lower critical concentrations of a particular nutrient, are the result of a number of physiologically based mechanisms which are enhanced in species or genotypes characterized by high efficiencies. The first of these mechanisms which contributes to defining nutrient use efficiency is thought to be the internal retranslocation of nutrients within the plant or tree. Retranslocation of nutrients within trees species has been the subject of many studies to further the understanding of nutrient cycling on sites where nutrient supply is limited (e.g. Blaser et al., 1967; Wikner, 1982; Nambiar and Fife, 1991; Hopmans and Clerahan, 1991; Saur et al., 1992; Chapin and Kedrowski, 1983; Millard, 1994; Miller et al., 1979). For example, Fife and Nambiar (1982) studied the accumulation and retranslocation of macronutrients in the foliage of radiata pine growing in southeastern Australia. They reported that approximately 86, 48 and 39% of the N, P and K requirements, respectively, of the current-year needles may have been translocated from the needles formed during the preceding growing season. In this regard, Miller et al. (1979) suggested that it might be useful to consider that there are essentially two levels of nutrient mobility. The first of these levels is represented by the nutrients that are available for uptake by either the roots or foliage. The second level is represented by the potentially mobile forms of nutrients within the plant or tree, that can retranslocated to meet the demands of the plant or tree that cannot be met by the first level.
The second mechanism proposed by Marschner (1990) is that nutrient use efficiency is linked to the ability of plants or trees to increase the proportion of the nutrients that are taken up from the rooting environment and made available for metabolic activities. Low nutrient use efficiency is thought to be attributed to a greater compartmentation or chemical binding of the nutrient in question (Marschner, 1990). The retention of P in roots of conifers due to the chemical binding of P as Al-phosphate in the free space of roots (McCormick and Borden, 1974; Cumming et al., 1986) is an excellent example of compartmentation on a whole-plant (i.e. root versus shoot level) basis. Indeed, Serrem (1991) reported that the superior growth of black spruce families was directly related to high rates of P use efficiency. He further reported that high growth rate was attributed to the ability of these families to retain Al in the roots and translocate the P to the shoot. Maliondo (1986), Cruikshank (1990) and White (1992) reported similar findings into the genetic aspects of black spruce nutrition. In their recent review, Sattelmacher et al. (1999) point out that the apoplast may serve as an important site for nutrient storage and that the characteristics of the leaf apoplast may contribute to defining a plant’s or tree’s nutrient use efficiency.

While the direct measurement of the translocation of nutrients from root to shoot, or the retranslocation of nutrients within foliage age classes, is relatively straightforward, studies into nutrient compartmentation at a cellular level are more problematic. The primary challenge, of course, is the measurement of changes in the spatial distribution of nutrient concentrations at a small scale without disturbing the plant system in a significant manner. In this regard, the development and availability of nuclear magnetic resonance (NMR) has allowed researchers to carry out experiments at the cellular level that were otherwise not possible. Indeed, NMR has many advantages, as noted by Roberts (1986): (1) only metabolites that are free to enter into metabolic processes are measured, (2) these free metabolites can be measured without disturbing the plant in any significant manner, (3) distribution of nutrient concentrations between different cell compartments can be obtained, and finally (4) sequential measurements in vivo can be made over time by changing the chemical environment within the NMR probe to understand the effects of the chemical or physical environment on aspects of the plant system under question.
Application of NMR in Plant Physiology

Despite the advantages of NMR, this approach has, surprisingly, received little attention by researchers within the field of tree nutrition. In sharp contrast, there has been a wide application of NMR spectroscopy within the field of plant physiology, including that of plant nutrition, since the early 1980's. These studies have mainly focused on issues related to P and N nutrition, intracellular pH, and the determination of the energy status of cells, which are briefly reviewed below.

NMR spectroscopy and the determination of intracellular pH

The internal regulation of cellular pH has long been recognized (e.g. Smith and Raven, 1979). As noted by Roberts (1986), there are essentially two questions that are of primary interest to plant physiologists. The first of these concerns the mechanisms responsible for the maintenance of cellular pH which optimizes plant function. The second question relates to the importance of changes in pH at the cellular level as a means for transmitting information from one cell to another. There have been many approaches to the measurement of pH at the cellular level but most have been problematic (e.g. Smith and Raven, 1979). More recently, NMR has been utilized by plant physiologists as the approach of choice as the advantages of NMR in general, noted above, lend this approach particularly well to the questions related to pH regulation.

The guiding principles of measuring pH using NMR are provided by Roberts (1986). What is noteworthy in this review, is that this approach differs somewhat from other applications on NMR. Measurements of pH are made by way of relating the chemical shift of a pH-sensitive shift to actual pH. The latter is achieved by using a titration curve of chemical shift to pH (Roberts, 1986).

Most recently, Vogel et al. (1999) used $^{31}$P NMR to determine the pH of the vacuolar and cytoplasmic compartments in cultured Catharanthus roseus cells. The chemical shift of the inorganic P (Pi) pool indicated a vacuolar pH of approximately 5.6 and a cytoplasmic pH of 7.2. Katsuhara et al. (1997) also used $^{31}$P NMR to gain an understanding of cellular mechanisms of salt tolerance of barley roots. In particular, they
were interested in the effect of salt stress on intracellular pH. They were able to
demonstrate that salt stress had no effect on the pH of the cytoplasmic compartment but
did result in a slight increase in the pH of the vacuolar compartment. The authors
speculated that this was due to the presence of a Na\(^+\)/H\(^+\) antiporter in the tonoplast, which
transported Na to the vacuolar compartment in exchange for H\(^+\). The latter was thought
to have been subsequently extruded to the external medium by means of a proton-
pumping ATPase in the plasma membrane. The authors concluded by stating that the H\(^+\)
-ATPase was the primary factor responsible for mitigating salt stress in barley roots.

Earlier, Sakano et al. (1992), in a \(^{31}\)P NMR study of cytoplasmic acidification in
*Catharanthus roseus* cells, reported that cytoplasmic pH is regulated by proton pumps
located in both the tonoplast and plasma membrane. Fox and Ratcliffe (1990)
investigated the relationship between the pH of the external environment and the pH of
both the cytoplasmic compartment and the vacuolar compartment in suspension cells of
oil palm (*Elaeis guineensis* Jacq.). Taking advantage of the ability to obtain sequential
measurements using NMR, the authors were able to demonstrate both short-term and
long-term responses in cytoplasmic pH to changes in the pH of the external medium.

Guern et al. (1989) and Mathieu et al. (1989) used \(^{31}\)P-NMR to study the mechanisms for
pH maintenance in isolated vacuoles, specifically through the activity of proton pumps in
the tonoplast. Sianoudis et al. (1987) also used \(^{31}\)P-NMR to study the effects of
photosynthesis on the pH of the cytoplasmic fraction in green alga (*Chlorella fusca*).
Similar studies into pH regulation of cellular compartments using \(^{31}\)P-NMR have been
reported by Guern et al. (1986), Ohmori et al. (1986), Enami et al. (1986), Reid et al.

**NMR spectroscopy and the study of N nutrition in plants**

Perhaps the most common application of \(^{15}\)N-NMR is in the understanding of
ammonium and nitrate uptake and incorporation into amino acids. For example,
Robinson et al. (1991) used sequential measurement with \(^{15}\)N–NMR to determine the
incorporation of labeled ammonium into amino acids in cell suspensions of carrots
(*Daucus carota* L. cv Chantenay). The oxygenated suspensions were labeled with 20
mM [$^{15}$N] ammonium chloride. The label first appeared in glutamine within the first hour of measurement and increased in concentration during the first 4 hours, then decreased rapidly and was undetectable during the 8-10 h measurement period. The label was detected in glutamate during the 1-2 h period and slowly increased during the duration of the experiment. The label next appeared in alanine during the 2-4 hour period, which also increased slowly in concentration throughout the remaining time periods. The label next appeared in GABA (gamma-amino-butryric acid) during the 6-8 h measurement period and remained constant. In a subsequent experiment, Amancio and Santos (1992) studied the assimilation of both ammonium and nitrate in the fine roots of maize plants. In their first experiment, the root apices were perfused with K$^{15}$NO$_3$. Sequential measurement over a period of twenty hours revealed a pattern of assimilation similar as to what was observed by Robinson et al. (1992). When the experiment was repeated using $^{15}$NH$_4$NO$_3$, similar results were obtained except that the rate of incorporation was much faster when $^{15}$NH$_4$ was used as the label and the pools of the labeled N were much greater. The authors attributed the slower time for the incorporation of N when supplied in the form of nitrate to the time required for the induction of the root nitrate uptake system and of nitrate reductase activity. Thorpe et al. (1989) also reported that the incorporation of ammonium was much faster than that of nitrate while working with cultures of white spruce (Picea glauca [Moench] Voss) using $^{14}$N and $^{15}$N-NMR. Additional examples of the application of NMR to the study of inorganic N uptake and assimilation are provided by Lee et al. (1992), Monselise et al. (1987) and Belton et al. (1985).

In their review of $^{15}$N-NMR, Martin and Driss (1986) noted that $^{15}$N-NMR also has an important application in the study of amino acid synthesis and turnover. For example, Skokut et al. (1982a) used $^{15}$N-NMR and $^{13}$C-NMR to study the utilization of glycine for the synthesis and storage of proteins. Similar studies into amino acid synthesis have been reported for allantoin (Coker and Schaefer (1985), asparagine (Schaefer et al., 1981; Skokut et al., 1982b), methionine (Coker et al., 1987) and glycine (Neeman et al., 1985).
NMR spectroscopy and the study of energy transformations

Roberts (1986b) noted that plant metabolism and growth essentially involve the coupling of light energy which is used for respiration, protein synthesis, and ion transport. Hence, factors that affect the production and turnover of ATP and its byproducts within different chemical and physical environments are of importance to plant physiologists. Nuclear magnetic resonance is one technique that has been utilized to study energy transformations as distinctive signals are detected for the three phosphates moieties of ATP (α, β and γ), inorganic phosphate, and monophosphate esters. For example, Hooks et al. (1994), using $^{31}$P-NMR, estimated the proportions of free and bound nucleotides in maize roots. Rebeille et al. (1985) used $^{31}$P-NMR to address the questions related to starch breakdown and sucrose hydrolysis in sycamore (Acer pseudoplatanus L.) cells. In an earlier study using $^{31}$P-NMR and sycamore cells, Rebeille et al. (1984) were again able to show that respiration rates were not limited by the supply of Pi in mitochondria. Jackson et al. (1986) and Yazaki et al. (1988) provide further examples of the application of NMR in the study of energy transformations.

NMR and the study of P nutrition

As noted by Roberts (1986), when plant tissues are placed in a Fourier transform NMR, a spectrum can be obtained. Each absorption line within the spectrum essentially represents a sub-population of $^{31}$P nuclei within the tissue. However, the spectrum does not represent compounds that comprise the entire P content of the tissue. Rather, only populations that are freely mobile, the proportion of total P that is available to enter into chemical reactions, are measured.

Ayling and Topa (1998) used $^{31}$P-NMR to fractionate total P in root tips of pond pine into vacuolar and cytoplasmic fractions. They reported that the P concentration in the cytoplasmic fraction remained relatively stable at 2 mM across a range of external P supplies. The relative stability of the cytoplasmic fraction at the sacrifice of the vacuolar compartment has been fairly well documented (Lee and Ratcliffe, 1993), Sakano et al. (1992), Tu et al. (1990) Lee et al. (1990) and Roberts and Testa (1988). It is interesting
to note that, despite the latter, the concentration that is maintained in the cytoplasmic fraction does not appear to be uniform between species. For example, while working with fine root tips of pea plants, Lee and Ratcliffe (1983) reported a cytoplasmic concentration of Pi to be approximately 18 mM. More recently, these same authors have reported that the Pi concentration in the vacuolar compartment of maize roots is approximately 8.8 mM. Similar studies have yet to be undertaken using conifers.

As noted in Chapter III, it would stand to reason that there would exist some form of sensory system within the cytosol that detects or perceives deviations in Pi. This system must also be capable of translating this information into a response involving mechanisms that increase or decrease the flux of P across the tonoplast. However, there currently exists no information of the sensory component of the regulation of P concentrations within the cells (Dietz and Harris, 1996). There is also no information on the potential signals that could affect the permeability of the tonoplast or the transport process. However, on the basis of the findings reported by Woodrow et al. (1984) and more recently by Mimura et al. (1990), there appears to be consensus that the vacuolar response is slow, suggesting that short-term cytosolic changes in Pi are ignored.

As noted in Chapter IV the chloroplast is a P-demanding organelle. The relationship between P flux across the tonoplast and possible species differences in the P concentration within the cytosol raises the question as to whether these factors may be related to inorganic P demand by photosynthesis. An interesting question is whether P availability within the cytosol, either the result of flux rates or feedback mechanisms that regulate P availability from the vacuolar pool, or the concentration of P that is maintained within the cytosol is related to shade tolerance or reliance on sunflecks. While species differences in their reliance and response to sunflecks have been studied (e.g. Hansen et al., 1998), their relationship to P nutrition has not yet been addressed.

**Determinant of Free P Metabolites using NMR**

Rolin et al. (1989) used in vivo $^{31}$P-NMR to assess not only the distribution of Pi, but also to study the major P metabolites. Similarly, Lohmeier-Vogel et al. (1986) used in vivo $^{31}$P-NMR to document changes in the major P metabolites in a study of glucose
metabolism. Ruyters et al. (1985) and Vogel et al. (1999) also used this approach to fractionate P. NMR may permit more rapid investigation of P allocation over a range of P supplies than the extraction methods used by Chapin et al. (1983).

Research Questions

The above review of literature and the finding of species differences in fertilization response as reported by White et al. (1999) raise a number of interesting, though challenging questions.

1) Do western hemlock and Douglas-fir maintain similar concentrations of Pi within the cytoplasmic fraction at similar supplies of P?

2) If these species maintain differing concentrations of inorganic P within the cytoplasmic pool:

   i) Are the critical concentrations of Pi that are maintained in the cytoplasmic compartment of each species, or the Pi$_{cyt}$/Pi$_{vac}$ ratios, related to differences in P use efficiency and P requirement?

   ii) Are lower concentrations of Pi in the cytoplasmic fraction compensated by increased rates of Pi flux across the tonoplast?

   iii) What is the feedback mechanism that regulates Pi transport from the vacuolar fraction to prevent the Pi concentration in the cytoplasm from declining below a critical concentration?

   iv) Are the differences between the species in P compartmentation related to differences in shade tolerance between these two species?
3) Does the distribution of total P amongst the major inorganic and organic fractions vary between western hemlock and Douglas-fir across a range of nutrient supplies?

4) If a species can be shown to maintain a higher concentration of inorganic P within the cytoplasmic pool, what are the metabolic costs associated with this requirement? While Raven (1987) briefly discussed the costs and benefits of vacuolation in plants, the potential costs, if any, for a species that maintains a higher cytoplasmic concentration of P have not yet been addressed. An interesting question in this regard is whether or not this nutritional requirement affects the inorganic P that can be made available for metabolic processes related to energy transformations which take place exclusively within the cytoplasmic fraction.

**Objectives**

A fertilization experiment was established in the spring of 1996. The objectives of this experiment were limited to the following:

1) To determine the Pi concentrations in cytoplasmic and vacuolar compartments in current-year foliage of western hemlock and Douglas-fir across a range of external P supplies.

2) To determine the distribution of total P amongst the major inorganic and organic fractions in the current-year foliage of both western hemlock and Douglas-fir across a range in nutrient supplies.

These objectives do not address many of the fundamental questions of interest which had been raised above. This present experiment is deemed to be a first step in a series of research investigations to address the above questions.
Methodology

Site Description

The experiment was conducted in the same plantation that had been used by White et al. (1996) in an earlier study contrasting the nutritional requirements of Douglas-fir and western hemlock (Figure 5.1 and Figure 5.2). The site is located approximately 50 km south of Port McNeill on Canfor’s TFL # 37 (Figure 5.3). The site has been classified as belonging to the very dry maritime coastal western hemlock subzone (CWHxm) with a poor to medium nutrient regime and a moderately dry soil regime (Green and Klinka, 1994). The shrub layer was dominated by dense salal (Gaultheria shallon Pursh) though red huckleberry (Vaccinium parvifollum) and bracken fern (Pteridium aquilinum) were also common.

The original old-growth stand was composed of western hemlock and Douglas-fir and was harvested in 1974 and 1975. The site was broadcast burned in 1976 and planted to Douglas-fir the following year. Significant ingress of hemlock has since occurred. Tree heights currently range from 6 to 10 m. The mean diameter at breast height of the planted Douglas-fir was approximately 10.1 cm while that of western hemlock was approximately 8.5 cm. Though the stand had not been thinned, care was taken to ensure ample opportunity existed for crown expansion.

The soil was classified as an Ortho Humo-Ferric Podzol (Expert Committee on Soil Survey, 1987) which overlies a granitic bedrock. Rooting depth is restricted to a depth of approximately 40 cm by the presence of a basal till. Chemical and physical properties of the mineral soil and forest floor are presented in Table 5.1.

Experimental Approach and Plot Establishment

The experiment consisted of four nutrient levels, which were imposed on 20 western hemlock and 20 Douglas-fir trees (Figure 5.4). Each of the latter represented a single experimental plot for a total of 40 single-tree plots. Each plot radius was 3 m and a 5-m buffer was maintained between plot boundaries. Only well-formed codominant
Figure 5.1. Study site located within the Nimpkish Valley on northern Vancouver Island, comprised of both immature western hemlock and Douglas-fir.
Figure 5.3. Map of Vancouver Island indicating location of study site which was approximately 50 km south of the town of Port McNeill.
Table 5.1. Mineral and forest floor properties by horizon.

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<td>Exchangeable Mg (me/100g)</td>
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Figure 5.4. Single-tree plots of western hemlock and Douglas-fir within the 18-year-old plantation.
trees were selected.

**Treatments**

The following treatments were applied: (1) control (no fertilizer added), (2) N (225 kg/ha), (3) N (225 kg/ha) and P (100 kg/ha), and (4) N (225 kg/ha) and P (500 kg/ha). Nitrogen was applied in the form of ammonium nitrate and phosphorus in the form of triple-super-phosphate. The fertilizers were broadcast applied during early May, 1996. The blend treatment that had been used in each of the aforementioned fertilization trials was not tested in the present study in order to minimize the number of samples required for NMR analysis.

**Foliage Collection**

Current-year foliage was collected from each of the 40 trees in early August of 1996 for NMR analysis. Shoots were collected from the base of the upper one-third of the live crown and transported to the laboratory the following day. Current-year foliage was also collected during October of 1996 and used for determination of foliar nutrient concentrations.

**NMR Analysis**

*In Vitro*

The procedure of Rolin *et al.* (1989) was adopted to study the internal Pi concentrations and major metabolites. Approximately 600 mg of foliage that had been previously ground using a Braun blender was extracted in 5 ml of 1M HClO₄ for 15 min at room temperature. The thick homogenate was centrifuged for 15 minutes to remove the particulate matter. The supernatant was titrated with 2M KHCO₃ to pH of 6.5 and centrifuged at 14 000 rpm to remove the KClO₄ precipitate. The supernatant was dried
using a Savant Speed Vac equipped with a Savant Refrigerated vapor trap. The freeze-dried material was re-dissolved in 3 ml. of 35 mM Mops buffer (pH 7.8). The latter included CDTA (trans-1, 2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), a strong chelator. Previous studies had shown that CDTA was more effective than EDTA in eliminating free divalent metal ions and led to sharper lines for specific P metabolites (Bligny et al., 1990). The solution also contained D$_2$O (20% by volume) to serve as a signal lock.

$^{31}$P spectrum was achieved using a Bruker 300-spectrometer equipped with a 5 mm probe and tuned to 16,000 hz frequency. Relaxation time was set at 0.4 seconds for an acquisition period of 15 min. Relaxation times were increased to 2 sec but were not found to increase the resolution of the spectrum. Similarly, the total acquisition times were increased from 15 min. to 30, 45 and 60 min. Though background noise decreased with increasing acquisition time, no additional points were identified than that at 2.9 PPM which was attributed to inorganic P (Pi).

**In Vivo**

Fresh needles were cut into 4 mm sections and placed within a Mops buffer under vacuum. The NMR spectrum was achieved using a Bruker 300-spectrometer equipped with a 5 mm probe and tuned to a 16 000 Hz frequency. Relaxation time was set for 0.4 seconds for an acquisition period of 15 minutes, which was later increased to one hour.

Spectrum separation between the vacuolar and cytoplasmic fractions became problematic using the above procedure. It was believed that the cytoplasmic fraction underwent anaerobic respiration within the NMR environment. The lack of pH differences in the two compartments would render the two pools indistinguishable, as there would be no phase separation. Consequently, we attempted to overcome this problem by aerating the Mops solution and not placing the needle sections in the solution until the moment before the NMR tubes were lowered into the spectrometer. These steps were not successful and it was not possible to differentiate between vacuolar and cytoplasmic pools.
Determination of foliar nutrient concentrations

Current-year foliage was collected from each tree at the end of the first growing season. As in earlier experiments, the foliage was dried for 48 hrs at 70°C and ground using a Braun coffee grinder. Chemical determination of foliar nutrient concentrations was undertaken using standard procedures noted in chapter II.

Soil Analysis

Samples were collected from the forest floor and mineral horizons from nine representative soil pits within the plantation. The MacMillan Bloedel Sciences Laboratory analyzed the individual samples for chemical and physical parameters using standard laboratory procedures (McKeague, 1978). Soil and forest floor pH was measured using CaCl₂ extractions. Soil organic matter content was determined using the Walkley-Black method. Exchangeable cations and CEC were determined using the ammonium acetate method. Total soil N was determined collimetrically following digestion with sulfuric acid. Available P was determined using the Bray-1 method (pers. comm., Arlene Gammel).
Results and Discussion

The fertilizer additions, as expected, significantly elevated foliar nutrient concentrations and the effect was greater in western hemlock trees than for Douglas-fir, \( P = 0.0001 \) (Table 5.2). Foliar N concentrations in the control trees of both western hemlock and Douglas-fir (Table 5.3) were within the range generally considered to be deficient for each species (Ballard and Carter, 1983). Similarly, the P treatments were effective in increasing the foliar P concentrations, with the greatest increase being achieved with the 500 kg/ha application. The addition of N induced a reduction in the P concentrations in Douglas-fir. The application of P at the rate of 100 kg/ha was also more effective at increasing the foliar P concentrations in western hemlock. Similarly, the addition of N alone resulted in a significant reduction in the P:N ratio indicating a condition of induced P deficiency. The addition of P at the rate of 100 kg/ha was not effective at increasing the P:N ratio to control values. The P:N ratio in the foliage from N alone plots and those that received P at the rate of 100 kg/ha had P:N ratios within a range that induced P uptake in excised roots (Chapter III). The applications of N and P had no effect on foliar K concentrations, nor did K concentrations vary between the two species. Interestingly, neither of the treatments had any effect on the foliar Ca and Mg concentrations, but Douglas-fir trees had significantly more Ca, \( P = 0.0001 \), and less Mg than, \( P = 0.0001 \) (Table 5.2) than western hemlock trees.

In chapter II it was reported that the organic P (Po) fraction increases to a mean of approximately 0.12% and further increases in the total P concentration results in a concomitant increase in the inorganic P (Pi) fraction. Hence, it would be expected that the Pi fraction would be lowest in foliage from the N alone, and greatest in foliage from trees that received P at the rate of 500 kg/ha. The fertilizer additions were therefore effective at supplying foliage material with a range of P concentrations for the NMR experiment.

Representative \(^{31}\)P spectra for the perchloric acid extracts by treatment are reported in Figure 5.5. This procedure was problematic. The only distinguishable P fraction that was detected in extracts from foliage collected from each of the treatments was that of inorganic P at the 2.9 region of the spectrum. The reason for the
Table 5.2. Mean nutrient concentrations by treatment and species: F ratios and level of significance.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>P</th>
<th>P/N</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>60.61</td>
<td>18.37</td>
<td>0.01</td>
<td>0.08</td>
<td>23.68</td>
<td>52.88</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0.0002</td>
<td>0.92</td>
<td>0.78</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td>123.90</td>
<td>266.22</td>
<td>142.40</td>
<td>0.22</td>
<td>0.25</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.88</td>
<td>0.86</td>
<td>0.42</td>
</tr>
<tr>
<td>Species*Treatment</td>
<td>12.54</td>
<td>5.78</td>
<td>12.45</td>
<td>0.96</td>
<td>1.18</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>0.003</td>
<td>0.0001</td>
<td>0.42</td>
<td>0.33</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Table 5.3. Concentrations (%) of macro nutrients and P:N ratio by treatment and species. Means with the same letter for each species do not differ significantly at the p=5%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>P</th>
<th>P/N</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western Hemlock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>1.05 (0.03) a</td>
<td>0.17 (0.01) a</td>
<td>0.17 (0.01) a</td>
<td>0.77 (0.03) a</td>
<td>0.29 (0.01) a</td>
<td>0.19 (0.01) a</td>
</tr>
<tr>
<td>N225</td>
<td>2.28 (0.08) b</td>
<td>0.15 (0.01) b</td>
<td>0.07 (0.00) b</td>
<td>0.74 (0.03) a</td>
<td>0.30 (0.02) a</td>
<td>0.21 (0.01) a</td>
</tr>
<tr>
<td>N225 P100</td>
<td>2.19 (0.08) b</td>
<td>0.25 (0.01) b</td>
<td>0.12 (0.00) c</td>
<td>0.73 (0.03) a</td>
<td>0.30 (0.02) a</td>
<td>0.22 (0.01) a</td>
</tr>
<tr>
<td>N225 P500</td>
<td>2.18 (0.08) b</td>
<td>0.40 (0.02) b</td>
<td>0.19 (0.01) a</td>
<td>0.76 (0.02) a</td>
<td>0.28 (0.01) a</td>
<td>0.19 (0.01) a</td>
</tr>
<tr>
<td>Control</td>
<td>1.16 (0.03) a</td>
<td>0.15 (0.01) a</td>
<td>0.13 (0.01) a</td>
<td>0.74 (0.03) a</td>
<td>0.34 (0.01) a</td>
<td>0.15 (0.01) a</td>
</tr>
<tr>
<td>Douglas-fir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N225</td>
<td>1.80 (0.04) b</td>
<td>0.11 (0.01) b</td>
<td>0.06 (0.00) b</td>
<td>0.75 (0.03) a</td>
<td>0.34 (0.01) a</td>
<td>0.13 (0.01) a</td>
</tr>
<tr>
<td>N225 P100</td>
<td>1.76 (0.06) b</td>
<td>0.18 (0.01) b</td>
<td>0.10 (0.01) a</td>
<td>0.78 (0.03) a</td>
<td>0.35 (0.02) a</td>
<td>0.15 (0.01) a</td>
</tr>
<tr>
<td>N225 P500</td>
<td>1.73 (0.05) b</td>
<td>0.41 (0.01) b</td>
<td>0.24 (0.01) c</td>
<td>0.76 (0.02) a</td>
<td>0.37 (0.03) a</td>
<td>0.16 (0.01) a</td>
</tr>
</tbody>
</table>
Figure 5.5. $^{31}$P spectrum of perchloric acid extracts of western hemlock foliage where (a) control, (b) N (225 kg/ha), (c) N (225 kg/ha) + P (100 kg/ha), (d) N (225 kg/ha) + P (500 kg/ha).
lack of peaks that could be attributed to the remaining free P metabolites remains unknown. Increasing the length of acquisition times did not aid in the identification of further peaks (results not shown). It may be that the concentrations of these fractions in the foliage of both western hemlock and Douglas-fir are too low for identification by NMR. Alternatively, as noted by Ratcliffe (1994), problems in spectrum identification or presence may be caused by high degree of polymerization, immobilization by gel formation or precipitation, or interactions of the free P metabolites with paramagnetic ions.

The peaks representing inorganic P in the foliage corresponded to the expected Pi concentrations noted above. A small peak was consistently identified in extracts of control foliage (Figure 5.5), regardless of species. A similar peak was often not present in extracts from foliage collected from plots that had received the N only additions. This finding is consistent with the corresponding reduction in the P/N ratio noted above. The additions of P resulted in a significant increase in the size of the peak thought to represent inorganic P, with the greatest peak documented in extracts from the P 500 kg/ha treatment. Despite apparent agreement between the results of the conventional foliar analysis and \textit{in vitro} determinations, there can be little question that this approach is inferior to conventional methods of diagnosing P status in either western hemlock of Douglas-fir unless additional fractions can be ascertained through additional experimentation.

The results of the \textit{in vivo} measurements of needles of western hemlock are reported in Figures 5.6 and 5.7. No peaks representing inorganic P could be assigned to either the cytoplasmic or vacuolar compartments in needles collected from the control plots. Several peaks were observed in the $^{31}$P-NMR spectra of needles that received P at 500 kg/ha. However, while one peak at 2.0 appears to represent the vacuolar fraction of inorganic P, the second peak at 3.2 remains unidentified. This second peak may represent the cytoplasmic fraction that has undergone acidification due to the presence of anaerobic conditions. The latter would have been the result of the failure to have the proper NMR tube apparatus to ensure continual aeration within the NMR tube. This interpretation would also explain a shift in the cytoplasmic P peak from 2.9 to 2.2.
Figure 5.6. $^{31}$P spectrum of western hemlock needles collected from control plots. Note lack of any recognizable peak indicative of low total P concentration.

Figure 5.7. $^{31}$P spectrum of western hemlock needles collected from plots that had been fertilized with P at the rate of 500 kg/ha. Note two peaks in the area of 2.0 and a small single peak at 3.2.
Conclusions

1) The only peak that was obtained in the \textit{in vitro} portion of this study was attributed to Pi. No peaks representative of the various organic-P fractions was obtained. The magnitude of the peaks attributed to Pi coincided with the foliar P concentrations and the expected Pi concentrations indicating that this method had no benefit over conventional procedures employed for the determination of Pi.

2) The fractionation of total P into cytoplasmic and vacuolar compartments was also not successful. Nor was it possible to determine if the concentration of P that was maintained within the cytoplasmic fraction differed between the two species.

3) Notwithstanding the problems encountered in this study, a review of the literature indicates that NMR offers researchers in the field of tree nutrition an approach that can be used to address fundamental questions. NMR is the only technique that allows one to study \textit{in vivo}, that is, to study the tree system without disturbing it in any significant way. In addition, NMR allows researchers to adopt an approach consisting of sequential measurements by altering the chemical environment within the NMR tube.
CHAPTER VI
Thesis Conclusions

The overall goal of the research studies reported in this thesis was to further our understanding of the nutritional requirements of western hemlock, in essence, to understand why certain stands respond to fertilization and why others do not respond. This basic understanding was recognized to be a prerequisite before large-scale operational fertilization programs involving this species could be contemplated.

1) The first question that was raised in this thesis was whether or not the response of western hemlock stands to N fertilization was limited by deficiencies of other elements such as P.

This question was first addressed in chapter II in which eight stands were fertilized with N, alone and in combination with P at two application rates, and a blend fertilizer. Measurement of the three-year basal area increment indicated that all eight stands showed some evidence of a growth response to the N only addition. However, a further response to P, at either of two application rates, or blend fertilizer, was not detected. This may have been due to the short response period that was documented. Accordingly, it is concluded that a response to the above question, on the basis of growth response data, should await a six-year re-measurement of the basal area increment in these stands.

The above thesis question was also tested using $^{32}$P uptake in excised roots (Chapter III). Uptake was increased in roots collected from three of the eight installations indicating that following N only additions, a secondary deficiency in P was induced in these stands. The latter, in turn, triggered what is believed to be a series of events within the tree that ultimately leads to an up-regulation of the P uptake apparatus. This is in agreement with the finding that N fertilization resulted in a decrease in the concentration of the inorganic P (Chapter II) which represents the metabolically active fraction of P. The results of the uptake study support the conclusion that the response of some stands to N additions will be limited by P, though the magnitude of this limitation can not yet be accurately ascertained.
The above thesis question was further tested by the fertilization of a five-year-old plantation (Chapter IV). The results clearly indicated that on this site P was a growth-limiting factor in addition to N. The addition of P increased height growth, photosynthetic rates and reduced carbon isotope discrimination.

Amino acid concentrations were determined in current-year foliage collected from trees at the eight-single-tree screening installations (Chapter II) and the five-year-old plantation trial (Chapter IV), a data set that included nine installations and in excess of 400 individual determinations. Without exception, arginine was the most sensitive amino acid to external nutrient supplies. Foliage collected from some 66 trees representing control plots had very low to undetectable concentrations of this amino acid. In sharp contrast, N additions alone resulted in a dramatic increase in arginine concentrations. Also consistent across the nine installations was the effect of the additions of P in reducing arginine concentrations to near control concentrations.

These results would seem to support the conclusion that the increased arginine concentrations in foliage collected from the N treatment plots represented a storage form of N. An increase in internal utilization of N following the addition of P is consistent with this interpretation. Though a relationship between ammonium toxicity and arginine concentrations cannot be dismissed, these results support the conclusion that N utilization may be increased with P additions.

2) It was noted in Chapter I that much of the research into the nutrition of western hemlock during the past three decades adopted a growth and yield approach. This body of research, arguably, did little to further our understanding of why certain stands responded and why others did not. Following the recommendation of Brix (undated), an additional thesis question was whether or not biological indicators could be identified which could aid in the diagnosis of nutrient status and in the prediction of long term growth response following fertilization.

As noted above, concentrations of arginine varied with fertilization treatment. An important question is whether or not arginine concentrations can be used to predict the long-term growth response of these stands to N fertilization. This question remains
unanswered. A more definitive answer may be possible following the long-term re-
measurement of the N response in the eight single-tree installations.

The findings of the root bioassay study indicated that this approach was a
sensitive indicator of P status. However, the results of this study were inclusive as to
whether this approach could be used in the prediction of long-term growth response to
fertilizer treatments. This shortcoming may in part have been the result of a short-term
growth response period as noted above. In the alternative, western hemlock may respond
to P deficiencies using a variety of response mechanisms, and that the up-regulation of
the P uptake apparatus is not a prominent response mechanism in western hemlock
during periods of P deficiency.

The relationship between carbon isotope discrimination and nutrition of western
hemlock was assessed in the 5-year-old plantation trial. This study represents what is
believed to be one of the first investigations into using carbon isotope discrimination
analysis in the assessment of nutritional status. The improvement in N status following
the addition of N alone in the 5-year-old plantation experiment resulted in a significant,
and consistent, decrease in discrimination. Similarly, the addition of each of the P and
blend additions resulted in a further reduction in discrimination. The latter reductions in
discrimination were mainly attributed to an increase in the photosynthetic rate over the
course of an entire growing season. Accordingly, carbon isotope discrimination values
were seen to be cumulative measurements of photosynthetic response rather than “point
in time” measurements obtained with conventional approaches.

These findings are similar to what had been reported by White et al. (1999).
These trials have shown that there is a clear and, seemingly, a repeatable relationship
between nutrient status and measures of carbon isotope discrimination. While this
approach has yet to be widely used within the field of tree nutrition, it is submitted that
this approach would seem to hold much potential.

The effect of improved nutrition following fertilizer additions on carbon isotope
discrimination raises a number of interesting questions. The first question is whether
mean $\delta^{13}$ values in western hemlock foliage collected from trees prior to any nutrient
additions could be used to ascertain the nutrient status of the stand. That is to say, could
mean $\delta^{13}$ values in western hemlock foliage be used in a similar fashion to that which
conventional foliar analysis is used to determine the nutrient status of Douglas-fir stands? For example, could one assume that hemlock stands that have a mean $\delta^{13}$ value in current year foliage above -29.00 are limited by N and would respond to fertilization? Unfortunately, this question can not be answered on the basis of the 5-year-old plantation. A large data set would need to be constructed and, to reduce the potential error attributed to moisture supply, the data set should be specific to site moisture.

Furthermore, could changes in carbon isotope discrimination following fertilization be used as an indicator of nutritional status and the long-term growth response the stand? For example, the mean $\delta^{13}$ value in current-year foliage in the 5-year-old plantation trial was reduced by a value of approximately 2 % following the addition of N. The growth of the western hemlock trees within these trials was increased with the improvement in N status. Essentially, this suggested approach is somewhat similar to that of vector analysis where screening trials are used and the first year response variable is used to predict long-term growth response. In the present scenario, the mean $\delta^{13}$ value is used instead of foliar N concentrations and total content. Unfortunately, this question also can not be adequately addressed on the basis of the 5-year-old plantation study. As noted above, a large data set would also need to be constructed before this question could be critically tested.

Inorganic P was found to be a more sensitive indicator of the P status of western hemlock than mere total P. The results of P fractionation in both the eight-single-tree screening installations and the 5-year-old plantation experiment, totaling some 324 individual determinations for a given growing season, revealed that the concentration of the organic fraction was relatively constant and remaining P was allocated to the inorganic fraction. As the organic fraction of P, a composite of nucleic acids, esters and lipid phosphates, was relatively constant, the inorganic pool of P could be accurately estimated from the total P determined by conventional foliar analysis. As such, P fractionation would seem to have little future benefit over that of total P in the diagnosis of P status of western hemlock stands. However, one benefit of determination of the inorganic pool of P, referred to by some as the "physiologically active fraction", is that it gives a greater appreciation for the understanding of changes in nutrient status on physiological processes such as photosynthesis and P acquisition strategies.
The results of the NMR investigations in this thesis proved to be inconclusive. Spectra representing the vacuolar and cytoplasmic compartments in the \textit{in vivo} experiment could not be identified. During the \textit{in vitro} experiment, spectra other than those attributed to inorganic phosphate could not be identified. These shortcomings limit any meaningful conclusions with respect to the possible relationship between the cellular compartmentation of P in foliage of western hemlock and Douglas-fir and P use efficiency of these species. The question related to the internal distribution of P amongst the free metabolites of P in the foliage of each species also remains unanswered. Notwithstanding the above shortcomings, the review of the applications of NMR to questions of interest to researchers in either the fields of tree or plant nutrition indicate clearly that the application of NMR in future studies will allow for new and exciting questions to be posed, questions that until now could not be addressed due solely to the technological difficulties, particularly those related to scale.
CHAPTER VII
RECOMMENDATIONS FOR FUTURE RESEARCH

The findings reported in this thesis have raised many interesting questions. The following recommendations are proposed to assist those researchers who share an interest in the nutrition of western hemlock.

1. The relationship between carbon isotope discrimination and foliar nutrient concentrations, or its applicability to assisting in the diagnosis of a stand's nutritional status, has received little attention within the field of tree nutrition. The results herein suggest that this technique has promise in furthering our understanding of the response mechanisms of trees to nutrient additions. Furthermore, and perhaps more importantly, the utility of $^{13}$C discrimination as an indicator of nutritional status, or as a predictor of future growth responses in screening trials, holds exciting promise and should be pursued. Studies to address these two questions may adopt one of two approaches. Retrospective studies of previous fertilization trials could be undertaken in which the changes in discrimination of stemwood are compared to long term response. This approach is favourable in so far as candidate stands are available throughout coastal British Columbia for a number of species and such a study would be of short duration since one is measuring past response rather than future. A second approach is the establishment of new trials in which the changes in discrimination in current-year foliage can be compared to short-term and long-term growth responses. This approach is more intensive in nature in so far as the measurement of long-term response, 3 to 6 years, necessitates a study length of equal duration. However, this approach has the benefit that the investigator can design his or her owns treatments and not be restricted to those used in past trials. In addition, changes in discrimination in foliage would be of more value to future investigators who rely on foliar analysis for diagnosing the nutritional status of stands.

2. These investigations have also demonstrated that several stands had shown evidence of an increase in growth following the addition of elements other than N and P. It is recommended that the role of K and S in limiting the response of western hemlock
following N additions be studied further. The assessment of K and S status may continue to be measured by conventional nutrient analysis and subsequently complemented by the root bioassay technique. Uptake of K can be measured in excised roots using Rd. Similarly, the potential to use $^{34}\text{S}$ to measure S uptake in excised roots should be investigated.

3. While the concentrations of enzymes in the foliage of western hemlock were not determined in this thesis, other researchers should adopt the physiological approach adopted by this thesis. The findings by Kolari and Sarjala (1995), who investigated the relationship between acid phosphatase activity in the foliage of Scots pine and P nutrition, indicate that this enzyme may have promise in complementing other measures of P status. In addition, the relationship between putrescine and K concentrations in current-year foliage of coastal conifers should be ascertained.

4. Future investigators may also wish to identify the precise cause of arginine accumulation following N additions in our conifer species and its relationship to P and the P/N ratio. In concert with a study into arginine, investigators may wish to document potential short-term changes in the foliar ammonium concentrations following N additions. These studies should ideally be carried out using field trials but it is recommended that initial studies be carried out under greenhouse conditions using seedling stock. While it is recognized that the nutritional requirements of seedlings and mature stands will differ, a range of foliar nutrient concentrations from deficient to concentrations indicative of luxury consumption or toxicity can be more accurately controlled under greenhouse conditions.

5. Further nutritional trials in immature hemlock stands should be established. Treatments should include P additions ranging from the conventional rate of 100 kg/ha to 300 – 500 kg/ha. In addition, treatments should include K and S additions, as noted above, in rates similar to those used in the present investigation. Ideally, an experimental design in any future trials would allow for a clear interpretation of the role of the latter two elements.
6. A number of research questions involving specific aspects of P nutrition were posed in Chapter 6 and remain unanswered. Of particular interest were questions related to the fractionation of P between the vacuolar and cytoplasmic pools and how this fractionation of P may be related to potential differences between species or genotypes in P requirement and use efficiency. Though meaningful use of nuclear magnetic resonance has been lacking in the field of tree nutrition, it is recommended that this approach be utilized in the near future to address the questions raised in this thesis. In this regard, it is also recommended that a concerted effort be put forth by researchers in the field of tree nutrition to more fully explore, or to reinvestigate previous assumptions, given the advantages of NMR.

7. Future research into assessing the P requirement of conifer species should also include defining the Pi pool required to maintain high rates of photosynthesis and productivity.

8. The higher foliar N concentrations in current-year foliage collected from a previous western hemlock trial located in the Nimpkish Valley again raises the question as to whether ammonium nitrate is a more effective source of N than the conventional application of N in the form of urea. While urea is preferred by industry as a cheaper source of N, additional based trials are recommended to ascertain if an enhanced growth response using ammonium nitrate may compensate for the higher initial costs of application.

9. The strong response of the 18-year-old western hemlock stand (Chapter 2) to both N and P additions, the findings of previous researchers in the Pacific Northwest, and the lack of response in many of the single-tree screening trials raises the question as to whether stand age may have an effect on response. The question of stand age has, arguably, yet to be fully addressed and other researchers interested in western hemlock nutrition may wish to pursue this question. Should experimentation prove that stand age has an effect investigators may wish to determine if N or P requirement
changes with stand age with particular reference to changes in nutrient retranslocation.

10. Future researchers studying western hemlock nutrition may wish to investigate whether or not a molecular-based study may compliment the more conventional approaches to the study of tree nutrition.

11. It was noted in the introduction (Chapter 1) that many previous researchers had limited their treatments to those which would have been operationally relevant. While many of the questions which have been of interest to researchers in the field of tree nutrition have been posed to addressed questions related to the economics of operational fertilization, it is important that researchers recognize the scope of their respective experiments to the broader study of tree nutrition.

12. Given the fact that the forest industry in the province of British Columbia will be under increasing pressure in the future to increase stand productivity, and given the unbalanced age class structure of many of its forests, the use of fertilization to increase growth can be expected to increase dramatically from current levels. Consistent with the recommendations noted above, steps should be initiated to re-develop and re-fund a tree nutrition cooperative within the province of British Columbia dedicated solely to increasing stand productivity.

13. While a tree nutrition cooperative, as suggested above, would necessarily direct much of its energies to address management questions, future research into tree nutrition should also focus on the development and further refinement of more physiologically meaningful parameters rather than merely establishing more growth and yield trials. This will require a collaborative approach that should involve leading scientists from the fields of soil science, silvicultural science, and physiology, managers representing each of the two levels of government that are relevant to forest management, and representatives from the forest industry.
CHAPTER VIII
LITERATURE CITED


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